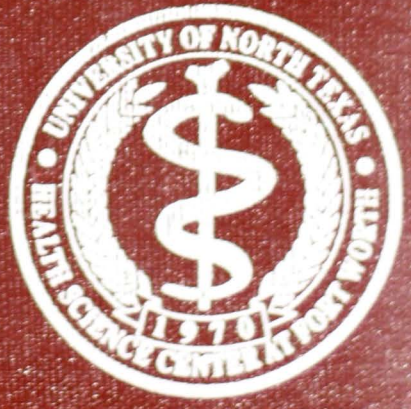
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The purpose of these studies was to evaluate the roles of IFN-γ and IL-4 during the development of immune responses of the upper and lower respiratory tracts, during mycoplasma respiratory disease. To study their roles, we took advantage of IFN-γ and IL-4 knockout (KO) mice. The loss of IL-4 did not impact the development of disease or the clearance of mycoplasma from either respiratory tracts during mycoplasma infection. However, IL-4 mediated responses dampen mycoplasma induced bronchial hyperresponsiveness (BHR), which are in direct contrast to theories that state that IL-4 is critical for the development of BHR. This suggests that mycoplasma exacerbation of asthma is a synergism between IL-4 and non-IL-4 mediated responses. Thus, IL-4 does not impact mycoplasma disease development, but dampens detrimental non-IL-4 mediated responses that exacerbate BHR during mycoplasma disease.

The loss of IFN-γ did not affect disease or the number of mycoplasma organisms in the upper respiratory tract; this is in contrast to the lungs where the loss of IFN-γ led to a defect in innate immune responses. A significant increase in mycoplasma organisms were seen by day 3 post-infection which led to exacerbation of disease pathology. By three days after infection, only the number of IFN-γ^+ NK cells increase in numbers in

response to mycoplasma infection. However, the depletion NK cells by anti-asialo GM1 antibody treatment did not affect the clearance of mycoplasma from lungs of BALB/c mice, however, NK cell depletion from IFN-γ KO mice lead to increased clearance of mycoplasma organisms from the lung. Further studies demonstrated that NK cells in an IFN-γ deficient environment lead to increased secretion of IL-10, G-CSF, and TNF-α and increased numbers of cell infiltrated into the alveoli and airways. These results suggest that NK cells of the lung have anti-inflammatory roles that IFN-γ counteracts in BALB/c mice. Regardless, these studies demonstrate that NK cells in an IFN-γ deficient environment impair innate immune responses from clearing mycoplasma organisms from the lung. These studies demonstrated diverse but novel functions for IFN-γ and IL-4 during respiratory infections that will have significant impact on future studies of respiratory immunology.

THE ROLES OF IFN-γ AND IL-4 IN THE UPPER AND LOWER RESPIRATORY TRACTS IMMUNE RESPONSES TOWARD MYCOPLASMA INFECTION

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CHAPTER I

INTRODUCTION TO THE STUDY

Mycoplasma is the smallest free-living organism identified to date. It possesses a small genome, lacks a true cell wall and requires cholesterol for membrane function and growth (1). Mycoplasma has been identified as the cause of several acute and chronic infections and as a possible co-factor in several other diseases (1). Mycoplasma infection is a leading cause of pneumonia worldwide. In the United States alone, *Mycoplasma pneumoniae* accounts for 30% of all cases of pneumonia and 100,000 hospitalizations a year (2-4). Chronic infections of *M. pneumoniae* have been identified as possible factors in asthma developed later in life, and has been clearly documented as exacerbating asthma disease severity (5, 6). Mycoplasma's are also a huge monetary burden on livestock, infecting large herds of cattle, swine and chickens leading to the development of pneumonia and mastitis (7). Antibiotic treatment of mycoplasma infections tend to clear acute symptoms, however, they fail to completely remove mycoplasma from the infection site leading to long-term chronic disease complications(8-10). The best hope for dealing with mycoplasma infections is the generation of a vaccine, however to date, no successful vaccine for human mycoplasmas have been manufactured. The problem is the immuno-pathological nature of the infection, where some aspects of the immune response are detrimental, and others are beneficial to clearance of the pathogen (11-15).

Although, which immune elements are pathological and which are protective against mycoplasma infection is still unclear. The purpose of this thesis is to determine what role interferon-gamma (IFN-γ) and interleukin-4 (IL-4) have in the modulation and generation of innate and adaptive immune responses towards mycoplasma respiratory disease.

The immune system

The immune system is a complex multi-layered system of cells and organs that are important in protecting hosts from a variety of insults. Because of its complexity, trying to characterize and categorize the immune system has been difficult. However, the immune response has been broken down into two major groups: innate and adaptive immune responses. Innate immunity is considered non-specific host defenses, being comprised of anatomic, physiologic, phagocytic, and inflammatory barriers (16). Physiology of the skin, the gut, and lungs provide protection by making it as inhospitipal as possible to invading organisms. If infectious agents get past these barriers, there is a group of molecules and cells that create inflammation and phagocytosis in a non-specific manner, such as complement, macrophages, NK cells and neutrophils (16). Adaptive immunity is comprised of B and T cells that react to specific antigenic challenges, and is characterized by antigenic specificity, diversity, memory and self/nonself recognition (16). However, adaptive and innate immune responses are connected in a variety of fashions. The type of inflammatory response generated and the cytokines that are released by the innate immune system in response to an infectious agent influence the type of adaptive immune responses. In return, antibodies generated by B cells and cytokine released by T cells aid phagocytic cells of the innate immune system. There are

numerous other examples of cross talk between the innate and adaptive immune system (16). Studies into both types of immunity are important in understanding host pathogen interactions, thus, allowing for the development of vaccines.

Cytokines

Cytokines are the communication network of the immune system. Cytokines are released by a variety of cells that act upon receptors, which are on the membranes of leukocytes. Cytokines exert three types of actions: Autocrine, where cytokines act upon the cell that released it, such as IL-2 that is released by T-cells to cause an increase in T-cell proliferation; Paracrine, where cytokines act on cells in the nearby area, such as IFN-γ released by NK cells which activate macrophages; and Endocrine, where cytokines act upon cells in distant parts of the body, such as IL-6 causing the increase of fever (17). Cytokines have a broad array of biological functions; the biggest being the modulation of innate immunity, adaptive immunity, inflammation, and hematopoieses, and new functions of cytokine, which are being identified daily (18).

Inflammation

Cytokines of importance in disease pathogenesis are those involved in pro- and anti-inflammatory responses. Inflammation is the physiologic response to stimuli such as infections and tissue injury. Inflammation is the acute rapid onset response that lasts for a short time interval. Complement, chemokines and cytokines play an important role in directing and dictating inflammation processes (11, 19-21). In general, at the site of an infection you get activation of tissue specific macrophages and complement. The macrophages release cytokines such as IL-1, IL-6 and TNF-α, as well as chemokines

(22). These mediators attract and activate neutrophils, as well as recruit bloodborne monocytes and lymphocytes to the area of infection(19, 22). At the site of infection, neutrophils release nitrogen and oxygen radicals as part of their respiratory burst to kill invading pathogens (19). Monocytes respond by releasing IL-12 and IL-18 (22). These cytokines act either alone or in concert upon NK cells and T cells to release IFN-γ (23-25). IFN-γ is a key inflammatory cytokine, as it activates macrophages to increase MHC II expression and microbicidal activity. IFN-γ also causes the phenotypic maturation of Th0 to Th1 cells, down regulates Th2 activity, increases MHC II expression on dendritic cells, activates NK cells, and causes antibody class switching of B cells to IgG2a production (26-28). All of these mechanisms create a robust immune response that is capable of dealing with an infectious agent. However, if left unchecked, due to genetic diseases or prolonged antigen stimulation, inflammation becomes chronic. Chronic inflammation leads to tissue damage, wasting, fibrosis and possible granuloma.

Dampening inflammatory processes

The release of anti-inflammatory cytokines is important in dampening inflammatory responses. Certain cytokines are critical in turning off inflammation. Without their effect, inflammation would run unchecked and lead to detrimental results. Key anti-inflammatory cytokines are IL-1 receptor antagonist, IL-4, IL-10, IL-11, IL-13 and TGF-beta (29). These cytokines block: macrophage activation, MHC II presentation, pro-inflammatory cytokine production, and the development of Th1 T cells (29). IL-4 and IL-10 are two of the more interesting of the anti-inflammatory cytokines as they have very potent affects on dampening innate and adaptive immune responses.

Interleukin 4

IL-4 is a pleiotrophic cytokine that is important in Th and B cell differentiation, as well as its ability to dampen inflammatory processes. B cells, after encountering IL-4, start to produce high levels of IgE and IgA (26, 30, 31). This IgE is captured by mast cells on the high affinity receptor (FCεRI). IL-4 also has a potent affect on the recruitment and activation of these mast cells to the site of an infection (32). When IgE antibodies bind to an antigen, which creates cross-linking, this leads to mast cells to degranulate: releasing of high levels of histamine, chemotactic factors, prostaglandin and leukotrienes (32). This type I hypersensitivity reaction causes increased vascular permeability and smooth muscle contraction. Along with the chemotactic factors, it also promotes the recruitment of inflammatory cells into the site of infection (32). These type I hypersensitivity reactions, if not properly controlled, are a major cause of allergies and asthma. IL-4 is also critical in the differentiation of Th0 cells to a Th2 phenotype. Th2 cells are important in mediating humoral responses, i.e. aiding in antibody secretion of B cells. IL-4 also directly down regulates Th1 responses, by blocking their secretion of IFN-γ and IL-2, therefore helping to dampen inflammatory processes (26). IL-4 plays a unique role in both aiding early inflammatory processes, while down regulating inflammation later in immune responses towards an infectious agent.

A major role of IL-4 in innate immune functions has not been well demonstrated. IL-4 does influence mast cell and eosinophil functions, through both recruitment and activation. However, mast cells and eosinophils react to invading pathogens by cross-linking of IgE antibodies that are bound to Fc receptors (32). Therefore, in order for

eosinophils and mast cells to response to an invading pathogen, they are dependent on B cells having to encounter the pathogen to produce IgE, making the contribution of IL-4 to first time infections minimal at best. There is current research looking for early bursts of IL-4 after infection, however, this IL-4 is most likely to modulate Th cell responses and really does not impact heavily on innate immune functions (33). Some new evidence is showing that NKT and γδT cells may release IL-4 early after Toxoplasma infections, which disrupts the generation of a robust inflammatory response (34). This work demonstrates that IL-4 may impact innate immune response, although, more studies must be characterized to definitively demonstrate what role IL-4 plays.

Interleukin 10

IL-10 was first discovered for its potent inhibition of IFN-γ and IL-2 secretion, and was initially named cytokine synthesis inhibition factor (35, 36). Th2 cells, monocytes and B cells secrete IL-10 (35, 36). It was also found to have profound effects on blocking monocyte/macrophage cytokine secretion and MHC II presentation. IL-10 also inhibits cytokine production by neutrophils and NK cells (37, 38). Overall, these actions severely dampen pro-inflammatory processes and protect the host from systemic inflammation after toxin-induced injury. However, incorrect expression of IL-10 at times when infections have not been cleared, lead to states of low-level chronic inflammation

(39). In several infectious disease models, the over-production of IL-10 renders the host more susceptible to lethal disease (40-42). Therefore, IL-10 is a critical cytokine to monitor in most infectious disease states.

Innate immune system

The cells of the innate immune system are comprised of phagocytic cells, inflammatory cells, antigen presenting cells, and immuno-modulatory cells. Monocytes, neutrophils, and tissue macrophages conduct most of the phagocytosis of invading pathogens (16). Several cells, such as mast cells, macrophages, NK cells, and neutrophils release oxygen radicals, cytokines, and inflammatory mediators that cause inflammation at the site of infection (16). The antigen presenting cells, such as dendritic cells and macrophages, are critical in directing and activating adaptive immune responses (16). All of these innate cells work in concert, to activate inflammation, kill invading pathogens, and direct adaptive immune responses.

Monocytes and Macrophages

Macrophages comprise an important cell of the innate immune system. Macrophages can be separated into two groups, bloodborne monocytes and tissue resident macrophages. Tissue macrophages, such as alveolar macrophages, kupffer cells, and microglial cells, play an important surveillance role in the immune system. These macrophages are longer lived than bloodborne macrophages, and are derived from a local progenitor cell, rather then recruitment of new monocytes into the tissue (43). Tissue macrophages are found in areas where there is a higher burden of antigen stimulation, because of which these macrophages become more of a monitoring cell, than an

inflammatory macrophage. Tissue macrophages release pro-inflammatory cytokines such as, IL-1, IL-6, IL-12, IL-18, and TNF-α, and hemopoetic cytokines GM-CSF, G-CSF, and IL-3, which are critical for the recruitment and initiation of inflammatory processes (22, 44), although, tissue macrophages also release anti-inflammatory cytokine, IL-10, which dampens early inflammatory events (45). Thus, these macrophages are important in monitoring and modulating localized immune responses, making sure to generate inflammation at times when it is necessary, yet insuring not to over respond to all antigen stimulations.

Monocytes, on the other hand, are mononuclear phagocytes that circulate through the blood for about eight hours. Monocytes have chemotactic, pinocytotic and phagocytic properties before entering tissue (44). However, once monocytes encounter inflammatory signals, which are normally generated by tissue-derived macrophages, they transverse through the tissue into the site of infection. Upon entering the tissue, monocytes go through a phenotypic change where they enlarge, as well as increase in organelle number, phagocytic ability, production of hydrolytic enzymes, and they also begin to secrete a variety of soluble factors (22). At this stage, they are integral to the inflammatory process. These activated macrophages release a variety of cytokines, such as IL-1, IL-12, IL-18, IP-10 and TNF-α, in order to aid in the generation of a robust inflammatory response (22). Of particular importance is IP-10, which helps in the recruitment of NK cells to the site of infection. Once NK cells enter the area of infection, IL-12 and IL-18 stimulate them to release IFN-γ (20). This IFN-γ further activates macrophages, thus increasing the phagocytic capabilities and antigen presentation. This

IFN-γ also is critical in aiding the phenotypic differentiation of Th0 cell to Th1 cells (26). Thus macrophages are an important innate immune cell that can directly phagocytize invading pathogens, as well as influence down stream innate and adaptive immune responses by cytokine secretion and antigen presentation.

Natural killer cells

A unique cell of the immune system is the NK cell, which was first identified for their ability to directly lyse tumor cells without prior stimulation (46). It soon became evident that NK cells play a much larger role in the immune system, than simply lysing tumor cells. NK activity has been attributed to tumor surveillance, dealing with virally-infected cells, intracellular bacteria infection, interfacing the immune system and reproductive system, and is done by a cell that does not have antigen specificity capabilities (46). NK cells utilize activation and inhibition receptors that lead to the release of degradative enzymes, granzyme B, perforin, and granulysin against a target cell (47). These actions have been studied extensively in response to cancer models, in hopes to utilize these cells to treat cancer. However, recent studies have identified an equally important role for NK cells, immune modulation. In both listeria and leishmania infections, recruitment and activation of NK cells is critical for clearance of the organism (48). The IFN-γ released by NK cells aid in the activation of bacteria laden macrophages. NK cells have also been documented to control CD4 T cell populations before and after stimulation (49, 50). Furthermore, NK cells have been demonstrated to cause apoptosis in activated T cells, as well as immature DCs (51). Possibly demonstrating a mechanism for NK cells to modulate adaptive immune responses before and after infection. NK

cells, modulate CD4 T cells in the lungs of male SJL mice, not allowing these mice to generate a Th1 cell response (50). Demonstrating that NK cells are more than just cells which conduct surveillance for tumor and virally infected cells, but is critical in modulating innate and adaptive immune responses through both direct cell-to-cell interaction and cytokine secretion.

Adaptive immunity

Specificity, memory, diversity and self-versus-nonself recognition characterize the adaptive immune response. T cell and B cell generated immune responses are responsible for these properties. These cell types have the ability to rearrange gene products that code for antibody structure and T cell receptor (TCR). Through gene re-arrangement, non-specific DNA splicing, and hyper variability, T and B cells can respond to possibly billions of different antigens (16). Even more important, T and B cells go through positive and negative selection, and need co-stimulation in order for them to become activated. Due to these fail-safes, generation of adaptive immune responses is delayed after the immune system encounters a new antigen. Seven to ten days after encountering an antigen, there will be the first generation of antigen specific T and B cells responses (16). After this first encounter though, specific T and B cells will be destined to be memory cells, these memory cells need lower levels of co-stimulation to be activated and respond faster and more robustly to a second infection. Cytokine environments coordinate the development and differentiation of adaptive immune responses.

The yin and yang of adaptive immunity

IFN-γ and IL-4 play intricate roles in adaptive immune responses. IFN-γ and IL-4 are the yin and yang cytokines of the adaptive immune system. IFN-γ leads to the development of cell mediated adaptive immune responses that aid macrophages in dealing with intracellular bacteria by further activating them (45). IFN-γ also leads to antibody class switching, to an IgG subclass that is the most suited for opsinization, further aiding the inflammatory response (26). IL-4 is necessary for the development of humoral adaptive immune responses. IL-4 pushes immune responses that aid antibody production to IgA and IgE subclasses (26). Each cytokine dampens the other cytokine mediated responses, in that IFN-γ dampens IL-4 mediated responses and vice versa.

T helper cell

T helper cell responses play important roles in controlling infectious disease. Correct Th cell responses are needed to properly respond to certain pathogens. In several diseases, it as been clearly demonstrated that one Th cell response exacerbates a disease while the other Th cell response clears the disease (52-54). The cytokines that each T helper cells subset release have contrasting down stream effects. Th1 cells secrete cytokines IL-2, IFN-γ, and TNF-β that aid in pro-inflammatory processes (26). These cytokines aid in proliferation of T cells and further differentiation of Th1 T cell subsets; however, the IFN-γ also important in aiding macrophages dealing with intracellular bacteria (22). Th1 subset is responsible for classic cell mediated responses, such as delayed-type hypersensitivity and the activation of cytotoxic T lymphocytes (CTL) (55). Th2 cells secrete cytokines IL-4, IL-5, IL-6, and IL-10 that help B cell activation and

antibody production (26). These cytokines are also important in dampening inflammatory responses. IL-10 directly dampens macrophage activation, while IL-4 blocks Th1 differentiation (16). Thus, Th cells subset play important roles in controlling disease.

Mycoplasma

Mycoplasma infection is a leading cause of pneumonia worldwide. In the United States, alone, *M. pneumoniae* accounts for 30% of all cases of pneumonia (2-4). Mycoplasma disease is also associated with the exacerbation of other respiratory diseases, such as asthma (5, 6). *M. pneumoniae* respiratory infection causes rhinitis, otitis media, laryngotrachetis and bronchopneumonia. The histopathology of lungs infected with *M. pneumoniae* demonstrates the accumulation of mononuclear and lymphoid cells along the respiratory airways (11, 56, 57). This suggests that the activation and recruitment of lymphoid and mononuclear cells is key in the development of both acute and chronic states of the disease. However, the immune response generated at the respiratory airways is inappropriate to clear the organism. As many *M. pneumoniae* infections become chronic, and even with the treatment of antibiotics airway physiology is affected for months after an infection (8-10). *M. pulmonis* causes a natural murine respiratory disease with high morbidity and low mortality. *M. pulmonis* is an excellent animal model of *M. pneumoniae,* as *M. pulmonis* infection has a similar histopathology to that of *M. pneumoniae* infection (57). The use of *M. pulmonis* in mice allows for the characterization of immune responses toward a pathogen in its natural host.

Mycoplasma and asthma

Acute mycoplasma infection, along with other respiratory infections, has been associated with the exacerbations of asthma. *M. pneumoniae* can be isolated from the respiratory tract of up to 25% of asthmatics experiencing acute exacerbations (58). Chronic *M. pneumoniae* infection has also been suggested as a possible contributing factor to the severity or development of asthma in humans. *M. pneumoniae* has been utilized to study bronchial hyperresponsiveness (BHR) in mice; however, *M. pneumoniae* is not a naturally occurring pathogen in mice (59-62). *M. pulmonis* infection in mice is similar to *M. pneumoniae* infection in humans in terms of both the nature of histologic inflammation generated and the chronic nature of the disease (2, 56, 57, 63, 64). This murine model allows us to study a natural host-pathogen interaction that is similar to *M. pneumoniae* in humans, and its affects on BHR.

Asthma is a common syndrome that is likely multifactorial and heterogeneous in etiology and pathogenesis. The physiological and inflammatory mechanism's that lead to increase BHR in asthmatic patients is not fully understood. Evidence suggests that allergic asthma is associated with a shift from Th1 immunological responses to a Th2 response, where IL-4 mediated inflammation causes BHR (65). Studies, in both humans and mice, demonstrate the pathogenic importance of IL-4 in the development of BHR seen in allergic asthmatic (65, 66). In fact, studies with IL-4 KO mice demonstrate an attenuation of allergic model BHR compared with wild type mice, yet few studies have examined IL-4 in an infectious model of BHR (65). Although, the current view is that

most BHR, in either infectious or asthmatic models, is Th2 dependent. IL-4 is the key cytokine in skewing the lung to a Th2 environment that leads to the development of asthma and BHR.

Immune response towards mycoplasma

It is clear that part of the adaptive immune response contributes to the pathology of mycoplasma disease, while part of it protects against mycoplasma disease. SCID mice, which lack T and B cells, do not develop as severe respiratory disease in response to *M. pulmonis* infections, as do corresponding immunocompetent mice. However, these SCID mice develop arthritis and have a higher rate of mortality, demonstrating there is a need for lymphoid immune responses to control mycoplasma infections. Reconstitution of these SCID mice with splenic lymphocyte results in similar disease compared to wild type mice (11). Work in T cell deficient hamsters, also demonstrates that these hamsters have less severe disease than hamsters with a full immune arsenal (13, 15). Both of these animal models demonstrate that T cell and B cell responses can play both pathological as well as protective roles.

Previous work has characterized the T cell environment within the lungs, and how this environment is modulated in the response of mycoplasma infection. The murine lung is classically a Th2 cell dominated environment, with high levels of IL-4 being detected (67). However, we demonstrated that in response to mycoplasma infection we see a shift to a mixed Th1/Th2 response, suggesting that IFN-γ from Th1 cells is playing an important role in mycoplasma respiratory disease (68). The depletion of all Th cells cause a less severe disease, once again demonstrating the pathological component of the

Th cell mediated immune response (68). To the contrary, depletion of $CD8^+$ T cells from mice, leads to an exacerbation of mycoplasma pulmonary disease (68). This suggests that $CD8^+$ T cells play a unique regulatory role within mycoplasma respiratory disease. Thus, T cell activation, and most likely the cytokines they produce, is instrumental in the pathogenesis of mycoplasma respiratory disease.

Innate immune responses are important in protection from mycoplasma respiratory disease. Work in C57Bl/6 mice, which are resistant to mycoplasma infections, demonstrates that their innate immune responses effectively protect them from disease pathology (69). C57Bl/6 mice have a short burst of pro-inflammatory cytokines shortly after infection, which quickly subside. This is in contrast to C3H/HeN mice; where early pro-inflammation is persistent for several days. Removal of macrophages from C57Bl/6 mice causes them to be susceptible to infection, demonstrating that the innate immune system is important (69, 70). However, removal of macrophages from C3H/HeN mice, a susceptible strain, does not change mouse susceptibility; thus, more than just macrophages of the innate immune system are important in controlling disease (70). Most likely the cytokine environment that is generated by innate immune cells is critical in activating innate immunity as well as determining the generation of protective or pathogenic adaptive immune responses towards *M. pulmonis*. Evidence has suggested that NK cells also play an important role, possibly through IFN-γ release that activates the macrophages (71, 72). In fact, IFN-γ along with surfactant-protein A has been

demonstrated to be critical for macrophages to kill mycoplasma (70, 73). Beyond this, very little is known of the innate immune system and its responses towards mycoplasma infections.

By utilizing IFN-γ KO and IL-4 KO mice, we hope to understand the contribution of these cytokines in the development of pathogenesis and/or protection in response to mycoplasma respiratory infection in both upper and lower respiratory tracts. Our lab has demonstrated that the upper and lower respiratory tracts seem to be immuno-logically separate in their response towards an infectious agent. Our previous studies also suggest that there is a difference in immune inflammatory mechanisms in the upper and lower respiratory tract. Mice immunized for, or infected with viruses, generate a higher titer of antigen specific antibody responses in the nasal passages than in the lungs (31, 74). This phenomenon of higher titers of antibody production in the upper, versus the lower respiratory tract, has also been demonstrated in rats in response to mycoplasma respiratory infection (75). The use of immunizations has shown that systemic immunization with *M. pulmonis* antigen can protect the lungs but fails to protect the nasal passages (76). These studies suggest differences in immune responses of the upper and lower respiratory tract in response to infectious agents. However, studies to characterize differences in cytokine control of the upper and lower respiratory tract have yet to be done.

The immune mechanisms that are generated towards mycoplasma infections are both pathogenic and protective. The work summarized in this dissertation uncovers several new and novel immune mechanisms of the respiratory tract. Studies conducted

demonstrate there is a difference in requirements of IFN-γ and IL-4 between upper and lower respiratory tracts immune responses towards mycoplasma infection. Further studies demonstrated the IFN-γ mediated immune responses, which are important in the lung, mask novel NK functions that dampen early inflammation, clearing mycoplasma from the lung. Also, this work begins to demonstrate that Th1 mediated adaptive immune responses protect the lungs from mycoplasma disease pathology, and IL-4 mediated responses protect against mycoplasma induced BHR. Overall, this work demonstrates many novel immune mechanisms that will have an impact on how respiratory immunology is viewed and studied.

CHAPTER II

The Upper and Lower Respiratory Tracts Differ in Their Requirement of IFN-γ and IL-4 in Controlling Respiratory Mycoplasma Infection and Disease

The purpose of this study is to evaluate the significance of IFN-γ and IL-4 production in controlling mycoplasma infection and the pathogenesis of disease in the upper and lower respiratory tract. By utilizing IFN-γ knockout (KO) and IL-4 KO BALB/c mice, we were able to study the contribution of these cytokines in the development of pathogenesis and/or protection in response to mycoplasma respiratory infection, in both the upper and lower respiratory tracts. The loss of either IFN-γ or IL-4 does not affect disease pathogenesis or mycoplasma organism numbers in the upper respiratory tract. However, in the absence of IL-4 the nasal passages developed a compensatory immune response, characterized by higher numbers of macrophages and $CD8^+$ T cells, which may be masking detrimental affects due to IL-4 deficiency. This is in contrast to the lower respiratory tract, where the loss IFN-γ, but not IL-4, leads to higher mycoplasma numbers and increased disease severity. The loss of IFN-γ impacted the innate immune systems ability to effectively clear mycoplasma, as the number of organisms was higher by day 3 post-infection. This higher organism burden most likely impacted disease pathogenesis, however, the development of Th2 cell-mediated adaptive

immune response most likely contributed to lesion severity at later time-points during infection. Our studies demonstrate that the upper and lower respiratory tracts are separate and distinct in their cytokine requirements for generating immunity against mycoplasma infection.

Introduction

Mycoplasma infection is a leading cause of pneumonia worldwide. In the United States, alone, *Mycoplasma pneumoniae* accounts for 30% of all cases of pneumonia (2-4, 76). Mycoplasma disease is also associated with the exacerbation of other respiratory diseases, such as asthma (5, 6). *Mycoplasma pulmonis* causes a naturally occurring murine respiratory disease with high morbidity and low mortality. *M. pulmonis* is an excellent animal model of *M. pneumoniae,* allowing the characterization of immune responses during the pathogenesis of mycoplasma respiratory disease. Both *M. pulmonis* and *M. pneumoniae* respiratory infections cause rhinitis, otitis media, laryngotrachetis, and bronchopneumonia. In terms of histopathology, both diseases are characterized by the accumulation of mononuclear, macrophages and lymphocyte cells along the respiratory airway (4, 56, 57, 63, 64). This suggests that the activation and recruitment of macrophages and lymphocytes is key in the development of both acute and chronic states of the disease. In support, several studies demonstrate a component of mycoplasma respiratory disease is immunopathologic (11-15).

It is clear that part of the adaptive immune system contributes to the pathology, while part is protective against *M. pulmonis* infections. SCID mice, which lack T and B cells, do not develop as severe respiratory disease in response to *M. pulmonis* infections

as do corresponding immunocompetent mice. However, these SCID mice eventually develop arthritis and a higher mortality rate, demonstrating there is a need for lymphoid immune responses to control mycoplasma respiratory infections. Reconstitution of these SCID mice with splenic lymphocytes result in similar disease compared to wild-type mice (76). Work in T cell deficient hamsters, also demonstrate that these hamster have less severe disease than hamsters with a full immune arsenal (13, 15). Both of these animal models demonstrate that T cell and B cell responses can contribute to both pathological as well as protective roles during mycoplasma respiratory disease.

Understanding the T cell environment within the lungs, and how this environment is modulated in the response to mycoplasma infection is important for identifying protective and pathological components of the immune response. The depletion of T helper cells (Th) result in less severe lung disease, demonstrating that a Th cell response contributes to disease pathology in the lung (77). To the contrary, depletion of $CD8^+$ T cell from mice, leads to an exacerbation of mycoplasma pulmonary disease. These results suggest that $CD8^+$ T cells play a unique regulatory role within mycoplasma disease in the lower respiratory tract. Our lab, as well as others, has demonstrated that the lung is a Th2 dominated environment (67, 78). However, in response to mycoplasma infections, there is a mixed mycoplasma specific Th1/Th2 response in the lung, suggesting that IFN-γ from Th1 cells is playing an important role in mycoplasma respiratory disease (77). Thus, T cell activation, and most likely the cytokines they produce, is instrumental in the pathogenesis of mycoplasma respiratory disease of the lower respiratory tract.

The generation of immune response in the upper respiratory tract (nasal passages) can contribute to the progression of mycoplasma respiratory disease. The upper respiratory tract is the initial and major site of antibody production after mycoplasma infection (75), a similar phenomenon has been shown for other infectious agents. Mice immunized for, or infected with viruses, generate a higher titer of antigen specific antibody responses in the nasal passages versus the lungs (31, 74). These studies suggest the upper and lower respiratory tracts, are immunologically separate in their response towards an infectious agent. In support, systemic immunization with *M. pulmonis* antigen can offer some protection to the lungs, but fails to protect nasal passages (76). In fact recent data, demonstrates local immunization of the upper and/or lower respiratory tracts is more effective in protection against mycoplasma disease. These studies suggest the importance as well as the difference in immune responses with in the upper and lower respiratory tracts in response to infectious agents.

IL-4 and IFN-γ are pleiotrophic cytokines that have strong immuno-modulatory roles on both innate and adaptive immune cells. Early after most infections, NK cells, γδ T cells, and NKT cells release IFN-γ (23-25). IFN-γ then activates macrophages, leading to increased cytokine secretion, and the up-regulation of nitric oxide and oxygen radicals production and secretion (79). This early source of IFN-γ is also important in directing adaptive immune responses. It causes the phenotypic maturation of Th0 cells to a Th1 phenotype, which aids macrophages in killing intracellular bacteria (26). IL-4 directs eosinophilic responses, IgE generations and Th 2 cell maturation (33). Th2 cells are important in aiding the humoral immune responses. IL-4 also plays a critical role in the

maintenance of mucosal immunity (80). Thus, IL-4 and IFN-γ are critical cytokines that are important in the modulation of innate and the generation of adaptive immune responses.

The purpose of this study is to evaluate the significance of IFN-γ and IL-4 production in controlling mycoplasma infection and the pathogenesis of disease in the upper and lower respiratory tracts. By utilizing IFN-γ knockout (KO) and IL-4 KO mice, we were able to study the contribution of these cytokines in the development of pathogenesis and/or protection in response to mycoplasma respiratory infection in both the upper and lower respiratory tracts. Studies to characterize differences in cytokine control of the upper and lower respiratory tracts have yet to be done. Information gained from these studies will give insight into the development of effective vaccines that lead to immunity of both the upper and lower respiratory tracts.

Materials and Methods

Mice. Viral- and mycoplasma-free BALB/c, IFN-γ (C.129S7(B6)-ifng^{tm1Ts} on a BALB/c background) knockout (KO) and IL-4 (BALB/c-Il4^{tm2Nnt} on a BALB/c background) KO mice were obtained from the Jackson Laboratories (79, 81) and breeding colonies were established. Mice were housed in sterile micro isolator cages supplied with sterile bedding, and sterile food and water was given, ad libitum. Mice used in the study were between 8-12 weeks of age. Female mice were used in all studies unless where noted in the results. Before experimental manipulation, mice were anesthetized with an i.m. injection of ketamine/xylazine.

Mycoplasma. The UAB CT strain of *M. pulmonis* was used in all experiments. Stock cultures were grown, as previously described (82), in mycoplasma medium and frozen in 1-ml aliquots at -80^0C. For inoculation, thawed aliquots were diluted to 10^5 CFU/20 μl. Nasal-pulmonary inoculations of 20 μl of diluted mycoplasma were given for experimental infections.

Cell Isolation. Mononuclear cells were isolated from lungs, as previously described (75, 83, 84). Lungs were fused with PBS without magnesium or calcium to minimize contamination of the final lung cell population with those from the blood. The lungs were finely minced. The tissues were suspended in RPMI 1640 medium (HyClone Laboratories, Logan, UT) containing 300 U/ml *Clostridium histolyticum* Type I collagenase (Worthington Biochemical, Freehold, NJ), 50 U/ml DNase (Sigma-Aldrich, St. Louis, MO), 10% FBS (Hyclone Laboratories), HEPES (Fisher Scientific, Pittsburgh, PA) and antibiotic/antimycotic solution (Life Technologies, Grand Island, NY). The tissues were incubated at 37^0C while mixing on a Nutator (Fisher Scientific) for 90-120 minutes. During the incubation period, the tissues were vigorously pipetted every 30 minutes. After incubation, the digestion mixture was passed through a 250-μm nylon mesh to remove undigested tissue. Mononuclear cells were purified from cell suspension by density gradient centrifugation using Lympholyte M (Accurate Chemicals, Westbury, NY).

Spleen cells were isolated after centrifugation of all suspensions, followed by red cell removal using ACK (ammonium chloride potassium) lysis buffer, as previously described (85).

Cells from the nasal passages were isolated as previously described (75). Briefly, the lower mandibles and skin were removed from the skull. The skull was longitudinally split, and the nasal passages were removed by scraping and transferred to collagenase-DNase digestion medium as used for isolation of lung cells. After 1 hour of incubation at 37°C while being mixed on a Nutator, the tissue was passed through a 250-μm nylon mesh, and the red cells were removed using ACK lysis buffer.

RNA isolation from nasal passages and lungs. Total RNA was isolated from both whole lungs and nasal passages of mice using the Ultraspec-II™ RNA Isolation System (Biotecx Laboratories, Inc. Houston, TX). Briefly, nasal passages and lungs were homogenized in the Ultraspec-II™ RNA reagent using a Pro 200 homogenizer (Pro Scientific, Monroe, CT). The RNA samples were frozen at –80°C until further isolation. Chloroform was added to the homogenate and centrifuged at 12,000 x g (4°C) for 30 minutes. The RNA was precipitated by adding isopropanol to the aqueous phase and centrifuging samples at 12,000 x g (4°C) for 10 minutes. The RNA pellet from each sample was washed twice with 75% ethanol by vortexing and subsequent centrifugation for 5 minutes at 7,500 x g and then resuspended in diethylpyrocarbonate (DEPC)-treated water. The concentration and quality of RNA in each sample was determined spectrophotometrically (GeneQuant II, Pharmacia Biotech, Piscataway, NJ.) and by gel electrophoresis. The RNA samples were stored at –80°C until ready for use.

Cytokine mRNA detection by Reverse Transcription-Polymerase Chain Reaction (RT-PCR). RT-PCR was performed using 100 ng RNA for each sample, as previously described (69). The sequences of the primers and the size of the resulting PCR

fragments (in parentheses) for IL-4, IFN-γ, and the housekeeping gene, β 2-microglobulin (β_2m) are given as follows (86): IL-4 (216 bp), 5'-CGGCATTTTGAACGAGGTC and 5'-GAAAAGCCCGAAAGAGTCTC; IFN-γ (227 bp), 5'-GCTCGAGACAATGAACGCT and 5'-AAAGAGATAATCTGGCTCTGC; and β_2m (222 bp), 5'-TGACCGGCTTGTATGCTATC and 5'-CAGTGTGAGCCAGGATATAG.

The increase in expression of cytokine mRNA after immunization was determined by the number of cycles of amplification that resulted in little or no PCR product for each cytokine in total lung RNA from sham-inoculated, control mice, as previously described (69, 87). For IFN-γ and β_2m, the samples were amplified for 30 cycles, and for IL-4, the samples were run for 35 cycles. The PCR products were separated on 1.8% agarose gels and stained with ethidium bromide. Gels were visualized using Alpha Image 2000 Documentation and Analysis System (Alpha Innotech, San Leandro, CA). The intensity of each band was determined using densitometry, and the relative cytokine mRNA reactions were compared after normalization to the housekeeping gene, β_2m.

Assessment of Gross Lesions and Histopathology. Lungs were removed, and each lobe was examined by two observers for the presence of gross lesions. The percentage of each lobe with gross lesions was recorded. The gross lesion scores were weighted by the percentage that each lobe contributes to the total lung weight in arriving at the gross lesion index for lungs (88).

Lungs and nasal passages were fixed in alcohol formalin (4% glacial acetic acid (Fisher), 6% formaldehyde solution (Fisher), 40% Di water , and 50% of 95% ethanol),

nasal passages were demineralized in acid decalcifying solution (Richard Allan Scientific, Kalamazoo, Michigan). Tissues were embedded in paraffin, sectioned at a thickness of 5μm, and stained with hematoxylin and eosin for light microscopy. Each lung lobe was sectioned separately, randomly coded, and subjectively scored at University of Alabama Birmingham for lesion severity (scale of 0 to 4) on the basis of the characteristic lesions of murine respiratory mycoplasmosis as described previously (89). Scores refer to (i) peribronchial and –vascular lymphoid hyperplasia or infiltration (peribronchial infiltrate), (ii) mixed neutrophilic and histiocytic exudate in alveoli (alveolar exudate), (iii) neutrophilic exudate in airway lumina (airway exudate), and (iv) hyperplasia of airway mucosal epithelium (epithelial) (89). A score for each lesion was weighted according to the percentage each lobe contributes to the total lung weight in arriving at a total lesion score for each set of lungs. For each of the four lesions, a lesion index was calculated by dividing the observed lesion score by the maximum lesion score possible. Thus, the maximum lesion index possible for any lesion was 1.0.

Characterization of Mycoplasma Numbers. The numbers of mycoplasma CFU in lungs and nasal passages were determined as previously described (90, 91). Briefly, lungs were minced and placed in mycoplasma broth medium. Nasal washes were collected with 1 ml of mycoplasma broth medium that was forced through the nasal passages of mice by inserting a syringe into the soft pallet. In some experiments, we also isolated nasal passage tissue for CFU determination. The samples were sonicated (Vibra cell sonicator; Sonics & Materials/Vibro Cell) for 2 min. at 50 amplitudes without pulsing. After sonication, serial dilutions (1:10) were prepared, and 20 μl of each

dilution was plated onto mycoplasma agar medium. After 7 days of incubation at 37^{0}C, the colonies were counted, and the CFU recovered from each tissue was calculated.

***M. pulmonis* Specific Antibody Levels.** To prepare antigen for ELISA, *M. pulmonis* was cultured at 37^{0}C in mycoplasma broth medium for 3 days and harvested. *M. pulmonis* broth was adjusted to 5 mg/ml protein concentration. Twenty ml of lysis buffer (4.2 g $NaHCO_3$/L and Na_2C0_3/L) pH 10.0 warmed to 37^{0}C were added to each 1 ml of *M. pulmonis* stock and incubated at 37^{0}C fro 15 minutes. Then 2.2 g of boric acid were added to 100 ml of lysis buffer and then frozen at –70^{0}C. Protein concentration was then determined by Bradford assay.

Falcon Microtest III assay plates (Becton Dickinson, Oxnard, Calif.) were coated with optimal concentrations of *M. pulmonis* antigen (100 µl at 10µg/ml) in PBS. After overnight incubation at 4°C, the plates were washed three times with PBS-0.05% Tween 20 and blocked with PBS-0.05% Tween 20 supplemented with 10% FBS overnight at 4^{0}C. Serum samples were initially diluted 1:100 and then serially (1:2) diluted with PBS-0.05% Tween 20-10% FBS, and 100 µl was placed in duplicate into wells of the antigen-coated plates. After overnight incubation at 4°C, the plates were washed four times with PBS-0.05% Tween 20. Secondary Ab (biotinylated anti-mouse Ab stock reagents of 0.5 mg/ml; Southern Biotechnology Associates, Inc., Birmingham, Ala.) were diluted 1:2000 (IgA, IgM, or IgG) or 1:500 (IgG_1 or IgG_{2a}) in PBS-0.05% Tween 20-10% FBS, and 100 µl were added to the appropriate wells. After overnight incubation at 4^{0}C, the plates were again washed four times with PBS-0.05% Tween 20, and a 1:2,000 dilution of horseradish peroxidase (HRP)-conjugated streptavidin (Neutralite avidin; Southern

Biotechnology Associates) in PBS-0.05% Tween 20-10% FBS was added to the wells (100 µl). The plates were incubated at room temperature for 2 h, and the plates were washed twice with PBS-0.05% Tween 20 and twice with PBS. The reaction mixtures were developed at room temperature by addition of 100 µl of 3,3'5,5'-tetra methylbenzidine peroxidase substrate (Moss Inc. Pasadena, Maryland) in each of the wells. Plates were read using MX80 plate reader (Dynatech, Chantilly, VA) at an absorbance of 630 nm. Endpoint Ab titers were expressed as the reciprocal dilution of the last dilution that gave an optical density (OD) at 630 nm of 0.1 U above the OD of negative controls after a 20-min incubation.

To detect antigen-specific IgA antibodies in nasal washes, samples were serially diluted 1:2 in PBS containing 10% FBS and added to the appropriate wells of antigen coated plates. The reactions were developed as for serum samples.

Cell Characterization By Flow Cytometry. Three-color immunofluorescent staining was preformed to identify T cell populations using FITC-labeled anti-murine CD4 mAb (L3T4, RM4-5; BD PharMingen, San Diego, CA), PCB-labeled anti-murine CD8 mAb (Lyt-2, 53-6.7; BD PharMingen), and PE-labeled anti-murine CD3 mAb (KT3; Beckman Coulter). PE-labeled anti-murine F4/80 mAb (F4/80; Caltag Laboratories, Burlingame, CA) was used to identify macrophages, and PE-labeled anti-murine B220 mAb (RA-6B2; Beckman Coulter) was used to identify B cells. Briefly, 10^6 cells per tube were incubated for 30 min at 4^0C in 100 µl of optimal concentration of

fluorescent Ab. The cells were washed in staining buffer [Mg^{2+}-free, Ca^{2+}-free PBS with 0.05% sodium azide, 1% FBS] and fixed with 2% paraformaldehyde in PBS for 30 min. After fixation, cells were resuspended in staining buffer for analysis.

The cells were analyzed using an EPICS XL-MCL flow cytometer (Beckman Coulter). Data collection was done using System 2 software (Beckman Coulter). Cell population gates and detector voltages were set using isotype stained (control) lung and splenic cells. The proportion of each cell population was expressed as the percentage of the number of stained cells. To determine the total number of a specific cell population, their percentages were multiplied by the total number of cells isolated from that tissue.

Statistical Analysis. Data was evaluated by ANOVA, followed by Fisher Protected Least Square Differences Multigroup comparison. These analyses were performed using the StatView (SAS Institute, CARY, NC) computer program. When appropriate, data was logarithmically transformed prior to statistical analysis, and confirmed by a demonstrated increase in power of the test after transformation of the data. A P value ≤ 0.05 was considered statistically significant. If data was analyzed after logarithmic transformation, the antilog of the means and standard errors of transformed data was used to present the data and are referred to as the geometric means (x/÷ standard error)

Results

Upper and lower respiratory tracts differ in their relative expression of IL-4 and IFN-γ mRNA after mycoplasma infection. To determine if there are qualitative

differences between upper and lower respiratory tracts in their cytokine expression after mycoplasma infection, IL-4 and IFN-γ mRNA expression was measured in the nasal passages and lungs 14 days post-infection using RT-PCR. After RT-PCR, the primers generated fragments of the predicted size.

Nasal passages expressed higher relative levels of IL-4 mRNA than IFN-γ mRNA after infection, while the lungs had a mixed expression of both cytokine mRNA's at day 14 post-infection, while uninfected control mice showed no detectable levels of mRNA for IFN-γ and IL-4 in both the nasal passages and the lungs (Fig 1). At 14 day post-infection, IL-4 and IFN-γ mRNA levels were increased, but mRNA expression for IL-4 was significantly greater relative to IFN-γ in the nasal passages. However, the lungs of mycoplasma infected animals at day 14 showed little shift in the relative expression of IL-4 and IFN-γ although, the levels were higher than naive mice.

Thus, there were relative differences in IL-4 and IFN-γ mRNA expression levels between nasal passages and lungs in mycoplasma infected mice. Thus, IL-4 mRNA levels are significantly increased, relative to IFN-γ mRNA levels in the nasal passages, as compared to lungs. This suggests a difference in the contribution of these cytokines in response to *M. pulmonis* disease in upper and lower respiratory tracts.

IFN-γ KO mice have more severe mycoplasma disease in the lungs than control mice. The previous study indicated that there was a difference in the contribution of IL-4 and IFN-γ responses in nasal passages and lungs of mice with mycoplasma

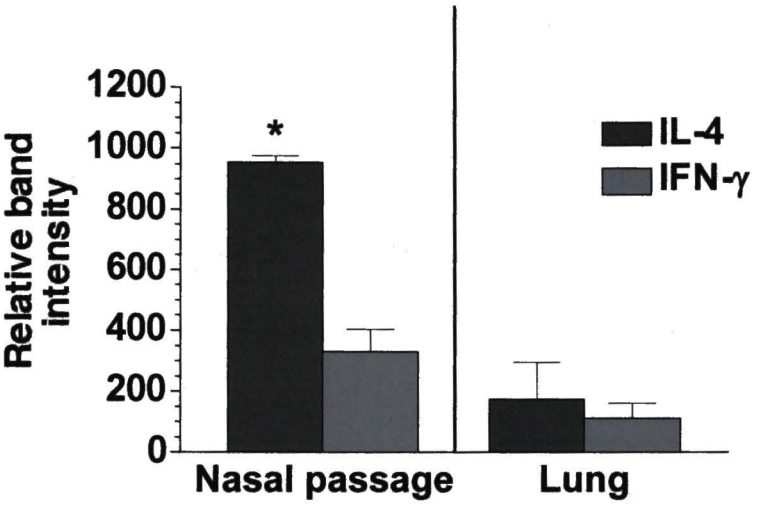


Figure 1. Cytokine mRNA expression in the respiratory tract after infection. Fourteen days following mycoplasma infection, lungs and nasal passages were removed, and total RNA was isolated. IL-4 and IFN-γ cytokine mRNA levels were measured in each tissue by using RT-PCR. Relative differences in mRNA expression of cytokines were determined by the relative increase in the ratio of cytokine to the house keeping gene β_2mGL. Vertical bars and error bars represent mean ± SE (n=6). "*" denotes statistical difference ($p \leq 0.05$) from all other groups.

respiratory disease. To determine the effect of IFN-γ and IL-4 on mycoplasma disease pathology, age matched IFN-γ KO and IL-4 KO mice were experimentally infected with *M. pulmonis*, and lungs and nasal passages were collected on days 3, 7, 10, and 14 post-infection. Lungs were first scored for the presence of gross lesions, then lungs and nasal passages were prepared in alcohol formalin for histological staining to determine disease pathology.

IFN-γ KO mice developed more severe mycoplasma disease than IL-4 KO or BALB/c (control) mice. Clinical signs of disease (lethargy and ruffled fur) became apparent in IFN-γ KO mice at a much earlier time point after infection (day 3 to 7), while IL-4 KO and control mice did not show signs of disease until day 10 to day 14. Consistent with clinical disease, IFN-γ KO mice had significantly higher pulmonary gross lesion scores by day 7 than control mice and continued through day 14 (Fig. 2). IL-4 KO mice, on the other hand, showed comparable lung gross lesion scores to control mice through day 10. However, by day 14 there was a trend for lower gross lesion scores in the lungs of IL-4 KO mice than in control mice.

To determine if the type of pulmonary lesions and disease severity was affected by cytokine deficiencies, lungs and nasal tissues were collected for histopathology from mycoplasma infected cytokine KO and normal mice. By day 3 post-infection, there were significantly higher scores in neutrophilic exudate and alveolitis in the lungs of IFN-γ KO mice, and on day 7 through day 14 post infection, all four histological scores (Airway Exudate, Alveolitis, Epithelial, and Peribronchial Infiltrate) were significantly higher in

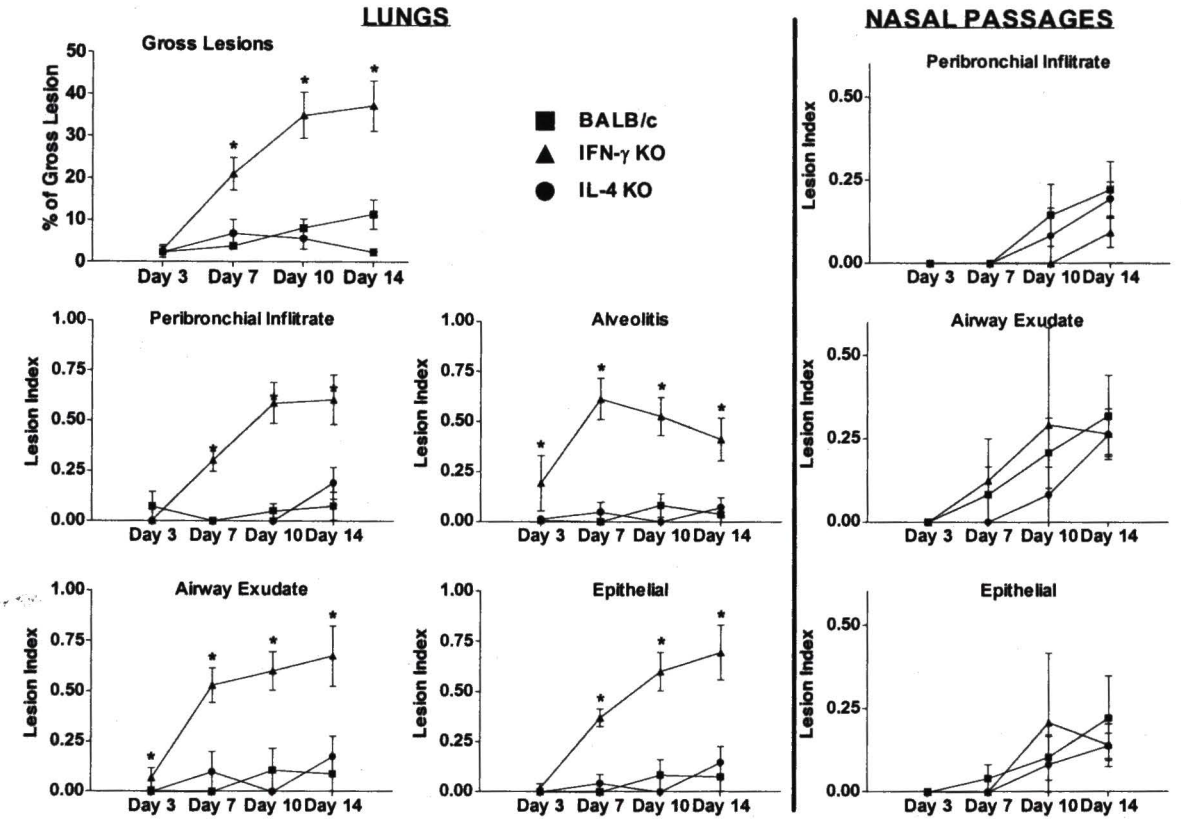


Figure 2. Lesion severity of lungs and nasal passages after infection. At days 3, 7, 10 and 14 after mycoplasma infection lungs and nasal passages were removed. Lungs were scored for % of gross lesion, while both lungs and nasal passages were formalin fixed for histology. Lesion index scores refer to (i) peribronchial and –vascular lymphoid hyperplasia or infiltration (peribronchial infiltrate) (ii) mixed neutrophilic and histiocytic exudate in alveoli (alveolitis), (iii) neutrophilic exudate in airway lumina (airway exudate), and (iv) hyperplasia of airway mucosal epithelium (epithelial). Mice in these studies were male and female. Vertical bars and error bars represent mean ± SE (n=6). "*" denotes statistical difference ($p \leq 0.05$) from BALB/c mice.

IFN-γ KO mice than in corresponding controls (Fig 2). IL-4 KO mice did not show any significant difference at any time point from control animals. Histological lesion scores of the nasal passages show no significant difference in any strain at any time point (Fig 2). Thus, IFN-γ KO mice, have more severe disease in the lungs than control or IL-4 KO mice.

IFN-γ KO mice have higher numbers of mycoplasma in the lungs than control mice. To determine how the loss of IFN-γ and IL-4 affects colonization of mycoplasmas in the lower and upper respiratory tracts, IFN-γ KO, IL-4 KO and corresponding BALB/c control mice were experimentally infected with *M. pulmonis*. On days 3, 7, 10, and 14 after infection, nasal washes and lungs were collected, and the number of mycoplasma CFU were determined in upper and lower respiratory tracts.

The IFN-γ KO, but not IL-4 KO mice, had significantly higher numbers of mycoplasmas in their lungs than control mice. By day 3 post-infection, the number of mycoplasma CFU in the lungs of IFN-γ KO mice was almost two logs higher than corresponding control mice (Fig 3.). By day 14, IFN-γ KO mice still had almost one log higher mycoplasma CFU in the lung than control mice. IL-4 KO mice on the other hand, tended to have a lower mycoplasma CFU burden within the lungs at days 7 and 10 post-infection; however, the lower CFU numbers in the lungs of IL-4 KO mice were not significantly different from control mice. On day 14 post-infection, mycoplasma CFU burden in the lungs was comparable between IL-4 KO and control mice.

Within the upper respiratory tract, the loss of either IFN-γ or IL-4 did not affect mycoplasma CFU numbers. This is in stark contrast to the lungs where IFN-γ is critical

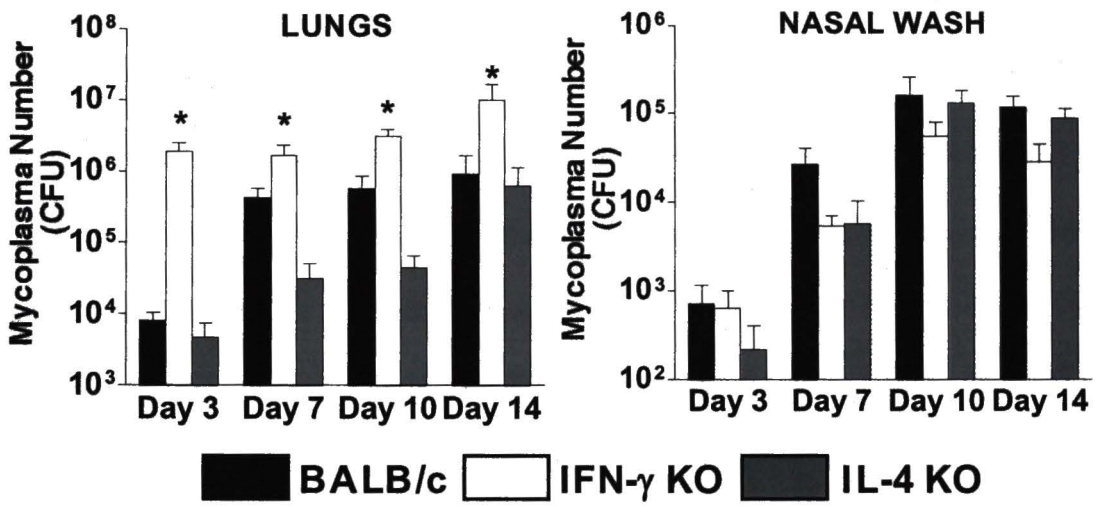


Figure 3. Mycoplasma CFU in respiratory tract after intranasal infection. BALB/c, IL-4 KO and IFN-γ KO mice were infected with *M. pulmonis*. At days 3, 7, 10 and 14 post-infection, the number of mycoplasma CFU in lungs and nasal passages (nasal wash) were determined. Mice used in these studies were male and female. Vertical bars and error bars represent mean ± SE (n=9). "*" denotes statistical difference ($p \leq 0.05$) from BALB/c mice.

for controlling mycoplasma CFU numbers. At all time points from day 3 to day 14 post-infection, there was no significant difference in CFU numbers obtained from nasal washes between IFN-γ KO, IL-4 KO, and control mice (Fig. 3). To ensure that the lack of differences was not due to the use of nasal washes for sampling, we collected nasal passage tissue in cytokine-deficient and control mice 14 days after infection. As with nasal washes, there were no significant differences in numbers of mycoplasma CFU recovered from the nasal passage tissue between the groups of mice (data not shown).

IFN-γ KO mice have higher levels of mycoplasma-specific serum IgG than control mice. To determine the generation of antibodies in response to *M. pulmonis* in the absence of IFN-γ and IL-4, mycoplasma-specific antibody levels were measured in IFN-γ KO and IL-4 KO mice infected with *M. pulmonis*. Serum was collected from control, IFN-γ KO and IL-4 KO mice on days 3, 7, 10 and 14 post infection, and the levels of *M. pulmonis* antigen specific IgA, IgM, IgG, IgG_1, and IgG_{2a} levels were determined. Nasal washes were also collected at day 14 post-infection, and mycoplasma-specific IgA titers were measured.

There were differences in Ab responses that developed in cytokine deficient mice in response to mycoplasma infection. At early time points (day 0 and 3), no detectable levels of any *M. pulmonis*-specific Ab classes were found in sera from the three mouse strains (Table I). At day 7, mycoplasma-specific Ab from all classes and subclasses were detectable, but there was no significant difference in the titers of any of the Ab classes between the three strains at this time point. By day 10, there was significantly higher titers of IgG, IgG_1, and IgG_{2a} in the sera of mycoplasma-infected IFN-γ KO mice, as

Table I. Comparison of mycoplasma-specific antibody response after infection[a].

		Mouse Strain		
Isotype[b]	**Day[c]**	**BALB/c**	**IFN-γ KO**	**IL-4 KO**
IgA	**3**	0(0)[d]	0(0)	0(0)
	7	885(1.3)	800(1.2)	800(1.1)
	10	1,233(1.3)	993(1.2)[e]	993(1.2)
	14	21,528(1.1)	27,825(1.2)	30,409(1.1)
IgM	**3**	0(0)	0(0)	0(0)
	7	2,075(1.7)	1,466(1.6)	1,466(1.5)
	10	2,382(1.5)	5,309(1.3)[e]	1,986(1.5)
	14	46,989(1.1)	46,989(1.1)	51,168(1.0)
IgG	**3**	0(0)	0(0)	0(0)
	7	475(1.4)	437(1.2)	337(1.2)
	10	497(1.2)	1,656(1.5)[e]	325(1.2)
	14	6,982(1.3)	28,708(1.3)[e]	4,932(1.4)
IgG1	**3**	0(0)	0(0)	0(0)
	7	337(1.3)	308(1.2)	218(1.2)
	10	497(1.2)	1,656(1.5)[e]	325(1.2)
	14	4,932(1.3)	19,724(1.5)[e]	4,932(1.4)
IgG2a	**3**	0(0)	0(0)	0(0)
	7	113(1.3)	104(1.6)	100(1.3)
	10	101(1.5)	3,097(1.2)[e]	993(1.2)
	14	2,075(1.2)	7,603(1.8)[e]	8,299(1.2)[e]

[a] BALB/c, IL-4 KO and IFN-γ KO mice were intranasally *M. pulmonis*. At days 3, 7, 10 and 14 post-Infection, serum was collected and antibody titers were determined by ELISA.

[b] mycoplasma-specific antibody responses

[c] days post-infection when serum was collected

[d] geometric mean x/÷ (standard error) N=12

[e] denotes significant difference ($p \leq 0.05$) from BALB/c

compared to control mice. In contrast, no differences were seen in sera titers of mycoplasma-specific IgM or IgA in IFN-γ KO mice as compared to control mice. These differences in Ab responses in IFN-γ KO mice at day 10 were also seen at day 14. IL-4 KO mice, on the other hand, had serum titer in all Ab classes comparable to that of control mice at days 7 and 10. By day 14, there were significantly higher titers of mycoplasma-specific IgG_{2a} in IL-4 KO than in control mice, although the overall levels of IgG were not significantly different from control mice.

Unlike the sera, IL-4 KO mice had significantly higher titer levels of mycoplasma-specific IgA in the upper nasal passages than control or IFN-γ KO mice (Fig. 4). By day 14 post-infection, IL-4 KO mice had one log higher titer of mycoplasma-specific IgA levels in the nasal passages than that of control or IFN-γ KO mice. Thus, IFN-γ KO mice had higher titers of mycoplasma-specific serum IgG Ab (and subclasses) responses than the control mice; while IL-4 KO mice had a shift in IgG responses from a mixed IgG_1 and IgG_{2a} mycoplasma-specific response, as seen in control, to a predominate IgG_{2a} antigen specific response.

IFN-γ KO mice have lower numbers of lymphocytes and macrophages in the lungs at day 14-post infection than control mice. To determine if lymphocytes or macrophages contribute to the differences in disease severity, BALB/c, IL-4 KO and IFN-γ KO mice were experimentally infected with *M. pulmonis*, and at 14 days post infection, cells were isolated from the lungs. Lung cells were stained with fluorescent Ab specific for CD3, CD4, CD8, B220, and F4/80 and analyzed by flow cytometry.

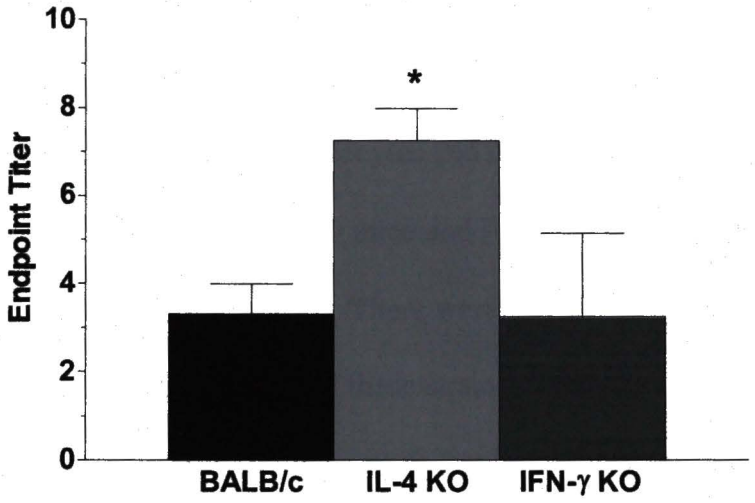


Figure 4. Mycoplasma-specific nasal IgA responses after intranasal infection. BALB/c, IL-4 KO and IFN-γ KO mice were intranasally inoculated with *M. pulmonis*. Nasal wash samples were collected 14 days after infection, and endpoint titers of mycoplasma-specific IgA were determined by ELISA. The data were collected from three experiments (n=12), and expressed as the means +/- standard error. "*" denotes statistical difference ($p \leq 0.05$) from BALB/c mice.

IFN-γ KO mice generated a different cellular response against mycoplasma infection in the lungs than seen in IL-4 KO and control mice. To determine if there were differences in resident pulmonary lymphocytes and macrophages, lungs cells were collected from naïve BALB/c, IFN-γ KO mice and IL-4 KO mice, and lymphocyte and macrophage populations were analyzed. There were no differences in either numbers or percentages of cell populations in any of these strains of naïve mice (data not shown). However, after 14 days of infection, there were significantly fewer numbers of cells isolated from the lungs of IFN-γ KO mice, than in lungs of control or IL-4 KO mice (Fig 5). There were also significantly lower numbers of T cell populations ($CD4^+$ Th and $CD8^+$ T cells) and macrophages ($F4/80^+$), present in mycoplasma infected IFN-γ KO lungs, than in control mice lungs. Interestingly, the percentage of each lymphocyte population in the lungs of IFN-γ KO mice was comparable to control mice; however, the percentage of macrophages was significantly lower in the lungs of IFN-γ KO mice than that seen in control mice (data not shown). IL-4 KO mice, on the other hand, showed no differences from control mice in the number of B cells, T cells or macrophages in the lungs after mycoplasma infection.

Mycoplasma infected IL-4 KO mice have higher numbers of $CD8^+$ T cells and macrophages in nasal passages at day 14 than control mice. To determine if lymphocyte or macrophage populations were affected by cytokine deficiencies in the upper respiratory tract, we similarly examined the cell populations of nasal passages from naïve and mycoplasma infected BALB/c, IL-4 KO and IFN-γ KO mice.

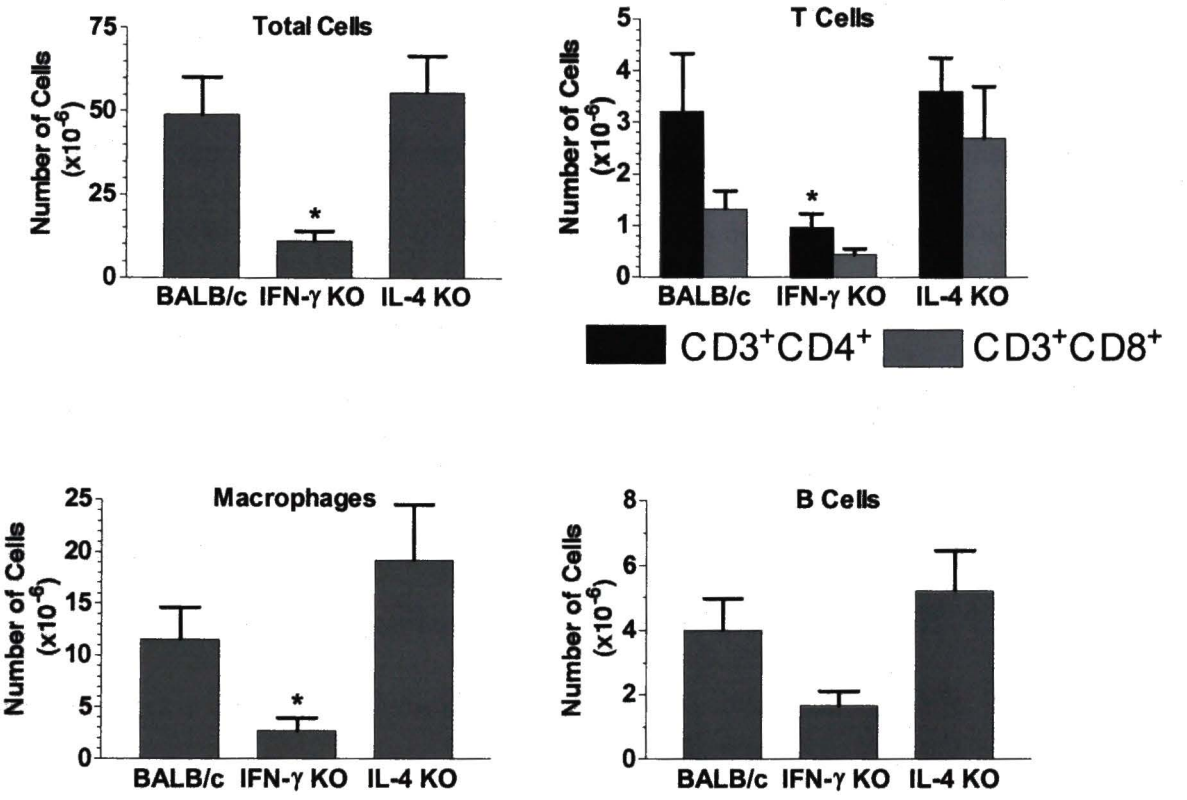


Figure 5. Numbers of immune cells in lungs after infection. BALB/c, IL-4 KO and IFN-γ KO mice were intranasally inoculated with *M. pulmonis*. At day 14 post-infection, lung leukocytes were isolated, and cell numbers were determined. The number of $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, $B220^+$ B cells, and $F4/80^+$ macrophages populations were determined using flow cytometry. Vertical bars represent mean ± SE (n=11). "*" denotes statistical difference ($p \leq 0.05$) from BALB/c mice.

Naïve IL-4 KO, IFN-γ KO and BALB/c mice had similar numbers of $B220^+$ B cells, $CD3^+$ T cells, and $F4/80^+$ macrophages residing in the upper respiratory tract. By day 14 post-infection, there were significantly more cells (approximately a million)isolated from IL-4 KO mice than from control or IFN-γ KO mice (Fig 6). Higher numbers of $CD3^+$ T cells (specifically $CD8^+$ T cells) and macrophages were found in IL-4 KO than in BALB/c or IFN-γ KO mice, while there were no difference in the numbers of B cells isolated from these mice. IFN-γ KO mice did not differ from control mice in numbers of T cells, B cells, or macrophages found in the nasal passages at day 14 post-infection. Thus, IL-4 KO mice have an increased number of macrophages and $CD8^+$ T cells in the nasal passages at day 14 post-infection as compared to control mice, while, IFN-γ KO mice have no differences.

Discussion

The purpose of this study was to determine the importance of IFN-γ and IL-4 in the upper and lower respiratory tracts after mycoplasma infection. IFN-γ and IL-4 are pleiotrophic cytokines that help to direct innate and adaptive immune responses (24, 80, 92-94). These cytokines are also important in the phenotypic development of lymphoid responses, as IFN-γ promotes a cell-mediated response while, IL-4 promotes a humoral response (26). Lymphoid responses are critical in mycoplasma lower respiratory tract disease, as they play both protective and pathological roles; however, immune responses of the upper respiratory tract are unknown (11-15). Studies in the upper respiratory tract do suggest that the nasal passages have a separate and distinct immune response from the lower respiratory tract (74-76, 91). Though unexplored in the upper respiratory tract, T

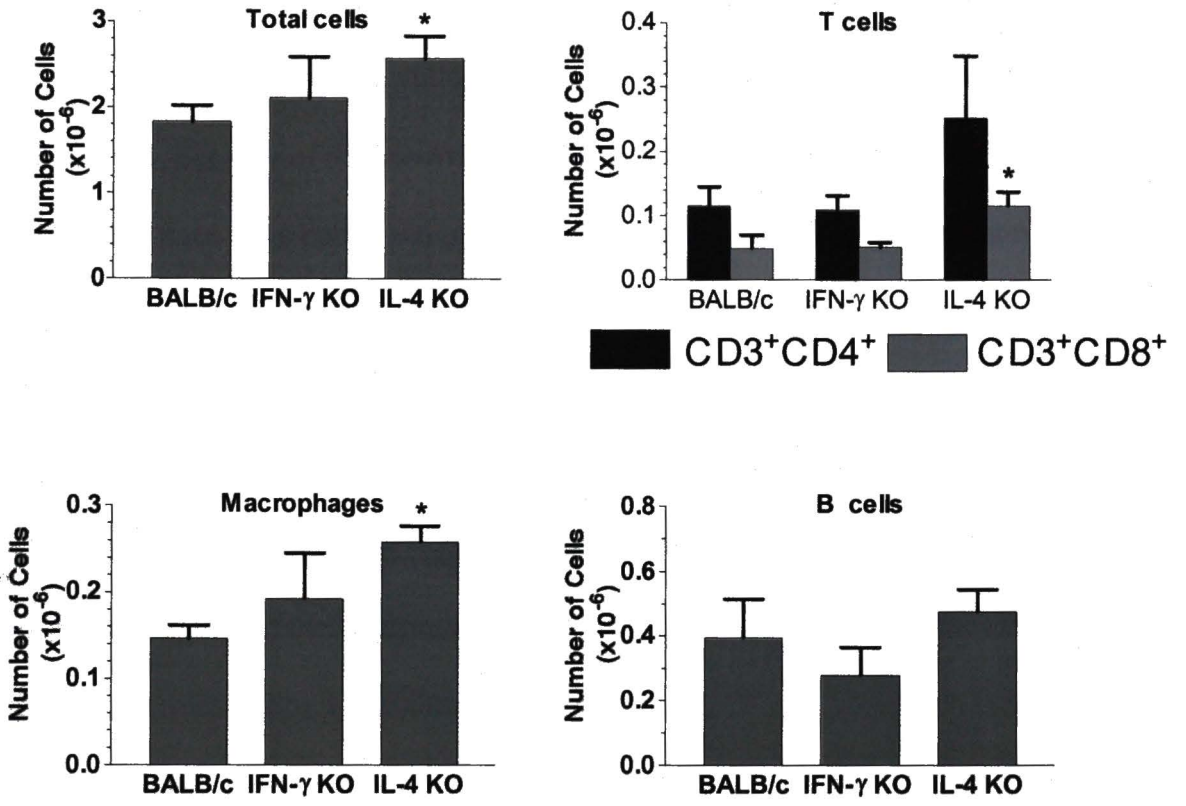


Figure 6. Number of immune cells in nasal passages after infection. BALB/c, IL-4 KO and IFN-γ KO mice were intranasally inoculated with *M. pulmonis*. At day 14 post-infection, nasal passage leukocytes were isolated, and cell numbers were determined. The number of $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, $B220^+$ B cells, and $F4/80^+$ macrophages populations were determined using flow cytometry. Vertical bars represent mean ± SE (n=11). "*" denotes statistical difference ($p \leq 0.05$) from BALB/c mice.

cells play a multifaceted role within mycoplasma lung disease. The depletion of $CD8^+$ T cells exacerbates lung disease, while depletion of $CD4^+$ T cells decreases lung disease severity (77). Because of the known roles of IFN-γ and IL-4 in Th cell maturation, IFN-γ and IL-4 KO mice have been used in a variety of disease models to begin examining the role of Th cell subsets (95-99), and the use of IFN-γ and IL-4 KO mice in these studies will address similar questions during mycoplasma infection. However, the roles of IFN-γ and IL-4 mediated cellular responses within mycoplasma respiratory disease have yet to be determined. Given the differences in upper and lower respiratory tract immunity, an in-depth description of these immune mediators within both compartments, will shed light on their roles during mycoplasma disease.

The importance of IFN-γ and IL-4 in controlling mycoplasma infections and disease is different between upper and lower respiratory tracts. The loss of either cytokine in the upper respiratory tract did not affect disease pathogenesis or the immune system's ability to control mycoplasma growth. This is contrast to the lungs, where the loss of IFN-γ results in more severe disease and a two-log increase in mycoplasma CFU. In support of differences in upper and lower respiratory tract immunity, several studies have demonstrated the upper respiratory tract is in a separate compartment of the immune system from the lung (31, 74, 75). The lung is protected from mycoplasma infection by nasal-pulmonary or by systemic immunization with or without an adjuvant, while the nasal passages are protected only with nasal immunizations that contain an adjuvant (76, 91). These studies demonstrated differences in upper and lower respiratory tract immune responses; however, we are unaware of any studies that look at differences in the

importance of cytokines in the upper and lower respiratory tracts in an infectious disease model. Our data demonstrates that the loss either IL-4 or IFN-γ does not affect disease pathology or controlling mycoplasma growth in the upper respiratory tract, while IFN-γ is critical in dampening disease pathology and mycoplasma growth within the lower respiratory tract.

Although the loss of IL-4 does not affect disease pathogenesis or mycoplasma growth in the upper respiratory tract, it does lead to a change in immune response. The fact that the loss of IL-4 does not affect disease pathogenesis was surprising as IL-4 mRNA relative levels were significantly higher than IFN-γ mRNA isolated from the nasal passages of mycoplasma infected mice. As opposed to, the relative levels of IL-4 mRNA and IFN-γ mRNA, which were equivocal in the lungs after infection, only the loss of IFN-γ lead to higher disease severity in the lungs. Though there was no difference in disease, the IL-4 KO mice appear to develop compensatory immune responses that included higher titers of mycoplasma-specific IgA, and higher numbers of $CD8^+$ T cells and macrophages. This increase in mycoplasma-specific IgA may be contributing to IL-4 KO mice being able to control mycoplasma upper respiratory tract infection. This would coincide with data that suggests that higher IgA levels play a protective role in *M. pneumoniae* infections (100, 101). Also, the increase in $CD8^+$ T cells and macrophages, which our and other labs have demonstrated to be critical cells in controlling mycoplasma disease and numbers (70, 73, 77, 102), may be masking any detrimental effects on upper respiratory tract immunity due to the loss of IL-4. Thus, IL-4 is likely an important contributor to the development of immunity in the upper respiratory tract towards

mycoplasma infection; however, the development of compensatory immune mechanisms masked the impact of IL-4 deficiencies on mycoplasma disease and infection. Further studies are needed to evaluate the contribution of IL-4 and related cytokines in upper respiratory tract immunity (96), as well as understanding compensatory mechanism which can replace or overcome IL-4 mediated immunity.

IFN-γ, but not IL-4, is critical in controlling the level mycoplasma infection within the lower respiratory tract. IFN-γ KO mice had a two-log higher number of mycoplasma CFU than control or IL-4 KO mice in the lungs by day 3 post-infection, and this trend of higher CFU continued up to day 14 post-infection. The higher number of mycoplasma at 3 days after infection suggests that innate immune mechanism's that clear mycoplasma from the lung in control mice are impaired in the absence of IFN-γ. This impairment subsequently contributes to the increase in disease severity in IFN-γ KO mice. In support that innate and not adaptive immune mechanisms are contributing to this phenomenon, there were no detectable mycoplasma-specific antibody responses at this time point in any group of mice, demonstrating little to no B cell activity at day 3 post-infection. Work in our lab demonstrated that T cell responses are not seen until day 7 post-infection, further supporting that no adaptive immune response are substantially activated at 3 days after infection (68, 77). Therefore, higher numbers of mycoplasma in the lung by this early time-point is likely due to impairment in innate immunity due to the absence of IFN-γ. These studies demonstrate the importance of IFN-γ; they do not however, identify the cell(s) critical in releasing IFN-γ at this early time point. IFN-γ can be released by NK, NKT, and γδ T cells early after infections with other infectious

agents, which activates macrophages and affects adaptive immunity (22-26, 92). In ongoing studies, we demonstrate that NK cells are the major cell population that increased intracellular IFN-γ in response to mycoplasma infection within the first 3 days after infection (manuscript in preparation). The loss of NK cell derived IFN-γ could impact upon macrophage activation, in which macrophages would be unable to kill mycoplasma (71, 72). However, ongoing studies from our laboratory demonstrate that the loss of NK cell-derived IFN-γ is not the cause of increased mycoplasma CFU, but instead NK cells in an IFN-γ deficient environment have activities that interfere with normal clearance mechanisms, presumably mediated by macrophages. Thus, IFN-γ plays a complex but instrumental role in the development of an effective innate immune response towards mycoplasma pulmonary infection.

IFN-γ does not affect innate immune responses solely, but likely contributes to the development of adaptive immune responses that reduce mycoplasma disease pathology. In the present studies, we found that IFN-γ KO mice had increased lung lesion severity compared to other mouse strains, and there was clearly a change in the contribution of lymphocytes and macrophages to the pulmonary inflammatory lesions in IFN-γ KO mice. In contrast to expectations, there were lower numbers of T cell (4-fold) and macrophage (4-fold) populations in the lungs of IFN-γ KO mice at day 14 post-infection. This decrease in the number of macrophages and T cells suggests that there is a change in the recruitment of cells into the site of infection. However, on-going studies show that at day 3 post-infection there is an increase in the numbers of neutrophils and associated chemokines (i.e. KC) in bronchial alveolar lavages of IFN-γ KO mice, and at later time

points, the larger contribution of neutrophils to the inflammatory lesions is likely to continue. This altered inflammation may be in part due to a change in the types of chemokines secreted by macrophages in an IFN-γ KO mice, as demonstrated in recently published work (97). Although this increase in disease severity may in part be due to the higher numbers of organisms, SCID mice, which are deficient in lymphocytes, develop little to no pulmonary disease at 14 days after infection (12, 15). These studies demonstrate, that lymphoid responses are critical mediators of the characteristic chronic inflammatory lesions in the lungs of mycoplasma infected mice. Therefore, the lymphocytes present in IFN-γ KO mice, though decreased in numbers, are likely more pathogenic than the adaptive immune response generated in control mice. This suggests that a shift from a mixed Th1/Th2 cell response (seen in control mice) in lungs toward a Th2 dominated response (seen in IFN-γ KO mice) contributing to the lesion severity (67). In support, pulmonary lymphocytes from 14 day post infected IFN-γ KO mice have enhanced (3-fold increase) IL-5 production in response to *in vitro* stimulation with mycoplasma antigen, demonstrating that there is indeed a shift in T cell responses (data not shown). Importantly, ongoing studies from our laboratory demonstrate that immunized IFN-γ KO mice develop much more severe disease than un-immunized, infected IFN-γ KO mice (Unpublished data). These results are consistent with pulmonary Th2-type responses generated in the mycoplasma infected IFN-γ KO mice, contributing to the more severe lung disease seen in the absence of IFN-γ. Thus, it is clear that there are changes in the type of inflammatory lesions in IFN-γ KO mice, and further studies are

needed to confirm that the severity of these lesions are mediated by Th2-type cell responses against mycoplasma.

The immune mechanisms examined in this study will provide a strong foundation for further studies into murine mycoplasma respiratory disease, and be beneficial in the understanding of detrimental effects of immune-mediated *M. pneumoniae* infections in humans. In summary, the present study provides insight into the potential mechanisms of immunity involved in the pathogenesis of mycoplasma respiratory disease. To our knowledge, this is the first study to examine the involvement of cytokines in the pathogenesis of mycoplasma disease in the upper and lower respiratory tracts. We demonstrated that the upper and lower respiratory tracts differ in the contribution of IL-4 and IFN-γ in the pathogenesis of murine mycoplasma respiratory disease. Based on our results, there are likely multiple (IL-4 and non-IL-4 mediated) immune mechanisms impacting the responses against mycoplasma infection in the upper respiratory tract. In contrast, IFN-γ production in the lung is clearly critical in developing beneficial innate and adaptive immune responses to control infection and inflammatory lesions. Most notably, we believe NK cells, in the absence of IFN-γ promote activities that are detrimental to the normal clearance of mycoplasma. Although these IFN-γ deficient NK cell-mediated activities influence disease progression, we also believe that results from the current and previous (77) studies indicate that mycoplasma-specific Th2 cell responses in the lung contribute to the development of immunopathologic reactions. In support, studies using other disease models demonstrate that Th2 mediated pulmonary responses can lead to more severe respiratory disease (38, 52, 103, 104). In mycoplasma

infections, the ability to generate a Th1 cell-mediated lung immune response may provide more protection against pulmonary infection (91). Additionally, as similar mechanisms may be present in other chronic respiratory diseases, we believe these studies will also yield insights to a greater understanding of respiratory disease pathogenesis as a whole, and by understanding the cytokine requirements in upper and lower respiratory tract immunity and how they differ, they will facilitate the development of nasal delivered vaccine strategies that can generated protective immunity along the entire respiratory tract.

CHAPTER III

Work in the previous chapter clearly showed a difference between cytokine requirements between upper and lower respiratory tract immune responses towards mycoplasma infection. IL-4 KO mice did not show any significant differences in the ability to clear mycoplasma or disease severity in the upper or lower respiratory tracts. The work does demonstrate that there is a compensatory immune response that is generated in the upper respiratory tract. However, the immune response generated is no more equipped to deal with mycoplasma infection than control mice, as there is no difference in disease. This compensatory immune response may be masking any detrimental effects that the loss of IL-4 has on upper respiratory tract immunity. Interestingly, the loss of IFN-γ leads to a significant increase in mycoplasma organisms and disease severity over BALB/c mice. However, the loss of IFN-γ did not impact the upper respiratory tract immune response towards mycoplasma infection. This work demonstrates that IFN-γ is important in the lower respiratory tract, while IL-4 affects the type of immune response generated in the upper respiratory tract.

The loss of IFN-γ impacted innate immune responses of the lung towards mycoplasma infection. The loss of IFN-γ leads to a two-log increase in mycoplasma organisms by day 3 post-infection. The first signs of an increase in disease severity were demonstrated at this time point as increases in alveolitis and airway exudate are seen. By day 7 gross lesions were significantly higher in IFN-γ KO mice. Furthermore, no mycoplasma specific antibody responses were detected by day 3 post-infection. This work brings forth several new questions. The biggest is the mechanisms by which IFN-γ is impacting the development of innate immune responses toward mycoplasma in the lung. My hypothesis for the next set of studies was that NK cells were releasing IFN-γ that activated macrophages, allowing them to kill mycoplasma. With the loss of IFN-γ, macrophages would not be properly activated and would be unable to kill the mycoplasma. The next paper examines the mechanisms by which IFN-γ impacts innate immune responses.

CHAPTER IV

Natural Killer Cell Function in an Interferon-Gamma Deficient Environment Dampens Innate Immune Clearance of Mycoplasma from the Lung

The purpose of this study was to understand the mechanism of IFN-γ mediated innate immune responses during mycoplasma pulmonary disease. Using IFN-γ KO mice, we will be able to study how IFN-γ impacts the development of innate immune responses toward mycoplasma infection in the lung. Interestingly, the depletion of NK cells with anti-asialo GM1 antibody treatment does not affect BALB/c mice from clearing mycoplasma from the lung. However, the depletion of NK cells from IFN-γ KO leads to an increased clearance of mycoplasma from the lung. NK cells in an IFN-γ deficient environment lead to significant increases in anti-inflammatory cytokines IL-10, G-CSF, and TNF-α at 3 days post-infection, furthermore, IFN-γ KO mice had a higher number of cells infiltrated into the alveoli and airways. The depletion of NK cells from IFN-γ KO mice decreased both anti-inflammatory cytokines and the number of cells infiltrated into the alveoli and airways and also changed the type of infiltrate from a neutrophil macrophage mixed response to a mainly macrophage response. This suggests that NK cells are playing a role in both dampening macrophages and in the recruitment of cells

into the site of infection. Interestingly, there is a correlation between the level of the chemokine KC and the number of neutrophils isolated from the bronchioalveolar lavage of IFN-γ KO mice before and after NK cell-depletion. Though the exact mechanism by which NK cells are interfering with innate immune responses in an IFN-γ environment are unclear; however, our studies clearly demonstrates the NK cells are detrimental to the clearance of mycoplasma organisms from the lung and IFN-γ counteracts these detrimental activities.

Introduction

Mycoplasma infection is a leading cause of pneumonia worldwide. In the United States, alone, *Mycoplasma pneumoniae* accounts for 30% of all cases of pneumonia (2-4). Mycoplasma disease is also associated with the exacerbation of other respiratory diseases, such as asthma (5, 6). *Mycoplasma pulmonis* causes a naturally occurring murine respiratory disease with high morbidity and low mortality. *M. pulmonis* is an excellent animal model of *M. pneumoniae,* allowing the characterization of immune responses during the pathogenesis of mycoplasma respiratory disease. Both *M. pulmonis* and *M. pneumoniae* respiratory infections cause rhinitis, otitis media, laryngotracheitis, and bronchopneumonia. In terms of histopathology, both diseases are characterized by the accumulation of mononuclear, macrophages and lymphocytes, cells along the respiratory airway (4, 56, 57, 63, 64). This suggests that the activation and recruitment of macrophages and lymphocytes is key in the development of both acute and chronic states of the disease. In support, several studies demonstrate a component of mycoplasma respiratory disease is immunopathologic (11-15).

Pulmonary mycoplasma infection is a complex and multi-faceted inflammatory process. Studies demonstrate that T cells and macrophages play critical roles in the pathogenesis developed during mycoplasma disease (11-15, 68, 70, 73, 77, 102). Macrophages play an interesting role in mycoplasma infection. Depletion of alveolar macrophages affects the clearance of organisms from mycoplasma resistant mice, but does not affect susceptible mice (70). Studies suggest that innate immune responses of the C57Bl/6 protect the mice from infection, and alveolar macrophages play a significant part in developing that innate immune response. However, the same alveolar macrophages are not critical in C3H/HeN mice (70). Suggesting that innate immune responses towards mycoplasma infections are complex. However, few studies have looked at cytokine involvement during innate immune functions. Previous work has clearly shown that interferon-gamma (IFN-γ) is important in innate immunity toward mycoplasma pulmonary infection, as the loss of IFN-γ leads to increased mycoplasma numbers in the lungs (Chapter II). Further studies must be done to identify the source of IFN-γ during mycoplasma infection, and how the loss of IFN-γ perturbs the innate immune function and its ability to clear mycoplasma infection.

Natural killer (NK) cell production of IFN-γ is thought to play a critical role in the activation of innate immune mechanisms. However, new functions for NK cells during immune responses are being discovered. Granules released from NK cells can kill cells infected with intracellular bacteria, as well as directly kill the bacteria (105, 106). NK cells can also modulate T cells before and after antigen presentation by causing apoptosis (49, 50). NK cells have also been implicated as being important in fetus development

(107-109). Beyond cell-to-cell interaction, NK derived cytokines play several intricate roles in the development of inflammation and adaptive immune phenotypic development. During leishmania infections, IFN-γ released from NK cells is critical for macrophage activation. Without this IFN-γ, macrophages are unable to kill intracellular leishmania (49, 110, 111). This early release of IFN-γ is critical for downstream phenotypic development of T cells and B cells during adaptive immune responses (112, 113). NK derived TNF-α is also critical in the development and recruitment of neutrophils and the oxidative burst into the site of infection (114). NK cells play an important role modulating several immune responses during bacterial infections.

Macrophages and NK cells interact early after encountering invading organisms to generate inflammatory process. Tissue-derived macrophages, such as alveolar macrophages, are key surveillance cells that monitor for infectious agents (22, 44). Detection of infectious agents through innate receptors, such as Toll-Like receptors, by macrophages trigger the release of pro-inflammatory cytokines, such as IL-6, IL-12, IL-18, IP-10 and TNF-α, as well as the release of leukotriens and prostaglandins (115). These cytokines have local and systemic affects from recruitment of inflammatory cells, permeabilization and relaxation of muscle, inducing fever, and many other key early inflammatory events (16). This first wave of inflammation will quickly subside without secondary signals. Bacteria-macrophage interactions lead to a level of macrophage activation, however without IFN-γ, macrophages fail to become activated to high enough state to kill intracellular bacteria, especially mycoplasma (26, 73, 102). NK cells are the first cell to release IFN-γ during an inflammatory response (20). This IFN-γ is critical for

enabling macrophages to become able to deal with phagocytized bacteria as well as furthering the inflammatory process (26). Without this important interaction of NK cells and macrophages, bacterial infections persist, leading to chronic infections.

The purpose of this study was to understand IFN-γ mediated mechanisms during innate immune responses during pulmonary mycoplasma infection. By utilizing IFN-γ KO mice, we were able to understand the contribution of this cytokine to the development of inflammation and clearance of mycoplasma from the lungs. There are few studies that examine innate immune responses during mycoplasma infection, and information gained from this study will shed light into these early events. Hopefully, these studies will lead to the development of new therapeutic strategies to deal with respiratory infections.

Materials and Methods

Mice. BALB/c and IFN-γ (C.129S7(B6)-ifng^{tm1Ts} on a BALB/c background) knockout (KO) viral- and mycoplasma-free mice were obtained from the Jackson Laboratories (79). Mice were housed in sterile microisolator cages supplied with sterile bedding, food, and water was given ad libitum. Mice used in the study were between 8-12 weeks of age. Female mice were used in all studies. Before experimental infection, mice were anesthetized with an i.m. injection of ketamine/xylazine.

Mycoplasma. The UAB CT strain of *M. pulmonis* was used in all experiments. Stock cultures were grown, as previously described (26), in mycoplasma medium and

frozen in 1-ml aliquots at -80^0C. For inoculation, thawed aliquots were diluted to 10^5 CFU/20 µl. Nasal-pulmonary inoculations of 20 µl of diluted mycoplasma were given for experimental infections.

Bronchoalveolar lavages (BAL). At time of harvest, mice were euthanized with 0.05ml of Ketamine/Xyalzine. The trachea was exposed and I.V. catheter (Beckton Dickinson, Sandy, Utah) was inserted. One ml of PBS was use to fuse into the lungs and then removed for analyses. BAL fluid was then spun at 200 g for 10 minutes to pellet BAL cells. Supernatants were removed and placed into new tubes and frozen at -80^0C for analyses later. Cells were either placed into Ultraspec-II™ RNA Isolation System (Biotecx Laboratories, Inc. Houston, TX) for mRNA characterization, or cells were stained with fluorescent antibodies or modified Wright-Giemsa stain (Diff-Quik; Baxter, McGaw Park, IL) for cell characteriztion. Corresponding lungs devoid of BAL cells were either placed into Ultraspec-II™ RNA Isolation System for RNA isolation or lymphocytes were isolated for characterization by flow cytometry. Differential counts on 300 cells were preformed by light microscopy, using a single-blind method after application of a modified Wright-Giemsa stain.

Cell isolation. Mononuclear cells were isolated from lungs, as previously described (25, 33, 80). Lungs were perfused with PBS without magnesium or calcium to minimize contamination of the final lung cell population with those from the blood. The lungs were finely minced. The tissues were suspended in RPMI 1640 medium (HyClone Laboratories, Logan, UT) containing 300 U/ml *Clostridium histolyticum* Type I collagenase (Worthington Biochemical, Freehold, NJ), 50 U/ml DNase (Sigma-Aldrich,

St. Louis, MO), 10% FBS (Hyclone Laboratories) HEPES and antibiotic/antimycotic solution (Life Technologies, Grand Island, NY). The tissues were incubated at 37^0C while mixing on a Nutator (Fisher Scientific, Pittsburgh, PA) for 90-120 min. During the incubation period, the tissue and were vigorously pipetted every 30 min. After incubation, the digestion mixture was passed through a 250-μm nylon mesh to remove undigested tissue. Mononuclear cells were purified from cell suspension by density gradient centrifugation using Lympholyte M (Accurate Chemicals, Westbury, NY).

Cell characterization by flow cytometry. Three-color immunofluorescent staining was performed to identify T cell, NK T cells, NK cell and intracellular IFN-γ using PE-CY7-labeled anti-murine CD3 mAb (Caltag), biotinylated-labeled anti-murine DX5 mAb (Caltag), PE-TC conjugated strepavidin (Caltag), and PE-labeled anti-murine IFN-γ mAb (Caltag). Briefly 10^6 cells per tube were incubated with Fc block (Caltag) for 30 minutes at 4^0C. The cells were washed in staining buffer [Mg^{2+}-free, Ca^{2+}-free PBS with 0.05% sodium azide, 1% FBS (Hyclone Laboratories)]. Cells were than stained with in a 100 μl cocktail of fluorescent Ab for NK and T cells (2 μg/ml) for 30 minutes at 4^0C. The cells were washed in staining buffer [Mg^{2+}-free, Ca^{2+}-free PBS with 0.05% sodium azide, 1% FBS (Hyclone Laboratories)]. Cells were then incubated with strepavidin PE-TC (1 μg/ml) for 30 minutes at 4^0C. The cells were washed in staining buffer [Mg^{2+}-free, Ca^{2+}-free PBS with 0.05% sodium azide, 1% FBS (Hyclone Laboratories)]. Cells were then fixed and permeabilized using Caltag fix and perm kit and stained for intracellular IFN-γ as per Caltag instructions. After fixation, cells were resuspended in staining buffer for analysis.

Two color immunofluorescent staing was preformed to identify apoptotic macrophage populations using PE-labeled anti-murine F4/80 mAb (Caltag) and FITC-VAD-FMK (Promega, Madison, WI). Briefly, 10^6 cells per tube were incubated for 30 min at 4^0C in 100 µl of fluorescent Ab (2 µg/ml). The cells were washed in staining buffer [Mg^{2+}-free, Ca^{2+}-free PBS with 0.05% sodium azide, 1% FBS (Hyclone Laboratories)] and fixed with 2% paraformaldehyde solution for 30 minutes. After fixation, cells were resuspended in staining buffer for analysis.

The cells were analyzed using an EPICS XL-MCL flow cytometer (Beckman Coulter). Data collection was done using System 2 software (Beckman Coulter). Cell population gates and detector voltages were set using isotype stained (control) lung and splenic cells. The proportion of each cell population was expressed as the percentage of the number of stained cells. To determine the total number of a specific cell population, their percentage was multiplied by the total number of cells isolated from the tissue.

RNA isolation from BAL cells and lungs. Total RNA was isolated from lungs and BAL cells of mice using the Ultraspec-II™ RNA Isolation System (Biotecx Laboratories, Inc. Houston, TX). Briefly, BAL cells were isolated; corresponding lungs were homogenized using a Pro 200 homogenizer (Pro Scientific, Monroe, CT); and both were placed in the Ultraspec-II™ RNA reagent. Chloroform was added to the homogenate and centrifuged at 12,000 x g (4^0C) for 30 minutes. The RNA was precipitated by adding isopropanol to the aqueous phase and centrifuging samples at 12,000 x g (4^0C) for 10 minutes. The RNA pellet for each sample was washed twice with 75% ethanol by vortexing and subsequent centrifugation for 5 minutes at 7,500 x g and

then resuspended in diethylpyrocarbonate (DEPC)-treated water. The concentration and quality of RNA in each sample was determined spectrophotometrically (GeneQuant II, Pharmacia Biotech, Piscataway, NJ.) and by gel electrophoresis. The RNA samples were stored at -80^0C until ready for use.

Cytokine mRNA detection by quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR). RNA from either BAL cells or corresponding whole lungs were transcribed using TaqMan® Reverse Transcription Reagents (Applied Biosystems, Branchburg, New Jersey) to generate cDNA. cDNA was subjected to real-time PCR using a Cepheid Real Time Smart Cycler I system (Cepheid, Kingwood, TX) and TaqMan® Universal PCR Master Mix kit (Applied Biosystems). Amplification conditions were a single cycle at 50^0C for 2 minutes and 95^0C for 10 minutes, followed by 50 cycles at 95^0C for 15 seconds and 60^0C for 1 minute using probes and primers for IL-4, IL-10, IL-12, IFN-γ and TNF-α purchased from Biosource (Biosource, Camarillo, CA). Quantification was determined by comparing cycle threshold (CT) values.

Granule preparation. Granules were isolated as previously described (116). The granules (117) were obtained from RNK-16 NK-like leukemia cells (118) grown as an ascites line in F344 rats obtained from the National Cancer Institute (USA). The ascites cells were washed with Borregard's relaxation buffer (119) and then ruptured using a nitrogen cavitation bomb (Parr instrument Co., Moline, IL) pressurized to 450 psi. The lysate was layered over 54% Percoll (Sigma Chemical Co., St. Louis, MO) which formed a gradient during a 20 minute centrifugation at 45,000 x g in a Beckman Ti50.2 rotor at 4^oC. The dense fractions (up to 1.068 g $\cdot$ cm^{-3}) were pooled. Nuclei were removed by

filtration through a 3 μm Nucleopore filter (Millipore, Bedford, MA) (118). Percoll was then removed by a high speed spin, 4 hours at 145,000 x g. Granules were collected from above the Percoll pellet, disrupted by three freeze-thaw cycles after adding NaCl to bring the salt concentration up to 1 M (119) and stored at –20°C. Protein concentrations were determined by a BCA assay (Pierce, Rockford, IL) using bovine serum albumin (BSA) for calibration.

Cytotoxicity assays. Cytolytic assays were done as previously described (116). Cytolytic activity was determined by the hemoglobin released from lysed red blood cells (RBC) (118, 120). Granules and varying concentrations were incubated with 0.5% (v/v) RBC at room temperature for twenty minutes in 0.2 ml round bottom microtiter plates (Falcon 3910, Beckton Dickinson Labware, Lincoln Park, NJ). The assay buffered contained 10 mM HEPES, 0.15 M NaCl, and 10 μg/ml BSA (Sigma A4503), pH 7.5 with addition of calcium to 1mM during incubation to start lysis (118). The reaction was halted by acidification with pH 6.0 2-[N-morpholino]ethane-sulfonic acid (MES, Sigma M-8250) (120). The microtiter plates were spun at 1500 x g for 10 minutes, the cell-free supernatants transferred to a second microtiter plate, and the released hemoglobin detected with a MX80 plate reader (Dynatech, Chantilly, VA)microplate reader at 412 nm. The percent lysis was calculated as [(% experimental hemolysis - % spontaneous hemolysis)/(% maximal hemolysis - % spontaneous hemolysis)] x 100. Addition of 0.01% saponin (Sigma) produced maximal RBC lysis.

NK cell depletion by anti-asialo GM-1 antibody treatment. To deplete mice of NK cells, mice were given an intraperitoneal injection of 100 μg in 100 μl of anti-asialo GM-1 (Wako, Osaka, Japan) on day -1 of infection. Depletion was confirmed by staining of lymphocytes with biotinylated anti-DX5 (NK marker) antibody (Caltag, Camarillo, CA.), followed by incubation with PE-TR conjugated strepavidin (Caltag) and analyzed using an EPICS XL-MCL flow cytometer (Beckman Coulter, Fullerton, CA.).

Characterization of mycoplasma numbers. The numbers of mycoplasma colony forming units (CFU's) in lungs and nasal passages were determined as previously described (83, 85). Briefly, lungs were minced and placed in mycoplasma broth medium. Nasal washes were collected by with 1 ml of mycoplasma broth medium that was forced through the nasal passages of mice by inserted a syringe into the soft pallet. In some experiments, we also isolated nasal passage tissue for CFU determination. The samples were sonicated (Vibra cell sonicator; Sonics & Materials/Vibro Cell, Newtown, CT) for 2 min. at 50 amplitudes without pulsing. After sonication, serial dilutions (1:10) were prepared, and 20 μl of each dilution was plated onto mycoplasma agar medium. After 7 days of incubation at 37^0C, colonies were counted, and the CFU recovered from each tissue was calculated.

Cytokine Assays. The levels of cytokines were measured by Bio-Plex suspension array. Levels of IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12 p40, IL-12 p70, IL-17, GM-CSF, IFN-γ, G-CSF, TNF-α, KC, MIP-1α, and RANTES was measured by Bio-Plex 18-plex cytokine panel (Bio-RAD, Hercules, CA). 96-well filter bottom plates were used. To each well 50 μl of anti-cytokine beads in assay diluent were

added to each well. Plates were washed with Bio-Plex washing buffer. 50 µl of sample or standard was added per well. Plates were washed with Bio-Plex washing buffer. Plates were incubated at room temperature while shaking in the dark for thirty minutes. Plates were washed with Bio-Plex washing buffer. 25 µl of biotinylated secondary antibodies were added to each well. Plates were incubated at room temperature while shaking in the dark for thirty minutes. Plates were washed with Bio-Plex washing buffer. 50 µl of strepavidin-PE was added to each well. Plates were incubated at room temperature while shaking in the dark for thirty minutes. Plates were washed with Bio-Plex washing buffer. Samples were read using Bio-Plex 100 system (Bio-Rad, Hercules, CA). Cytokine levels were determined by comparison with standard curves generated from murine recombinant cytokines and analysed using Bio-Plex Manager Software (Bio-Rad, Hercules, CA).

Statistical Analysis. Data was evaluated by ANOVA, followed by Fisher Protected Least Square Differences Multigroup comparison. These analyses were performed using the StatView (SAS Institute, CARY, NC) computer programs. When appropriate, data was logarithmically transformed prior to statistical analysis, and confirmed by a demonstrated increase in power of the test after transformation of the data. A P value ≤ 0.05 was considered statistically significant. If data was analyzed after logarithmic transformation, the antilog of the means and standard errors of transformed data was used to present the data and are referred to as the geometric means (x/÷ standard error).

Results

IFN-γ mRNA levels increased along the airway after infection. To determine if mycoplasma infection caused a change in IFN-γ transcript levels, total RNA was isolated from cells from the BAL and the corresponding BAL treated lungs of naïve and 3-day infected BALB/c mice. Real-time RT-PCR was used to quantify the IFN-γ mRNA transcript levels.

After infection, the levels of IFN-γ mRNA transcripts levels increased in BAL cells, but not in the corresponding lungs (Fig 1a). Transcript levels of IFN-γ was approximately 3 fold higher in cells isolated from the BAL of 3-day infected animals when compared to transcript levels of cells from the BAL of uninfected animals. The corresponding lungs, on the other hand, showed no difference in IFN-γ transcript levels after infection. Thus IFN-γ mRNA is increased in cells within the respiratory airways alveoli, not the cells within the parenchymal lung tissue.

Intracellular IFN-γ is increased in NK cells isolated from the lungs of mycoplasma-infected mice. To determine which cells are responding to mycoplasma infection by increasing intracellular levels of IFN-γ, lymphocytes were isolated from naïve and 3 day infected BALB/c mice. Lymphocytes were stained with fluorescent-tagged antibodies for cell surface expression CD3 (T cell) and DX5 (NK cell), as well as intracellular IFN-γ. Cells were characterized by flow cytometry.

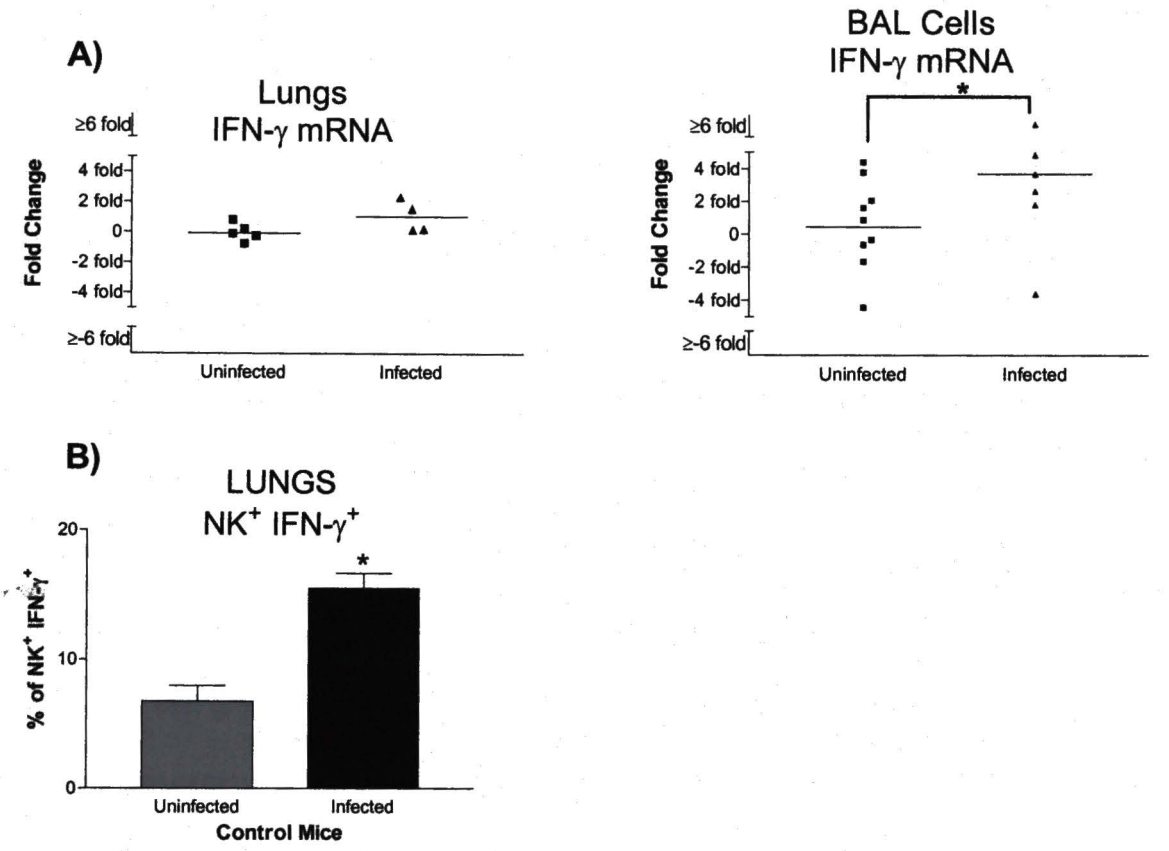


Figure 1. mRNA and intracellular level of IFN-γin BALB/c mice three-days after infection. A) Three days after infection cells of the BAL and corresponding lungs were isolated and total RNA was collected. IFN-γ transcript levels were measured by real time RT-PCR. Levels of mRNA were standardized to GAPDH housekeeping gene. "*" denotes statistical difference ($p \leq 0.05$) from uninfected mice.

B) Three days after infection lung cells were isolated and stained for CD3 (T cell), DX5 (NK) and intracellular IFN-γ, and analyzed by flow cytometry. Vertical bars represent mean ± SE (n=8). "*" denotes statistical difference ($p \leq 0.05$) from uninfected mice.

Only the numbers of NK cells with intracellular levels of IFN-γ increased after infection (Fig 1b). IFN-γ positive NK cells were increased by about 100% after infection in BALB/c mice. Neither T cells nor NK T cells significantly increased in intracellular IFN-γ after infection. Therefore, only IFN-γ^+ NK cells are increasing in response to mycoplasma infection in the lung.

Granules isolated from NK cells do not kill *M. pulmonis*. To determine if NK cells could directly kill mycoplasma organisms with granules, granules isolated from NK cells were incubated with a known number of *M. pulmonis* CFU, and the number of mycoplasma CFU were determined after incubation with granules.

NK cell granules did not kill mycoplasma organisms. When 10^5 CFU of mycoplasma organism were incubated with a concentration of NK cell granules, which lysed 100% of target red blood cells in twenty minutes, there was no loss in mycoplasma organisms (Fig 2). Demonstrating that NK cell granules cannot kill mycoplasma. To determine if mycoplasmas can interact with granules and block their activity, varying concentrations of mycoplasmas were incubated with NK cell granules and target red blood cells for 20 minutes. After twenty minutes, regardless of the concentration of mycoplasma, 50% of target cells were lysed (Fig 2). These results demonstrate that mycoplasma do not inhibit NK cell granule functions, nor are NK cell granules able to kill mycoplasmas.

Depletion of NK cells from IFN-γ KO mice allows them to clear mycoplasma organisms from the lungs. We previously showed that NK cells are the only cells with an increase in intracellular IFN-γ after mycoplasma infection. To determine if the loss of

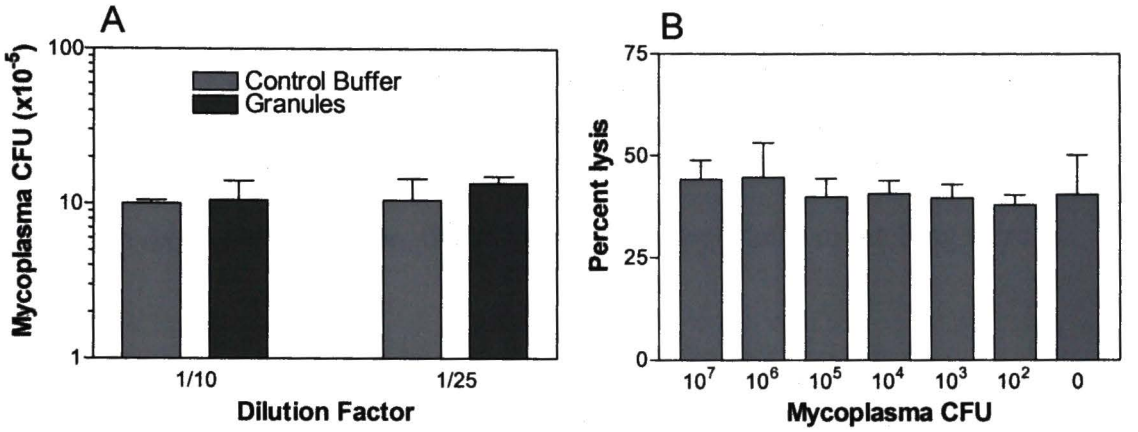


Figure 2. Interaction of mycoplasma and NK cell derived granules. A) 10^5 CFU of mycoplasma were incubated with either control broth or NK cell-derived granules for twenty minutes, the number of mycoplasma organisms were determined. B) Varying numbers of mycoplasma CFU were incubated with a constant concentration of NK cell-derived granules and sheep blood for twenty minutes, afterword the percentage of red blood cell lyses was determined.

this NK derived IFN-γ is responsible for the inability to control mycoplasma growth within the lungs, NK cells were depleted by anti-asialo GM1 antibody treatment one day prior to mycoplasma infection. IFN-γ KO mice were also depleted of NK cells as a control. On day 3 post-infection, the numbers of mycoplasmas in the lung were determined.

Contrary to our hypothesis, the loss of NK cells did not affect the ability of BALB/c mice to clear mycoplasma organisms from the lung (Fig 3). Surprisingly, the depletion of NK cells from IFN-γ KO mice allow these mice to clear mycoplasma organisms as well as control mice. Demonstrating that NK cells dampen immune responses during mycoplasma disease in an IFN-γ deficient environment. To determine if anti-asialo GM1 antibody treatment affected other lymphoid cell populations, lymphocytes were collected from the lungs of an extra group of anti-asialo GM1 treated BALB/c mice, and cells were labeled with fluorescently-tagged antibodies for T cells and NK cells. Only the NK positive fraction was depleted, while the number of T cells was unchanged by treatment.

IFN-γ KO mice have a significant increase in transcript levels of IL-12, IL-10 and TNF-α in cells from the BAL. It appears that more than simply the loss of IFN-γ is affecting IFN-γ KO mice from clearing mycoplasma from the lungs. To determine if there is a change in the type of immune response generated, mRNA transcript levels were determine before and after infection in both BALB/c and IFN-γ KO mice. Mice were either infected with mycoplasma or sham-inoculated, and at 3 days post-infection, total RNA from BAL cells and corresponding lungs was isolated and the levels of IL-10, IL-4,

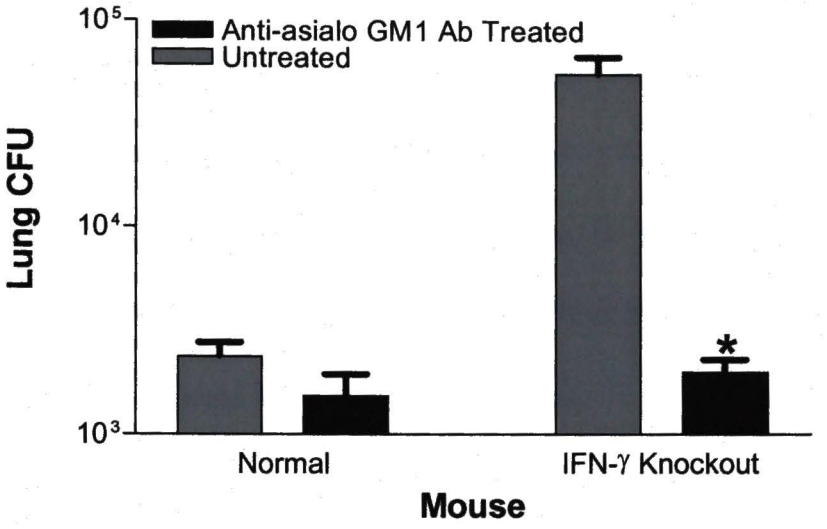


Figure 3. Mycoplasma CFU found in lungs of anti-asialo GM-1 treated and untreated BALB/c and IFN-γ KO mice. BALB/c and IFN-gamma KO were depleted of NK cells on day -1. Three days post-infection the number of mycoplasma organisms in lungs were determined. Vertical bars and error bars represent mean ± SE (n=8). "*" denotes statistical difference ($p \leq 0.05$) from strain control.

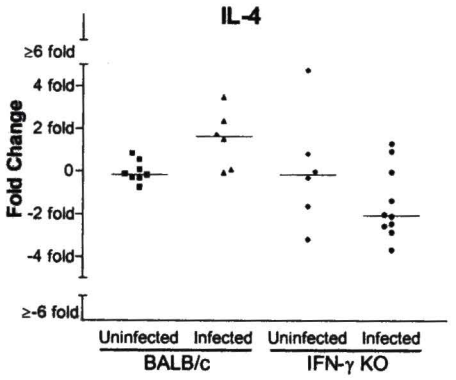
TNF-α, and IL-12 were determined. mRNA isolated from the BAL or corresponding lungs of BALB/c mice showed no change in any cytokine level determined from uninfected to day 3 infected BALB/c mice (Fig 4a). IFN-γ KO mice on the other hand showed an increase in mRNA transcript levels of TNF-α, IL-12 and IL-10 in cells isolated from the BAL after infection (Fig 4a). Only TNF-α transcript levels increased in corresponding lungs of infected IFN-γ KO mice (Fig 4b).

There is a no change in the number of apoptotic macrophages in IFN-γ KO mice depleted of NK cells. NK cells play a detrimental role in the clearance of mycoplasma in IFN-γ KO mice. Further, we have demonstrated that NK cell granules are unable to kill mycoplasma. Therefore, we examined the possibility that NK cells were causing bystander damage in IFN-γ KO mice during mycoplasma infection. NK cells were depleted from BALB/c and IFN-γ KO mice and then infected with mycoplasma. Three days after infection, cells were isolated from the BAL and corresponding lungs and stained for macrophages and activated caspases to determine apoptosis.

There was no significant difference in the number of apoptotic macrophages isolated from either IFN-γ KO or BALB/c mice (Fig 5). Furthermore, the depletion of NK cells from either mouse strain did not significantly affect the levels of apoptotic macrophages. Depletion of NK cells from BALB/c mice did not affect apoptosis levels in the cells isolated from the BAL. The corresponding lungs of all tested groups showed no changes in levels of apoptotic cells.

Figure 4. Cytokine mRNA expression of the BAL cells and corresponding lungs before and after infection. Total RNA was isolated from the BAL cells and corresponding lungs of infected and uninfected BALB/c and IFN-γ KO mice. Cytokine mRNA levels were measured using real time RT-PCR. Cytokine levels were standardized to GAPDH housekeeping gene. A) Lung cells mRNA. B) BAL cells mRNA. "*" denotes statistical difference ($p \leq 0.05$) from uninfected mice.

A)


IL-4
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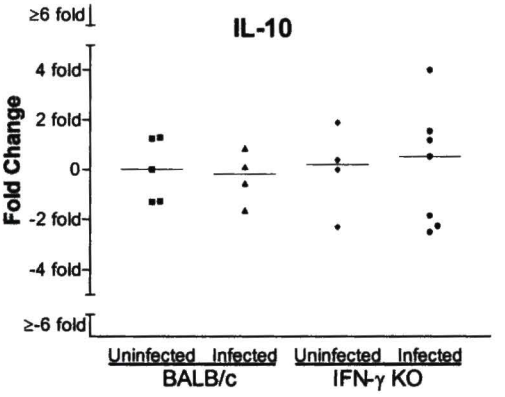
-2 fold
-4 fold
≥-6 fold
Fold Change
Uninfected Infected Uninfected Infected
BALB/c
IFN-γ KO


IL-10
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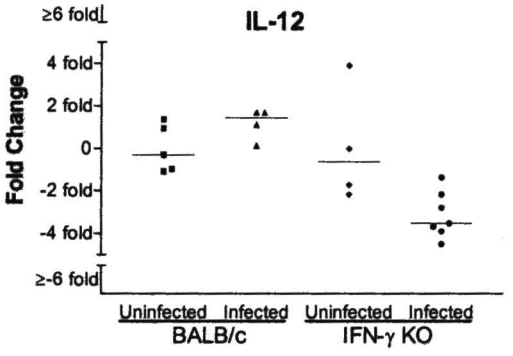
Uninfected Infected Uninfected Infected
BALB/c
IFN-γ KO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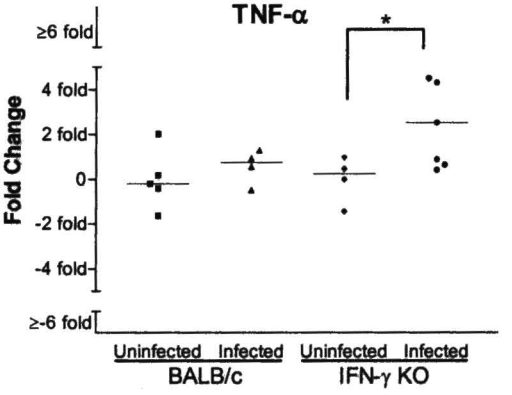
Uninfected Infected Uninfected Infected
BALB/c
IFN-γ KO


TNF-α
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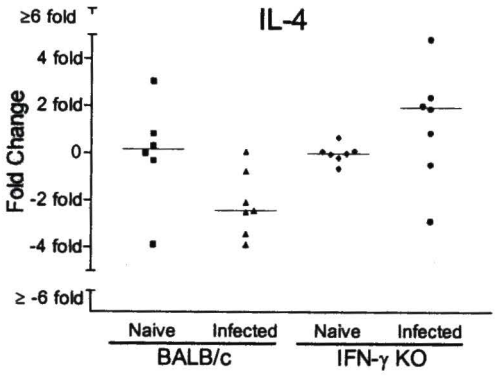
Fold Change
Uninfected Infected Uninfected Infected
BALB/c
IFN-γ KO

B)


IL-4
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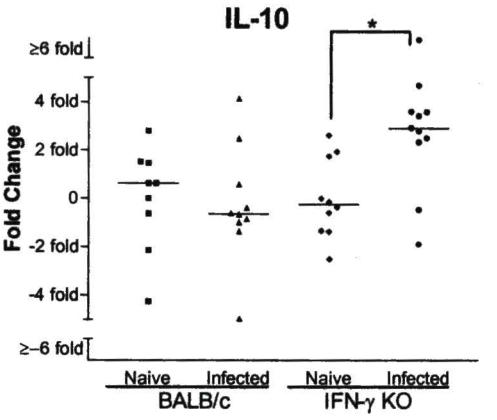
Naive
Infected
Naive
Infected
BALB/c
IFN-γ KO


IL-10
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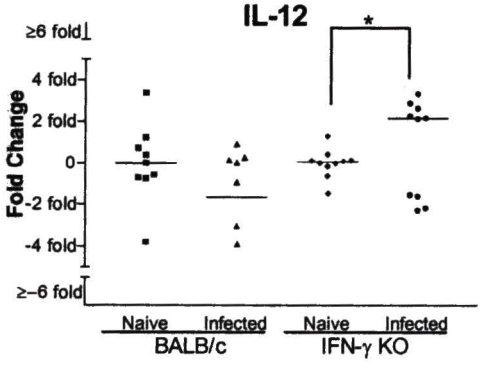
IFN-γ KO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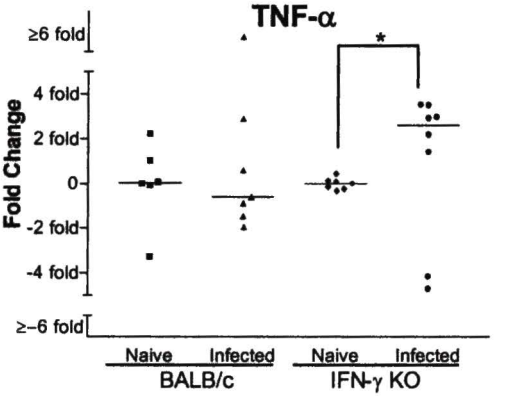
TNF-α
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Naive
Infected
Naive
Infected
BALB/c
IFN-γ KO

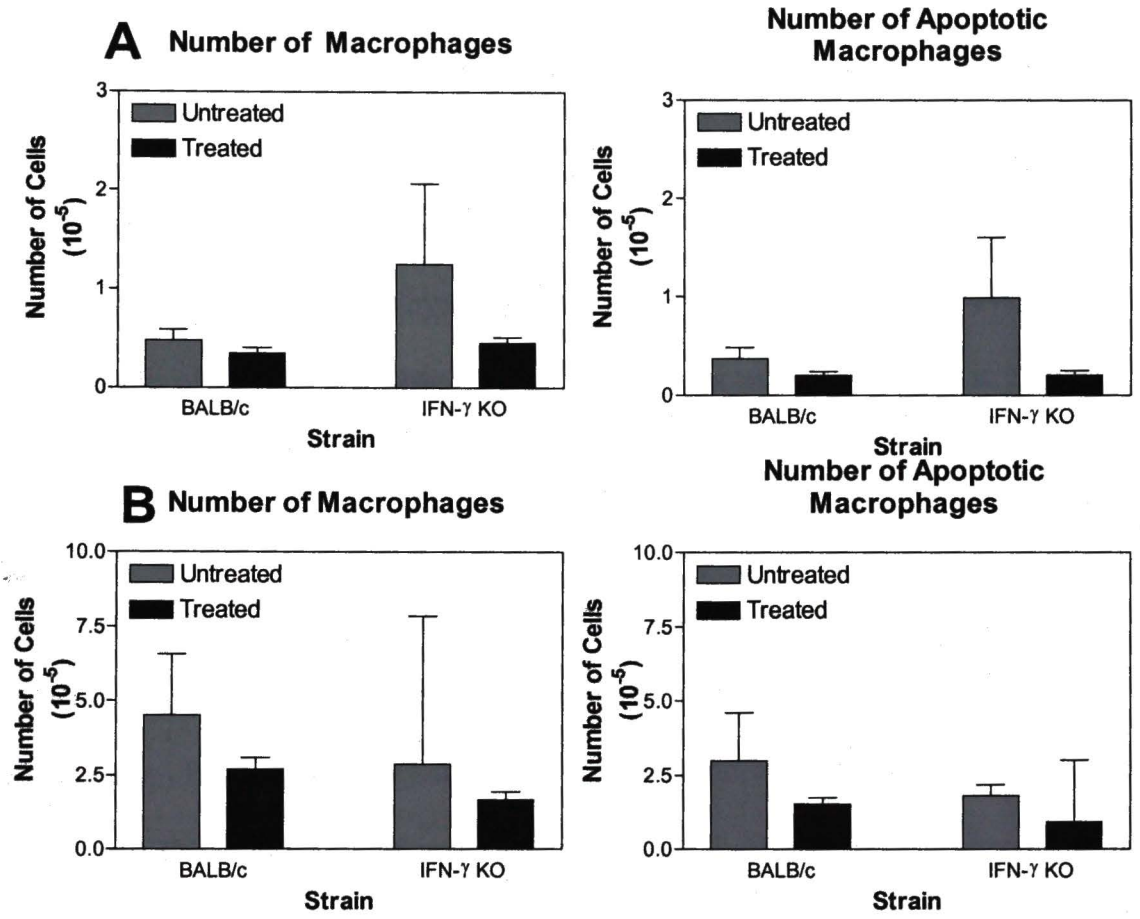


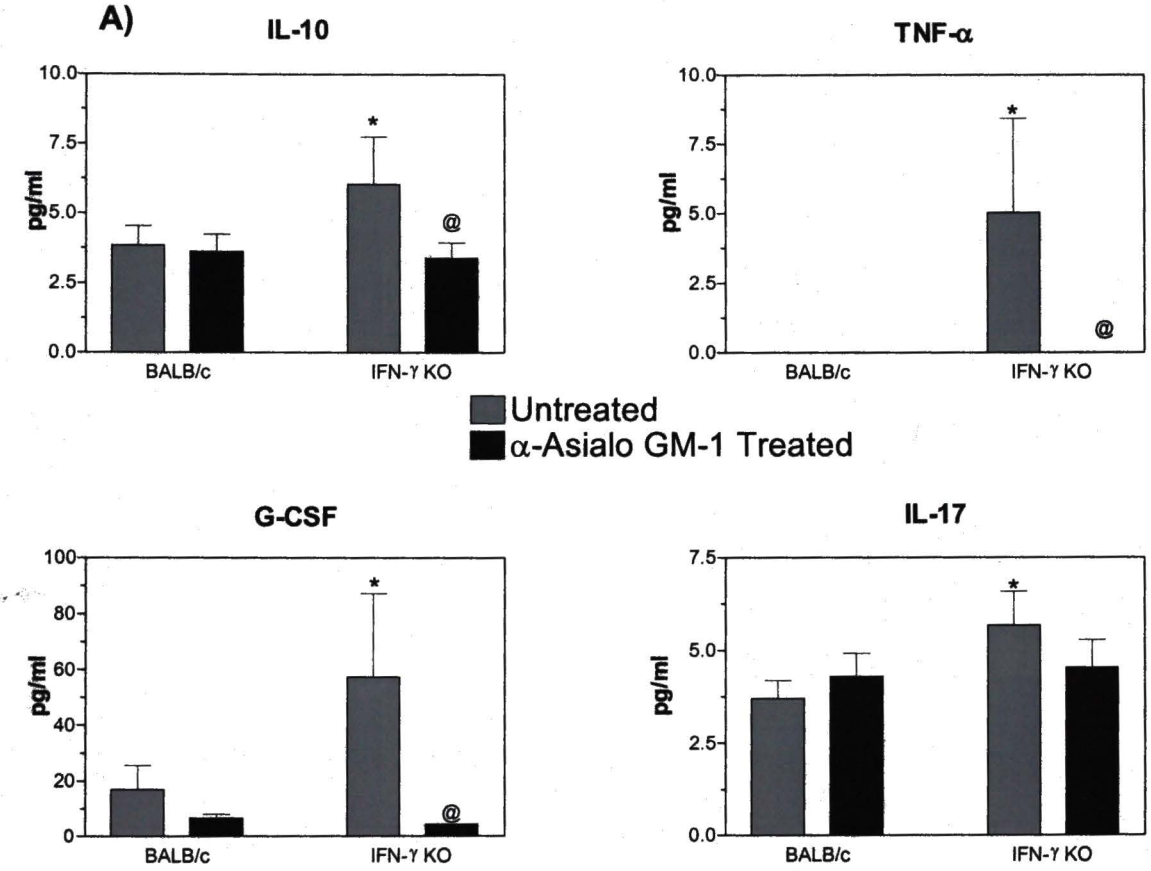
Figure 5. Number of Macrophages and Apoptotic Macrophages in BAL and corresponding Lung before and after NK depletion. On day -1 BALB/c and IFN-γ KO mice were either i.p. injected with anti-asialo GM-1 Ab or sham treated. Three days after infection BAL cells and corresponding lung cells were collected and stained for F4/80 (macrophages) and FITC-VAD-FMK (apoptosis) and analyzed by flow cytometry. A) The number of macrophages and apoptotic macrophages in the BAL. B) The number of macrophages and apoptotic macrophages in the corresponding lung. Vertical bars and error bars represent mean ± SE (n=8).

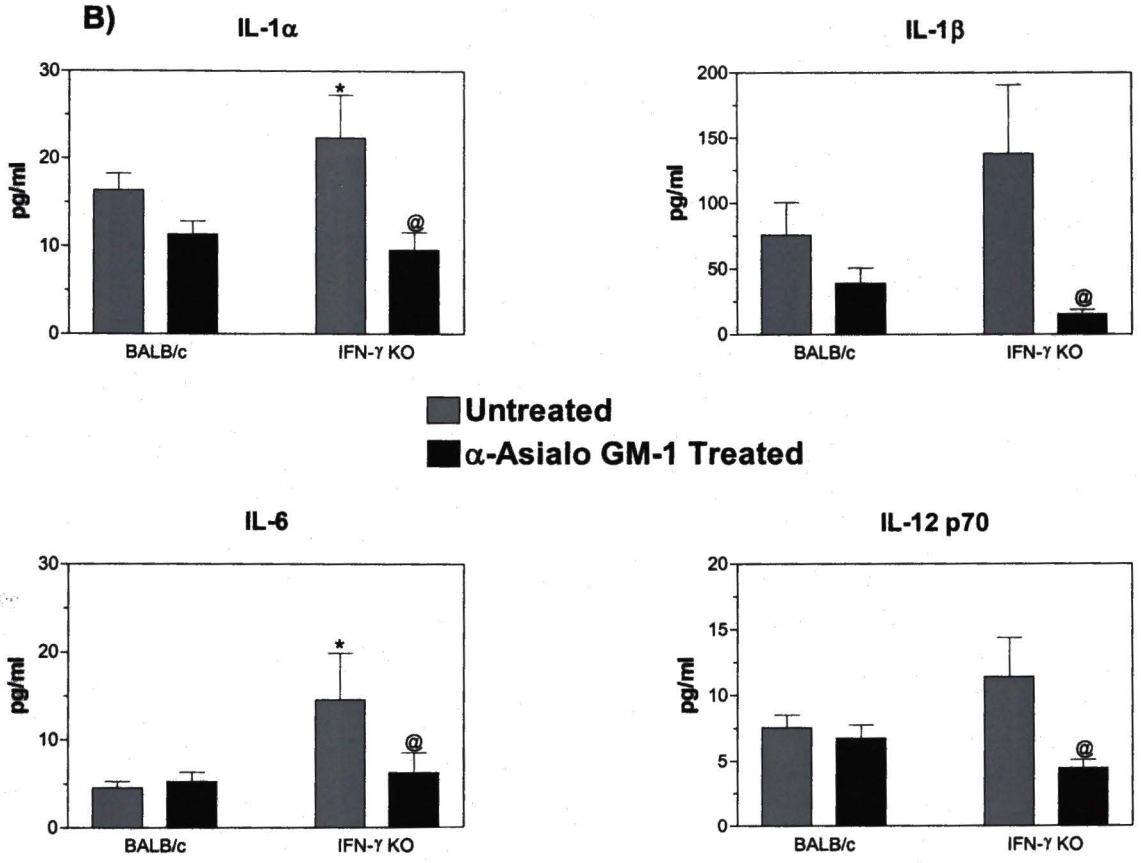
Depletion of NK cells from IFN-γ KO mice leads to a decrease in anti-inflammatory cytokine secretion. To further understand the cascade in which NK cells are involved in BALB/c and IFN-γ KO mice after infection, BALB/c and IFN-γ KO mice were anti-asialo GM1 antibody treated to deplete NK cells. After three days of infection, BAL were performed to isolate cytokine from the alveolar spaces and airways. The protein levels of 18 cytokines were determined to characterize differences in cytokines secreted during mycoplasma infection in BALB/c and IFN-γ KO with or without NK cells.

Depletion of NK cells from IFN-γ KO mice affected a variety of cytokines. The cytokines levels that were affected were placed into three broad categories: Anti-inflammatory, Pro-inflammatory, and Chemokines. Anti-inflammatory cytokines IL-17, G-CSF, and TNF-α were significantly elevated while IL-10 tended to be elevated in IFN-γ KO mice when compared to BALB/c mice (Fig 6a). After depletion of NK cells, IL-10, G-CSF and TNF-α were significantly lower than IFN-γ KO mice with NK cells. However, levels of cytokines in NK cell-depleted IFN-γ KO mice were equal to control animals. The depletion of NK cells from BALB/c mice did not significantly affect the levels of any anti-inflammatory cytokine tested.

Several pro-inflammatory cytokines were significantly elevated in IFN-γ KO mice when compared to control animals. IL-1α and IL-6 were significantly higher, and IL-1β and IL-12 p70 tended to be higher in IFN-γ KO mice than in BALB/c mice (Fig 6b). The depletion NK cells affected levels of IL-1α, IL-1β, IL-6 and IL-12 p70. Each of these

Figure 6. Cytokine levels in BAL of anti-asialo GM1 treated or untreated BALB/c and IFN-γ KO mice after infection. Mice were depleted of NK cells by anti-asialo GM1 Ab treatment on day -1. After three days of infection, BALF was collected and cytokines were analyzed by Bio-Plex suspension array. A) Levels of anti-inflammatory cytokines. B) Levels of pro-inflammatory cytokines. C) Levels of chemokines. Vertical bars and error bars represent mean ± SE (n=8). "*" signifies significant difference ($p \leq 0.05$) from BALB/c untreated. "@" signifies significant difference ($p \leq 0.05$) from corresponding strain control.


A)
IL-10
TNF-α
G-CSF
IL-17
pg/ml
BALB/c
IFN-γ KO
Untreated
α-Asialo GM-1 Treated


B)
IL-1α
IL-1β
pg/ml
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20
10
0
200
150
100
50
*
@
BALB/c
IFN-γ KO
Untreated
α-Asialo GM-1 Treated
IL-6
IL-12 p70
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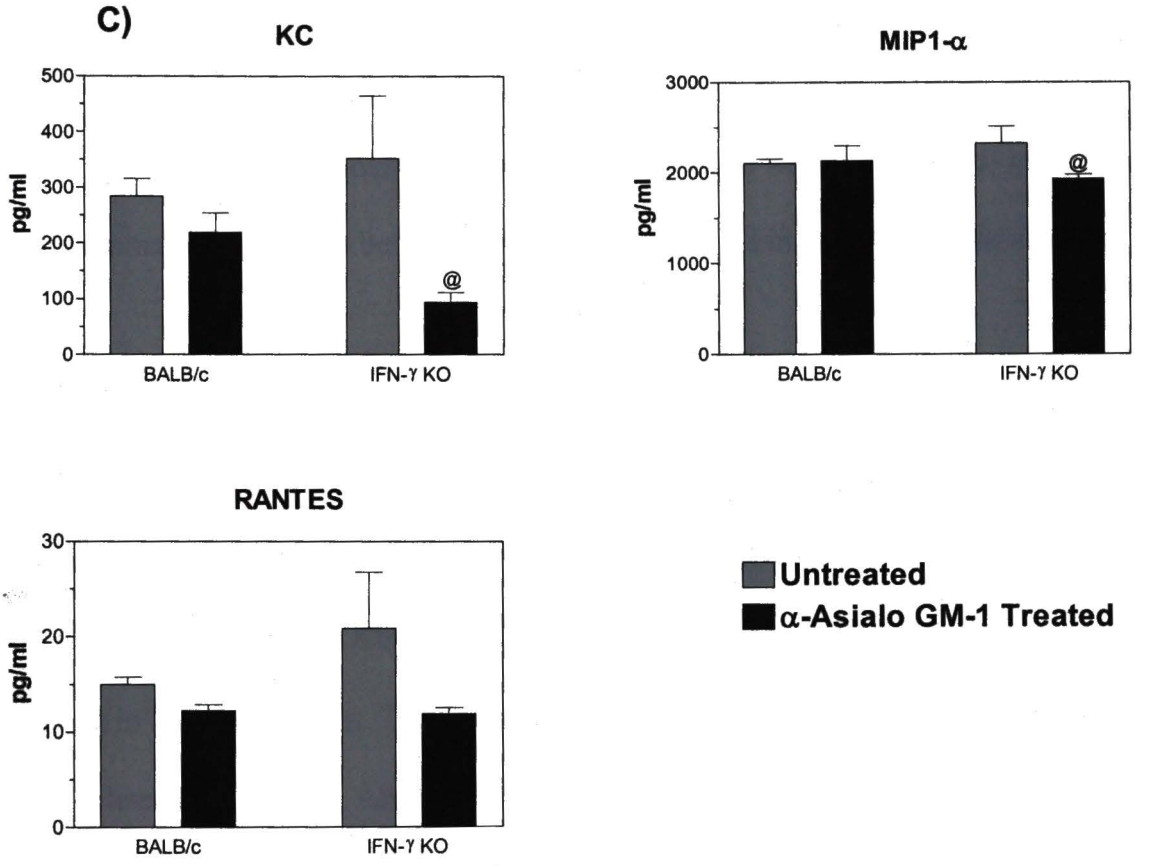
C)
KC
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400
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pg/ml
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MIP1-α
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1000
0
pg/ml
@
BALB/c
IFN-γ KO
RANTES
30
20
10
0
pg/ml
BALB/c
IFN-γ KO
Untreated
α-Asialo GM-1 Treated

cytokines was significantly lower in NK cell-depleted IFN-γ KO mice than in un-depleted IFN-γ KO mice. NK cell-depleted IFN-γ KO mice cytokine levels were comparable to wild type cytokine levels. Once again there was no difference in levels of pro-inflammatory cytokines between NK cell-depleted and immuno-competent BALB/c mice.

The chemokines KC, MIP1α, and RANTES were also characterized in non-depleted and NK cell-depleted mice 3 days post-infection. All three chemokines tended to be elevated in non-depleted IFN-γ KO mice when compared to BALB/c mice (Fig 6c). Of these three chemokines, KC and MIP1α were significantly decreased after NK depletion in IFN-γ KO mice. The levels of RANTES tended to be lower in IFN-γ KO mice depleted of NK cells. All three chemokines were slightly lower than levels found in BALB/c mice. There was no significant difference between non-depleted and NK depleted BALB/c mice.

NK cells affect the recruitment of neutrophils into the airways of infected mice. IFN-γ KO mice have increased cytokine protein levels in response towards mycoplasma infection that is brought down to BALB/c protein levels by the depletion of NK cells. To determine if NK cells also impacted the recruitment of inflammatory cells to the BAL after mycoplasma infection, BALB/c and IFN-γ KO mice were either anti-asialo GM1 ab treated or sham-treated to deplete NK cells. At day 3 post-infection, modified Wright-Giemsa staining was used to characterize BAL cells.

There was no significant difference in the types of cells isolated from the BAL in either BALB/c or IFN-γ KO mice at day 3 post-infection. However, there were

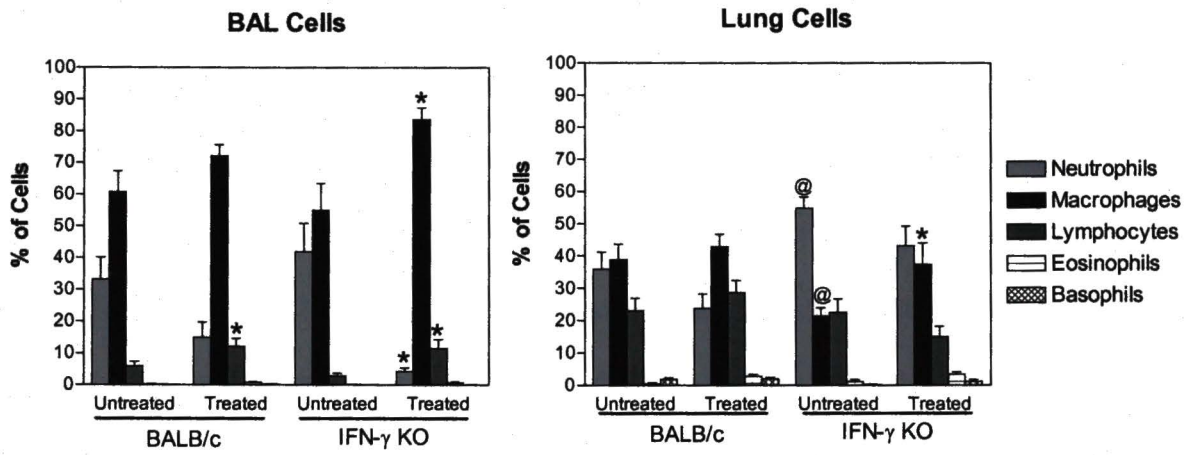


Figure 7. Cell differentiation of the lungs BAL in BALB/c and IFN-γ KO mice before and after NK depletion. On day -1 BALB/c and IFN-γ KO mice were either i.p. injected with anti-asialo GM-1 Ab or sham treated. Three days after infection BAL cells and corresponding cells were collected and stained by a modified Wright-Giemsa stain. Vertical bars and error bars represent mean ± SE (n=8). "@" signifies significant difference ($p \leq 0.05$) from untreated BALB/c mice. "*" signifies significant difference ($p \leq 0.05$) from corresponding untreated strain mice.

significant differences in the cells isolated from the lungs between BALB/c and IFN-γ KO mice after infection (Fig 7). IFN-γ KO mice show significantly higher numbers of neutrophils and significantly lower number of macrophages in the corresponding lungs than seen in BALB/c mice. After NK cell-depletion, there is no change in the types of cells in the lung of BALB/c mice. A significant increase in lymphocytes and eosinophils was seen in the BAL of NK cell-depleted BALB/c mice with a trend decrease in neutrophils when compared to untreated BALB/c mice. IFN-γ KO mice showed a similar trend in cell levels of the BAL after NK cell depletion, with significant increase in lymphocytes and eosinophils and a significant decrease in neutrophils. There was also a significant increase in the number of macrophages also isolated from the BAL of NK cell depleted IFN-γ KO mice then in untreated IFN-γ mice.

Discussion

The purpose of this study was to identify how IFN-γ affects the innate immune mechanisms towards pulmonary mycoplasma infection. IFN-γ is a pleiotrophic cytokine that has a very intricate role in the development of innate immune responses (26, 48). Early after infection, macrophages release a variety of cytokines and chemokines that attract more inflammatory cells into the site of infection (16, 22, 44). NK cells and neutrophils are some of the first cells to be recruited into the area of infection. NK cells are activated by IL-12 released from macrophage, causing them to release IFN-γ, TNF-α, and granules (20). The NK cell-derived IFN-γ activates macrophages, leading to an increase in MHC presentation, pro-inflammatory cytokine release, and oxygen and nitrogen radicals (26). This increased activation of macrophages as well as NK cell

cytokine release allows for a large increase in the recruitment of inflammatory cells such as neutrophils. This early release of IFN-γ plays an important role in the early development of this inflammatory response, and several studies demonstrate that the loss of IFN-γ leads to more severe disease (48, 110, 111). Therefore, IFN-γ is critical in the development of innate immune responses, however the mechanisms behind IFN-γ mediated responses towards mycoplasma infections is poorly understood. This study will examine how the loss of IFN-γ impacts the development of innate immune responses towards pulmonary mycoplasma infection.

NK cells in an IFN-γ deficient environment impair pulmonary innate immune response's ability to clear mycoplasma organisms. Our lab previously demonstrated that IFN-γ KO mice have a higher number of mycoplasma organisms in the lungs than in BALB/c mice within 3 days after infection (Chapter II). In examining the source of IFN-γ at day three post-infection, only the number of IFN-γ^+ NK cells were significantly higher after infection. Previous research has suggested that NK cells are important in controlling mycoplasma in lung, through their release of IFN-γ (71, 72). However, we have demonstrated that the depletion of NK cells does not impair BALB/c mice from clearing mycoplasma from the lung. Suggesting that NK derived IFN-γ is not critical for the clearance of mycoplasma from the lung. It is possible that other cells such as $CD8^+$ or γδ T cells are becoming activated in the absence of NK cells and releasing IFN-γ early, as both have been documented to do, or cytokines with similar function of IFN-γ such as

IFN-α or IFN-β are activating macrophages (23-25, 121). Importantly, we have demonstrated a novel role for NK cell mediated activity in the lung. The depletion of NK cells from IFN-γ KO mice enhanced the ability to control mycoplasma infection in the lungs; in fact, the number of mycoplasma in the lung was similar to that of BALB/c mice, thus NK cells impair innate immune function in the absence of IFN-γ.

Macrophages of IFN-γ KO mice are not being removed from the BAL by bystander damage from NK cell granules. Mycoplasma can activate NK cells leading to cytokine and granule secretion (122-124). Now, we clearly show that NK cell granules do not affect mycoplasmas and mycoplasmas in return, do not interfere with granule activity. Furthermore, we found that NK granules can cause apoptosis of macrophages *in vitro*, and the addition of mycoplasma enhances macrophage apoptosis (data not shown). It is possible that *in vivo* NK cell activation is leading to degranulation and bystander damage to macrophage in the absence of IFN-γ, causing apoptosis and clearance of the cells that effectively clear mycoplasma from the lung. IFN-γ activity on macrophages may protect them from NK granule mediated apoptosis. However, IFN-γ KO mice do not have an increase in apoptotic macrophages in the BAL or corresponding lung, and the depletion of NK cells from IFN-γ KO mice does not lead to a decrease in apoptotic macrophages. Thus, NK cell killing of macrophages in an IFN-γ deficient environment is likely not responsible for the impairment of innate immunity. Possibly, NK cells are dampening innate immune responses in IFN-γ KO mice through the modulation of cytokine environment and not through innocent bystander damage of macrophages.

NK cells influence the anti-inflammatory cytokine environment generated in response towards pulmonary mycoplasma infection in IFN-γ KO mice. The clearance of mycoplasma from the lung is dependent on macrophage activity (70, 102). Cytokines influence the activity of macrophages; as pro-inflammatory cytokines such as IFN-α, IFN-β, or IFN-γ activate macrophages, while anti-inflammatory cytokines such as IL-10 and G-CSF can dampen macrophage activity (22, 37, 125). As discussed above, NK cells do not directly kill mycoplasma, nor kill macrophages by bystander damage in IFN-γ KO mice. Therefore the cytokine cascade that NK cells participate in must dampen innate immune mechanisms from clearing mycoplasma, which IFN-γ normally overrides. By utilizing an 18-plex cytokine suspension array, we were able to characterize the protein levels of 18 different cytokines in the BAL of three day infected BALB/c and IFN-γ KO mice before and after NK cell depletion. IFN-γ KO mice showed significant increases in levels of anti-inflammatory and pro-inflammatory cytokines and chemokines over BALB/c mice. Levels of IL-10, IL-17, TNF-α, and G-CSF were all significantly elevated in IFN-γ KO mice over BALB/c mice. This corresponds with mRNA data demonstrating increases in IL-10 and TNF-α in BAL cells isolated from IFN-γ KO mice. All of these cytokines have the ability to dampen or are involved in a cascade that dampens macrophage activation (22, 37, 125). Of these, IL-10 is well known for its ability to down-regulate macrophage's ability to phagocytize bacteria, as well as dampen inflammation (22, 37). At appropriate times, IL-10 protects tissue from being damaged by the inflammatory responses, but incorrectly express IL-10 can lead to chronic

infections and failure to clear invading pathogens (38, 126). TNF-α can stimulate macrophages to express G-CSF, which can dampen macrophage activity and cause a strong Th2 adaptive immune response (127-129). NK cells can release anyone of these three cytokines (130-133), and the depletion of NK cells lead to a decrease in IL-10, G-CSF, and TNF-α in IFN-γ KO mice. Further work needs to be done to determine if NK cells are directly responsible for the release of these cytokines in IFN-γ KO mice. Regardless, NK cells are involved either directly or through a cascade which leads to the secretion of anti-inflammatory cytokines in IFN-γ KO mice.

Pro-inflammatory cytokines are increased in IFN-γ KO mice during mycoplasma infection. Several pro-inflammatory cytokines, IL-1α and IL-6 significantly increase and IL-1β and IL-12 p70 show a trend towards an increase of elevation in IFN-γ KO mice. All four of these cytokines are cytokines which macrophages first release in response to invading pathogens to initiate inflammation (22, 44). All four cytokines are significantly decreased in IFN-γ KO mice after NK-cell depletion. This first demonstrates that macrophages are initially being activated and are trying to generate inflammatory responses to clear mycoplasma from the lung. However, I believe this increase in pro-inflammatory cytokine levels is due to the significantly higher mycoplasma organism present in the lungs, which leads to more mycoplasma macrophage interaction, causing increased cytokine secretion. With the depletion of NK cells, IFN-γ KO mice can clear the mycoplasma from the lung, leading to a decrease in macrophage-mycoplasmas interaction and subsequent decrease in pro-inflammatory cytokine levels.

IFN-γ KO mice have a different cellular inflammatory response in the alveoli and bronchial airways than seen in BALB/c mice. In normal BALB/c and IFN-γ KO mice, we see a mixed neutrophilic macrophage infiltrate in alveoli and bronchial tubes. Though the number of cells isolated from the BAL of IFN-γ KO mice was significantly higher than that of BALB/c mice. It is possible for neutrophils to dampen macrophage activity (134), it is possible that without IFN-γ neutrophils significantly inhibit macrophages in the lung from killing mycoplasma. Furthermore, the respiratory burst of neutrophils is unable to kill mycoplasma (135, 136). The depletion of NK cells in IFN-γ KO mice leads to a significant decrease in neutrophils and increase in macrophages. This suggests that NK cells are involved in the recruitment of neutrophils into the alveoli and airways. In fact, the levels of the chemokines, KC, are elevated in IFN-γ KO mice and are decreased by NK cell-depletion. KC has a similar function to IL-8 in humans, as it is a chemoattractant for neutrophils (137, 138). Interestingly, the level of KC correlates well with the number of neutrophils isolated from the BAL. KC has also been documented to be secreted by NK cells, however further research needs to be done to determine if NK cells directly secrete KC during mycoplasma infection. Thus, these studies demonstrate that NK cells are involved in recruitment of neutrophils into the site of infection either by chemokine secretion or involved in a cytokine cascade that recruits neutrophils.

NK cells in IFN-γ KO mice have a novel role during mycoplasma respiratory infection. We had previously demonstrated that IFN-γ was important for the development of innate immune responses and clearance of mycoplasma from the lung (Chapter II). Previous work by Lai et. al. suggests that NK cells role during mycoplasma

infection, is through the release of IFN-γ leading to activation of macrophages (71, 72). However, in contrast, we clearly show that NK cells are not required for the clearance of mycoplasma from the lungs of BALB/c mice. Since the discovery that NK cells can kill tumor cells without prior stimulation, there have been many novel roles discovered for NK functions (49, 50, 105-109). We show a novel role for NK cells in IFN-γ KO mice, where NK cells inhibit innate immunes ability to clear mycoplasma from the lung. We believe that the role of NK cells seen in IFN-γ KO mice is present in the lungs of BALB/c mice; however, IFN-γ is able to counteract these anti-inflammatory properties. In unpublished data, the depletion of NK cells before immunization confers better protection than immunization alone. Therefore, we may have identified a function for a subpopulation of NK cells that are present in the lung. The function of these NK cells needs to be examined in other respiratory infection models. If present, the utilization or neutralization of these NK cells functions in the lungs can open a new strategy for vaccines and therapies for respiratory infections.

CHAPTER V

Asthma is a common syndrome that is likely multifactorial and heterogeneous in etiology and pathogenesis. Evidence has clearly demonstrated that bronchial hyperresponsiveness and airway reconstructioning that is characteristic of asthma is Th2 dependent (65). Studies in mice clearly demonstrate that the loss of IL-4 blocks asthma induce BHR in an ovalbumin model. Work in humans, correlates IL-4 levels with severity of asthmatic attacks (66). Infectious respiratory disease can lead to exacerbation of acute asthmatic attacks, and one key infectious agent is *M. pneumoniae*. Up to 25% of asthmatics experiencing acute attacks have *M. pneumoniae* isolated from their respiratory tracts, and mycoplasma infections are a possible contributing factor in the severity and/or development of asthma in humans (58). Studies with *M. pneumoniae* in mice further demonstrate that mycoplasma infections can exacerbate BHR in ovalbumin asthma model (61). The mechanism behind mycoplasma infection exacerbating asthma is poorly understood.

In previous research, I have demonstrated that IL-4 does not impact the development of mycoplasma disease severity in the upper or lower respiratory tract (Chapter II). However, further studies were needed to truly demonstrate that IL-4 does

not impact immune generation against mycoplasma respiratory infection. By utilizing IL-4 KO mice, I began to examine how IL-4 contributes to mycoplasma mediated BHR. This study would give insight into the mechanisms by which mycoplasma infections can exacerbate asthma attacks. I believed that in line with other asthma studies, that the loss of IL-4 would block or dampen BHR that is developed during mycoplasma disease. The next paper begins to answer the contribution of IL-4 during the development of BHR during methacholine induce BHR.

CHAPTER VI

IL-4 Dampens Methacholine Induced Bronchial Hyperresponsiveness During Pulmonary Mycoplasma Infection

The purpose of this study was to evaluate the role of IL-4 during methacholine induced bronchial hyperresponsiveness (BHR) during mycoplasma pulmonary infection. Research has long demonstrated a correlation between respiratory infections and exacerbation of BHR in asthma patients. Asthmatic induced BHR has long been demonstrated to be an IL-4 mediated event. *Mycoplasma pneumoniae* can be isolated form the respiratory tracts of up to 25% of asthmatics experiencing acute exacerbations. The mechanism behind this acute exacerbation is poorly understood. By using *Mycoplasma pulmonis*, we can study a natural murine respiratory disease that is similar to *M. pneumoniae* infection in humans. By utilizing IL-4 knockout (KO) mice, we can follow methacholine induced BHR during *M. pulmonis* infection using whole-body plethysmography. We infected BALB/c and IL-4 KO mice with *M. pulmonis*, and then monitored Penh scores before and after methacholine inhalation with whole-body plethysmography. IL-4 KO mice showed no difference in histopathology of the lungs before or after mycoplasma infection when compared to BALB/c mice. IL-4 KO mice

showed no difference in airway obstruction during mycoplasma infection when compared to BALB/c control mice, as both had increased airway obstruction from days 7 to 21. However, IL-4 KO mice had significantly higher methacholine induced BHR during mycoplasma respiratory infection when compared to BALB/c mice. Uninfected mice showed no difference in methacholine induced BHR between strains. This demonstrates that mycoplasma induced BHR is not IL-4 mediated, in fact IL-4 dampens this mycoplasma induced BHR. Suggesting, that the mechanism by which mycoplasma exacerbates asthmatic attacks, is both IL-4 and non-IL-4 mediated responses.

Introduction

Mycoplasma pneumoniae is a common cause of both upper and lower respiratory tract infections in humans. Acute mycoplasma infection, along with other respiratory infections, is associated with the exacerbations of asthma. *M. pneumoniae* can be isolated from the respiratory tract of up to 25% of asthmatics experiencing acute exacerbations (58). Chronic *M. pneumoniae* infection is suggested as a possible contributing factor to the severity or development of asthma in humans (58). *M. pneumoniae* was utilized to study bronchial hyperresponsiveness (BHR) in mice (60, 61); and Chu et al. (2003) demonstrated that *M. pneumoniae* infection in mice after sensitization and challenge with ovalbumin, increases BHR (61). Demonstrating *in vivo* that mycoplasma infections exacerbate allergic asthma. However, *M. pneumoniae* is not a naturally occurring pathogen in mice and does not precisely mimic human disease. *Mycoplasma pulmonis* is a natural murine respiratory pathogen with high morbidity and low mortality that is well characterized. *M. pulmonis* infection in mice is similar to *M.*

pneumoniae infection in humans in terms of both the nature of histologic inflammation generated and the chronic nature of the disease (57). This murine model allows us to study a natural host-pathogen interaction that is similar to *M. pneumoniae* in humans, and its affects on BHR.

Asthma is a common syndrome that is likely multifactorial and heterogeneous in etiology and pathogenesis. The physiological and inflammatory mechanism's that lead to an increase of BHR in asthmatic patients is not fully understood. Evidence suggests that allergic asthma is associated with Th2 responses, where IL-4 mediated inflammation causes BHR (65). Studies, in both humans and mice, demonstrate the pathogenic importance of IL-4 in the development of BHR seen in allergic asthmatic (65, 66). In fact, studies with IL-4 KO mice demonstrate an attenuation of allergic model BHR compared with wild type mice; yet, few studies have examined IL-4 in an infectious model of BHR (65). However, the current view is that most BHR in either infectious or asthmatic models are Th2 dependent. Evidence demonstrates that people who live in developed countries are at a higher risk of developing asthma, leading to the development of the hygiene model, were recurrent respiratory infections Th1 mediated inflammatory response that shift the lung away from a Th2 environment (61). Without these recurring stimuli, the lung skews to a Th2 laden environment, allowing for the development of allergic asthma. IL-4 is the key cytokine that leads to a Th2 environment in the lung and to the development of asthma and asthma-induced BHR.

Since the 1970's, epidemiological data has shown a correlation between asthma exacerbations and respiratory infections in humans. However, few studies have

characterized the mechanisms of respiratory infections impact on BHR. In the present study, IL-4 KO mice are utilized to identify the impact of IL-4 on methacholine induced BHR during *M. pulmonis* infection.

Materials and Methods

Mice. Viral- and mycoplasma-free BALB/c, and IL-4 (BALB/c-Il4^{tn2Nnt} on a BALB/c background) knockout (KO) mice were obtained from the Jackson Laboratories (81). Mice were housed in sterile microisolator cages supplied with sterile bedding, and sterile food and water were given ad libitum. Female mice used in the study were between 8-12 weeks of age. Before experimental infection, mice were anesthetized with an i.m. injection of ketamine/xylazine.

Mycoplasma. The UAB CT strain of *M. pulmonis* was used in all experiments. Stock cultures were grown, as previously described (82), in mycoplasma medium and frozen in 1-ml aliquots at -80^0C. For inoculation, thawed aliquots were diluted to 10^5 CFU/20 µl. Nasal-pulmonary inoculations of 20 µl of diluted mycoplasma were given for experimental infections.

Plethysmography. Whole-body, unrestrained plethysmography (Buxco, Troy, N.Y.) was utilized to monitor the respiratory dynamics of mice in a quantitative manner before and after methacholine exposure. Prior to methacholine exposure, previously inoculated mice were allowed to acclimate to the chamber, and then plethysmography readings were recorded to establish baseline values. Next the mice were exposed to aerosolized methacholine (50 mg/ml); after exposure, plethysmography readings were recorded again. Enhanced pause (Penh) is a dimensionless value that represents a

function of the ratio of peak expiratory flow to peak inspiratory flow and a function of the timing of expiration. Penh correlates with pulmonary airflow resistance or obstruction. Penh as measured by plethysmography has been previously validated in animal models of airway hyperresponsiveness (59). Baseline and methacholine challenge Penh was evaluated at days 7, 14, and 21 post-infection.

Statistical Analysis. Data was evaluated by ANOVA, followed by Fisher Protected Least Square Differences Multigroup comparison. These analyses were performed using the StatView (SAS Institute, CARY, NC) computer program. A P value ≤ 0.05 was considered statistically significant.

Results

Control and IL-4 KO mice had increased airway obstruction after infection with *M. pulmonis*. To determine if IL-4 had an effect on pulmonary airway resistance and obstruction, control and IL-4 KO mice were experimentally infected with *M. pulmonis* or sham inoculated, and then baseline Penh scores were monitored on day 7, 14, and 21 post-infection by whole-body unrestrained plethysmography.

Mycoplasma infection leads to a time dependent increase in baseline Penh values in both control and IL-4 KO mice (Fig 1a). By day 14 post-infection a significant increase in baseline Penh scores was detected in both strains of mice used and stayed elevated through day 21 post-infection, this increase was not seen at earlier time-points (day 7). There was no difference between infected control and IL-4 KO mice in airway obstruction.

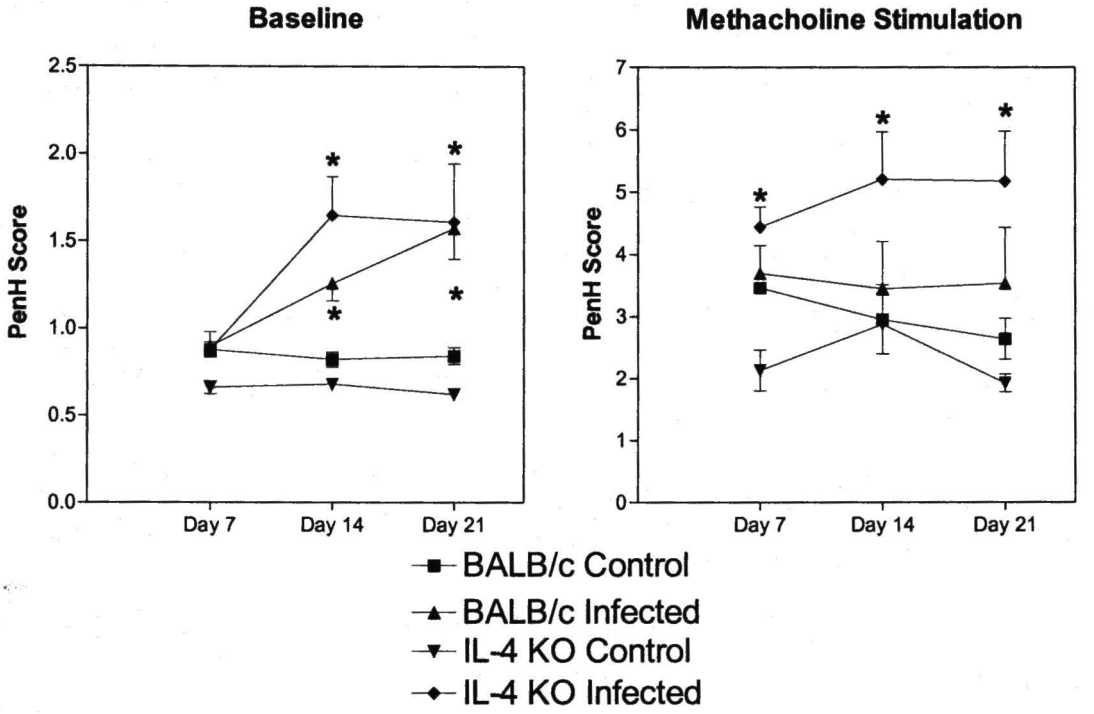


Figure 1. Airway obstruction and BHR scores of BALB/c and IL-4 KO mice. At days 0, 7, 14 and 21 post-infection Penh scores were monitored in BALB/c and IL-4 KO mice before and after methacholine inhalation by unrestrained whole-body plethsymography. A) "*" denotes statistical difference ($p \leq 0.05$) from uninfected mice. B) "*" denotes statistical difference ($p \leq 0.05$) from all other groups.

IL-4 KO mice had increased airway hyperresponsiveness after mycoplasma infection. To determine if IL-4 had an effect on BHR, control and IL-4 KO mice were experimentally infected with *M. pulmonis* or sham inoculated, and then methacholine induced hyperesponsiveness Penh scores were monitored on days 7, 14, and 21 post-infection by whole-body unrestrained plethysmography.

IL-4 KO Mycoplasma-infected mice had a significant increase in the methacholine-induced hyperresponsiveness Penh scores over sham inoculated IL-4 KO mice and both uninfected and infected control mice (Fig 1b). There was no significant difference between infected and uninfected control mice methacholine induced BHR.

Discussion

The purpose of this study was to determine the involvement of IL-4 in the development of methacholine-induced BHR during mycoplasma infection. Mycoplasma has long been associated with the asthma exacerbations (58). It has also been implicated as a chronic pathogen that may act as a co-factor in the severity or the development of asthma in an individual (58). IL-4 mediated responses have been demonstrated to be important in the development of asthma in both murine and human studies (65). Studies have examined the role of infectious agents and the development of a BHR. Studies with *M. pneumoniae* clearly demonstrate that mycoplasma infections can exacerbate BHR in an ovalbumin asthma model, when the infection is introduced after sensitization to OVA (61). *M. pneumoniae* is a human pathogen that creates a strong acute infection in mice, and a low level chronic infection, however it fails to generate a robust chronic infection that is typical in human infections (59) However, *M. pulmonis* infection in mice creates

both similar histologic inflammation as well as a robust chronic infection that is characteristic of human mycoplasma infections. By utilizing *M. pulmonis* infection in mice, we hope to understand the involvement of IL-4 in the development of a BHR during mycoplasma infections. These studies will help in the understanding of infectious agents and how they exacerbate airway hyper reactivity in asthmatic patients.

Airway obstruction increases during the development of mycoplasma disease in both IL-4 KO and control mice. In chapter II I demonstrated that the loss of IL-4 does not impact disease histopathology by day 14 post-infection, at time points in the disease state where BHR is exacerbated. Demonstrating that the loss of IL-4 did not affect airway obstruction during mycoplasma disease. Furthermore, mice infected with *M. pulmonis* did not demonstrate airway obstruction until day 7 to day 14 post-infection and continued through day 21, at which time both IL-4 KO and control mice showed increased obstruction. This development of airway obstruction coincides with the development of a strong T cell response, as publish in previous papers by our lab (68). Therefore airway obstruction that is developed during mycoplasma infection is adaptive immune dependent that is not IL-4 mediated.

IL-4 is important in dampening BHR during mycoplasma infection. IL-4 KO mice had an overall significant increase in methacholine induced airway hyper reactivity after mycoplasma infection when compared to all other groups tested. Interestingly, IL-4 mediated inflammatory responses are necessary for the development of asthma in humans and mice (65, 66). However, this study demonstrates that during mycoplasma infection, IL-4 dampens detrimental inflammatory responses that lead to exacerbated BHR. This

suggests that unlike asthma, IL-4 does not mediate BHR during mycoplasma infection, but rather protects against it. Suggesting that non-IL-4 mediated events, possibly Th1 inflammation, are the cause of increased BHR during mycoplasma infection. This coincides with recent human studies, were Th1 cytokines are isolated from the BAL of patients experiencing acute asthmatic episodes (66). Suggesting that Th1 mediated responses may contribute to BHR in asthmatics. This is contrary to current theories that claim that IL-4 is absolutely necessary for BHR. However, this study clearly demonstrates that BHR during mycoplasma infections is IL-4 independent.

Asthma is a multi-billion dollar a year impact on the economy (139). Research has been done to understand and treat this important disease. However, the etiology of respiratory disease on asthma is poorly understood. Evidence clearly demonstrates a correlation between several respiratory infectious agents, notably mycoplasma, and either development or exacerbation of asthmatic episodes (5). This research begins to uncover the mechanisms by which pulmonary disease can affect and exacerbate BHR. In contrast to current theories on asthma induced BHR, we show, that non IL-4 mediated events lead to increased BHR during mycoplasma infections, and that IL-4 is important in dampening these reactions. This suggests that there is a synergistic affect of IL-4 and non-IL-4 mediated responses seen in asthmatics with mycoplasma infections that lead to exacerbated BHR.

CHAPTER VII

T Helper 2 Mediated Responses Exacerbate Mycoplasma Pulmonary Disease Severity

The purpose of this study was to identify the roles of Th1 and Th2 mediated immune responses during pulmonary mycoplasma infection. Immunization studies in *Mycoplasma pulmonis* infected mice have conferred protection from disease. By immunizing IFN-γ knockout (KO) and IL-4 KO mice we were able to determine the involvement of Th1 and Th2 in the development of pathogenesis and/or protection during mycoplasma respiratory infection. The T cell responses of spleen cells were Th1 for IL-4 KO and Th2 for IFN-γ KO. T cells of the lung on the other hand were not as clearly skewed Th1 or Th2 as they were only deficient in their respective cytokine that was knocked out. However, immunization suggest that the adaptive immune responses of IFN-γ KO and IL-4 KO mice have different contributions to the pathogenesis of mycoplasma respiratory disease. Immunization of IFN-γ KO mice leads to an increase in gross lesion severity of the lungs, that is not seen in immunized BALB/c or IL-4 KO mice. Suggesting that Th2 mediated responses significantly contributes to pathogenesis of mycoplasma respiratory disease. However the mechanism, this work demonstrates that IFN-γ is important in the development of beneficial adaptive immune responses during mycoplasma pulmonary disease.

Introduction

Mycoplasma infection is a leading cause of pneumonia worldwide. In the United States, alone, *Mycoplasma pneumoniae* accounts for 30% of all cases of pneumonia (2-4). Mycoplasma disease is also associated with the exacerbation of other respiratory diseases, such as asthma (5, 6). *Mycoplasma pulmonis* causes a naturally occurring murine respiratory disease with high morbidity and low mortality. *M. pulmonis* is an excellent animal model of *M. pneumoniae,* allowing the characterization of immune responses during the pathogenesis of mycoplasma respiratory disease. Both *M. pulmonis* and *M. pneumoniae* respiratory infections cause rhinitis, otitis media, laryngotracheitis, and bronchopneumonia. In terms of histopathology, both diseases are characterized by the accumulation of mononuclear, macrophages and lymphocytes, cells along the respiratory airway (4, 56, 57, 63, 64). This suggests that the activation and recruitment of macrophages and lymphocytes is key in the development of both acute and chronic states of the disease. In support, several studies demonstrate a component of mycoplasma respiratory disease is immunopathologic (11-15).

The adaptive immune response during mycoplasma respiratory disease is both protective and pathological. SCID, which lack T and B cells, fail to develop disease pathology in the lung as seen in wild type mice (11-15, 68, 140). However, SCID mice develop arthritis and have a higher rates of mortality than corresponding immuno-competent mice (11). Interestingly, SCID mice do not show clinical signs of disease before day 14, after which they become extremely ill and can die, this unlike control mice, which begin to show signs of disease at day 7 to 14. Reconstitution of SCID mice

whole splenocytes confers protection and an increase in disease severity comparable to immunocompetent mice. Our lab has further shown that T cell immune responses do not develop until days 10 to 14, corresponding to the time points when control mice begin to show clinical signs of disease (68, 77). Data clearly suggests that adaptive immunity contribute to both pathogenesis and protection during mycoplasma respiratory infection.

Adaptive immune responses are dictated by cytokine interactions. Interferon-gamma (IFN-γ) and Interleukin-4 (IL-4) are pleiotrophic cytokines that have an impact on both innate and adaptive immune responses (26, 33, 79). IFN-γ is key in aiding macrophage activation during innate immune responses and causing T helper (Th) 1 cell phenotypic development (26, 79). IL-4 aids in the development of antibody production and Th2 T cell phenotypic development(33). Work in our lab has demonstrated that the requirements of IFN-γ and IL-4 differ between upper and lower respiratory tract immune responses. The loss of either IL-4 or IFN-γ did not affect mycoplasma disease or organism numbers in the upper respiratory tract, this in direct contrast to the lower respiratory tract where higher mycoplasma organism number and increased disease severity was seen in IFN-γ KO mice (Chapter I). A disruption in innate immune response, most notable NK cell function, was attributed to the increase in mycoplasma CFU (Chapter IV). This early innate immune disruption precludes any effect that adaptive immune response may contribute to disease severity. However, research into T cell responses during mycoplasma infection and further research with IFN-γ and IL-4 KO mice will provide valuable information into mycoplasma disease.

T cell responses can be broken into two classic phenotypes, Th1 and Th2. Th1 cells are characterized by IL-2 and IFN-γ secretion, and aid macrophages, by activating them when laden with intracellular bacteria through IFN-γ secretion (26, 79). Th2 T cells are characterized by IL-4, IL-5, and IL-10 secretion and aiding B cell in antibody production (52). Other and our labs have demonstrated that the lungs are predominately Th2 environment (67, 78). After mycoplasma infection a mixed Th1 Th2 response is generated (77). However, which T cell response is beneficial and which is detrimental is unknown. Research into understanding the T cell responses during mycoplasma respiratory infection will aid in the generation of effective vaccine strategies.

The purpose of this study was to identify the involvement of Th1 and Th2 mediated responses during mycoplasma respiratory infection. By utilizing immunization strategies in IFN-γ and IL-4 KO mice, we can prime T cells prior to infection. Memory T cells, Th2 in IFN-γ KO and Th1 in IL-4 KO, would be activated at the time of infection. Therefore the disruption in innate immune responses would be minimal, and characterization of T cell responses would be possible. This study demonstrated that Th2 mediated T cell responses are pathogenic during mycoplasma respiratory infection.

Materials and Methods

Mice. Viral- and mycoplasma-free BALB/c, IFN-γ (C.129S7(B6)-ifng^{tm1Ts} on a BALB/c background) knockout (KO) and IL-4 (BALB/c-Il4^{tn2Nnt} on a BALB/c background) KO mice were obtained from the Jackson Laboratories (79, 81) and breeding colonies were established. Mice were housed in sterile micro isolator cages supplied with sterile bedding, and sterile food and water was given, ad libitum. Mice

used in the study were between 8-12 weeks of age. Female mice were used in all studies unless where noted in the results. Before experimental manipulation, mice were anesthetized with an i.m. injection of ketamine/xylazine.

Mycoplasma. The UAB CT strain of *M. pulmonis* was used in all experiments. Stock cultures were grown, as previously described (82), in mycoplasma medium and frozen in 1-ml aliquots at -80^0C. For inoculation, thawed aliquots were diluted to 10^5 CFU/20 μl. Nasal-pulmonary inoculations of 20 μl of diluted mycoplasma were given for experimental infections.

Immunogens and adjuvants. Cholera toxin (CT) was purchased from List Biological Laboratories, Inc. (Campbell, CA), and 0.1 μg was used for intranasal and intraperitaneal immunizations. *M. pulmonis* antigen was a crude membrane preparation as previously described (91). Briefly, thawed *M. pulmonis* stocks were cultured at 37°C in PPLO broth and harvested at pH 7.0. Cells were then centrifuged at 10,000 rpm for 20 min and pellets were re-suspended in 5 ml of sterile NaCl. Following a second centrifugation at 9000 rpm for 20 min, pellets were resuspended in a total of 4 ml 2M glycerol at 37°C for 10 min. Cells were then sonicated at highest setting for 15 sec, followed incubation at 37°C for 10 min. For cell lysis, 0.5 ml of cell preps were then forced through a 27 gauge needle into 25 ml aliquots of distilled water. To remove unlysed organisms, cells were centrifuged at 10,000 rpm for 20 min. Supernatants were again centrifuged at 20,000 rpm for 1 hour. The crude preparations of mycoplasma membrane was resuspended in 5 ml of PBS (Hyclone, Logan, UT). The absence of

viable organisms was verified by culture, and the *M. pulmonis* antigen was stored at –80°C. All centrifugations were done at 20°C. 5 μg/ml of *M. pulmonis* membrane antigen was used for immunizations as indicated in results.

Assessment of Gross Lesions. Lungs were removed, and each lobe was examined by two observers for the presence of gross lesions. The percentage of each lobe with gross lesion was recorded. The gross lesion scores were weighted by the percentage that each lobe contributes to the total lung weight in arriving at the gross lesion index for lungs (88).

Cell Isolation. Mononuclear cells were isolated from lungs, as previously described (75, 83, 84). Lungs were perfused with PBS without magnesium or calcium to minimize contamination of the final lung cell population with those from the blood. The lungs were finely minced. The tissues were suspended in RPMI 1640 medium (HyClone Laboratories, Logan, UT) containing 300 U/ml Clostridium histolyticum Type I collagenase (Worthington Biochemical, Freehold, NJ), 50 U/ml DNase (Sigma-Aldrich, St. Louis, MO), 10% FBS (Hyclone Laboratories), HEPES (Fisher Scientific, Pittsburgh, PA) and antibiotic/antimycotic solution (Life Technologies, Grand Island, NY). The tissues were incubated at 37^{0}C while mixing on a Nutator (Fisher Scientific) for 90-120 min. During the incubation period, the tissue and were vigorously pipetted every 30 min. After incubation, the digestion mixture was passed through a 250-μm nylon mesh to remove undigested tissue. Mononuclear cells were purified from cell suspension by density gradient centrifugation using Lympholyte M (Accurate Chemicals, Westbury, NY).

Spleen cells were isolated after centrifugation of all suspensions, followed by red cell removal using ACK (ammonium chloride potassium) lysis buffer, as previously described (85).

Preparation of M. pulmonis antigen for *In vitro* stimulation. To prepare antigen for stimulation, *M. pulmonis* was cultured at 37^0C in mycoplasma broth medium for 3 days and harvested. *M. pulmonis* broth was adjusted to 5 mg/ml protein concentration. Twenty ml lysis buffer (4.2 g $NaHCO_3$/L and Na_2C0_3/L) pH 10.0 warmed to 37^0C were added to each 1 ml of *M. pulmonis* stock and incubated at 37^0C fro 15 minutes. Then 2.2 g of boric acid were added to 100 ml of lysis buffer and then frozen at -70^0C. Protein concentration was then determined by Bradford assay.

Ag-specific in vitro stimulation of mononuclear cells. Lymphoid cells were cultured in 96-well round-bottom microtiter plates in RPMI 1640 (HyClone Laboratories) supplemented with 10% FBS (Hyclone Laboratories), HEPES, antibiotic/antimycotic solution (Life Technologies). Lymphoid cells were stimulated at 37°C and 5% CO_2. Cells were stimulated with or without 25 μg/ml prepared M. pulmonis Ag in a final volume of 200 μl/well culture media at a cell concentration of 2×10^6 cells/ml. Supernatants were collected 4 days later and stored at –80°C until assayed for cytokine concentrations.

Cytokine Assays. The levels of cytokines were measured by Bio-Plex suspension array. Levels of IL-2, IL-4, IL-5, IL-10, IL-12p70, GM-CSF, IFN-γ and TNF-α was measured by Bio-Plex Th1 Th2 cytokine panel (Bio-Rad, Hercules, CA). 96-well filter bottom plates were used. To each well 50 μl of anti-cytokine beads in assay

diluent were added to each well. Plates were washed with Bio-Plex washing buffer. 50 µl of sample or standard was added per well. Plates were washed with Bio-Plex washing buffer. Plates were incubated at room temperature while shaking in the dark for thirty minutes. Plates were washed with Bio-Plex washing buffer. 25 µl of biotinylated secondary antibodies were added to each well. Plates were incubated at room temperature while shaking in the dark for thirty minutes. Plates were washed with Bio-Plex washing buffer. 50 µl of strepavidin-PE was added to each well. Plates were incubated at room temperature while shaking in the dark for thirty minutes. Plates were washed with Bio-Plex washing buffer. Samples were read using Bio-Plex 100 system (Bio-Rad, Hercules, CA). Cytokine levels were determined by comparison with standard curves generated from murine recombinant cytokines and analysed using Bio-Plex Manager Software (Bio-Rad, Hercules, CA).

Statistical Analysis. Data was evaluated by ANOVA, followed by Fisher Protected Least Square Differences Multigroup comparison. These analyses were performed using the StatView (SAS Institute, CARY, NC) computer program. When appropriate, data was logarithmically transformed prior to statistical analysis, and confirmed by a demonstrated increase in power of the test after transformation of the data. A P value ≤ 0.05 was considered statistically significant.

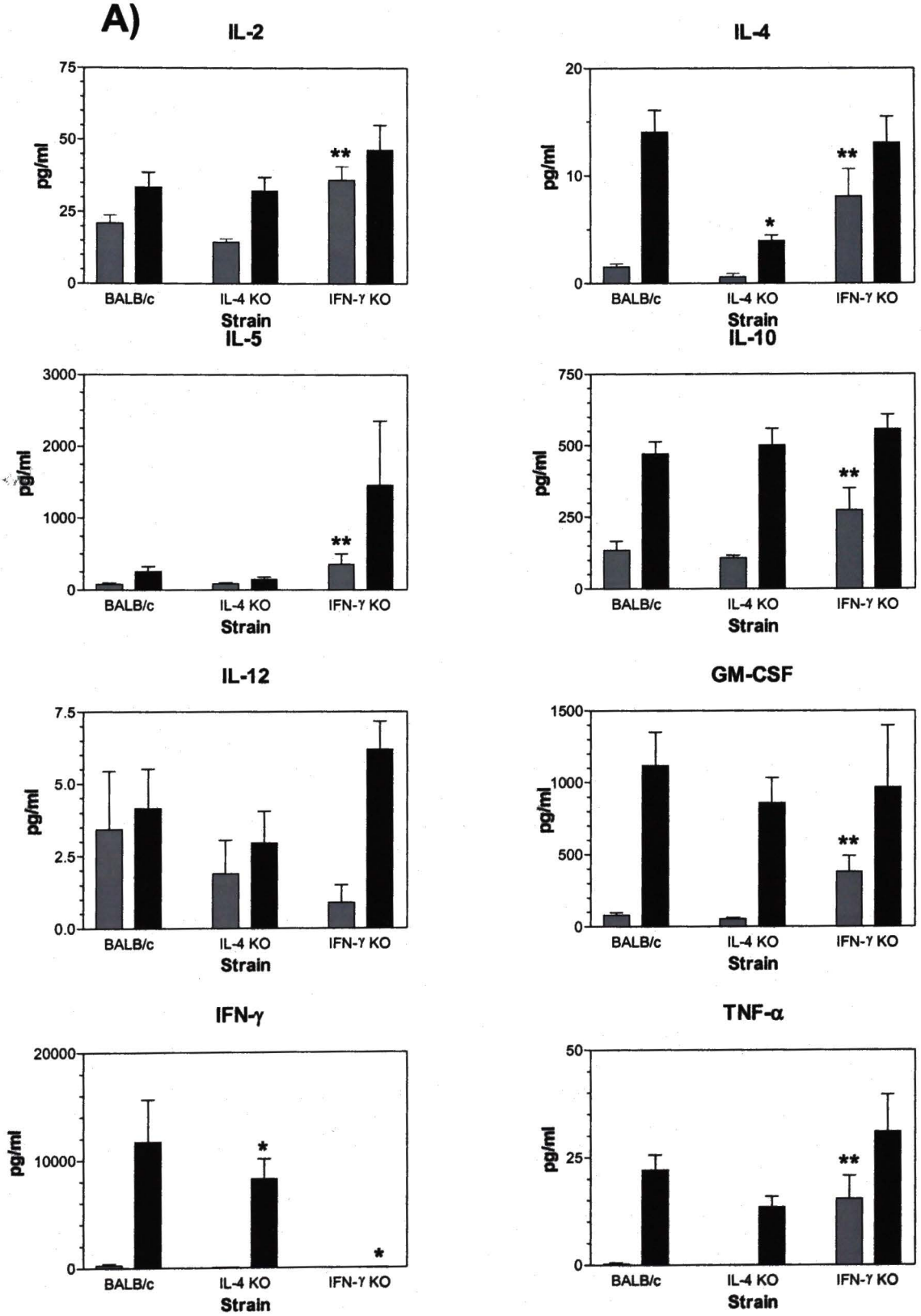
Results

T cell isolated from the lungs of IFN-γ and IL-4 KO mice significantly differ than those from BALB/c mice in levels of cytokines secreted. To characterize the T cell environment that is developed in BALB/c, IL-4 and IFN-γ KO mice were infected

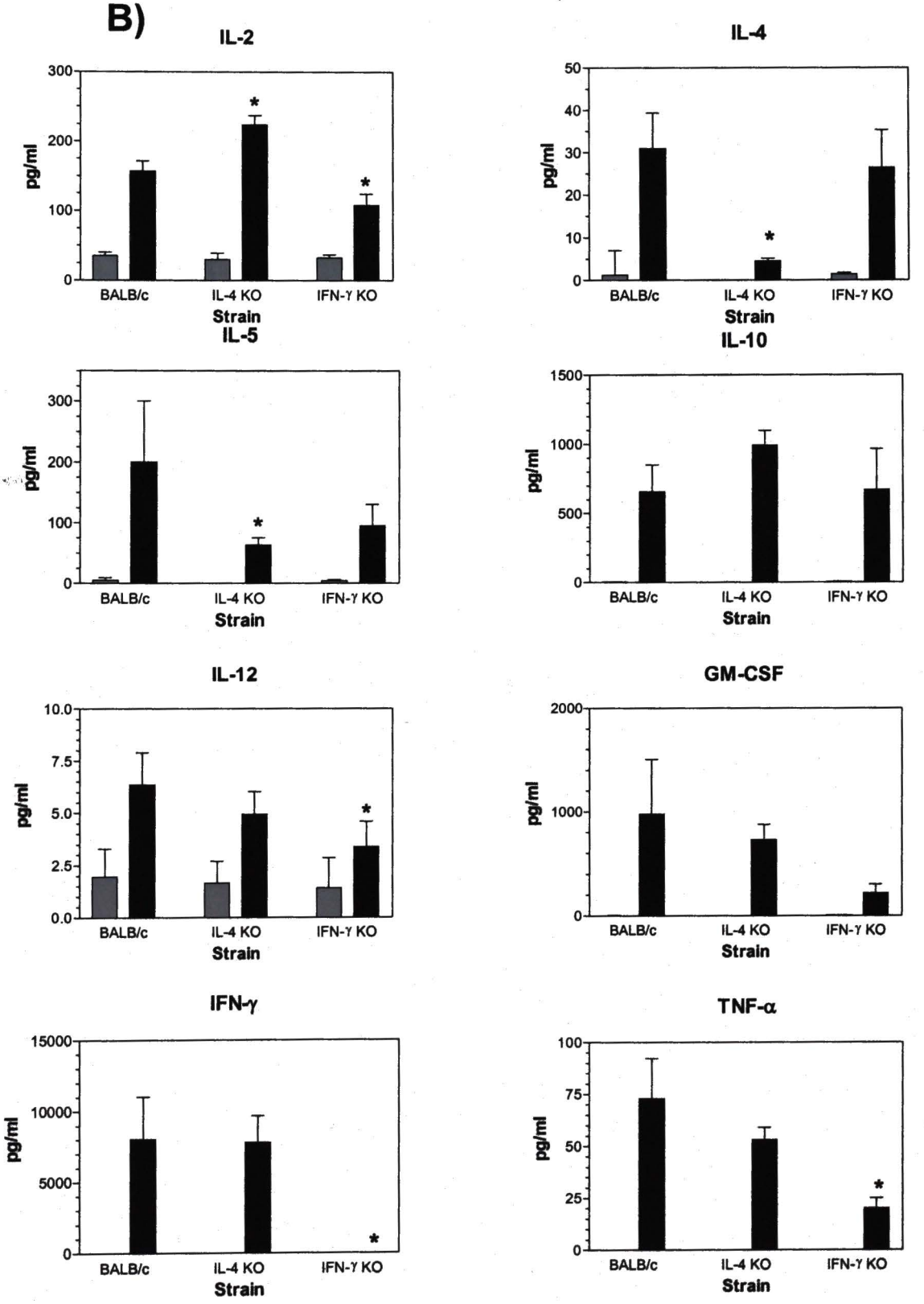
with *M. pulmonis*. On day 14 post-infection total cells were isolated from the lungs and spleen, and were incubated with mycoplasma antigen to stimulate the T cells. After 4 days, the supernatants were collected and Th1 and Th2 cytokine concentrations were determined by luminex suspension bead array. Levels of IL-2, IL-4, IL-5, IL-10, IL-12, GM-CSF, IFN-γ and TNF-α were measured. Spleen lymphocytes of BALB/c mice show a significant increase in levels of IL-2, IL-4, IL-10, IFN-γ and TNF-α after stimulation (Fig 1a). Levels in IFN-γ KO spleen stimulated lymphocytes differ in Th1 mediated cytokines IL-2, IL-12, IFN-γ and TNF-α being significantly lower. The spleens of IL-4 KO mice only differed in their levels of IL-2 and IL-4. Stimulated lung lymphocytes of BALB/c mice showed significant increase in levels of IL-4, IL-5, GM-CSF, IFN-γ, and TNF-α (Fig 1b). The lymphocytes of IFN-γ KO mice were only different in IFN-γ levels, while IL-4 KO lymphocytes had decreased secretion in IL-4 and IFN-γ.

Immunizing IFN-γ KO mice lead to increase disease severity in the lungs. To determine if Th1 and Th2 T cell environments are beneficial or detrimental, BALB/c, IFN-γ KO and IL-4 KO mice were immunized with mycoplasma antigen. Mice were then challenged with *M. pulmonis* and 14 days post-infection lungs were isolated and gross lesions were determined. Intranasal immunization lead to a trend in decreased lesion severity in BALB/c and IL-4 KO mice. However, IFN-γ KO mice had a significant increase in gross lesion severity after intranasal immunization. No other immunization strategy had any significant affect of lesion severity.

Figure 1. Cytokines secretion of stimulated cells isolated from the spleens and lungs of infected mice. Lymphoid cells were isolated from the lungs or spleens of 14 day infected BALB/c, IL-4 KO and IFN-γ KO mice. Cells were stimulated with mycoplasma antigen and after 4 days supernatants were collected. Supernatants were analyzed for cytokine concentration utilizing Bio-Plex suspension array. A) Lung lymphoid cytokine levels. B) Spleen lymphoid cytokine levels "**" signifies significant difference ($p \leq 0.05$) from BALB/c un-stimulated. "*" signifies significant difference ($p \leq 0.05$) from corresponding BALB/c stimulated (n=7).


A)
IL-2
IL-4
IL-5
IL-10
IL-12
GM-CSF
IFN-γ
TNF-α
pg/ml
BALB/c
IL-4 KO
IFN-γ KO
Strain
**
*

B)


IL-2
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Strain

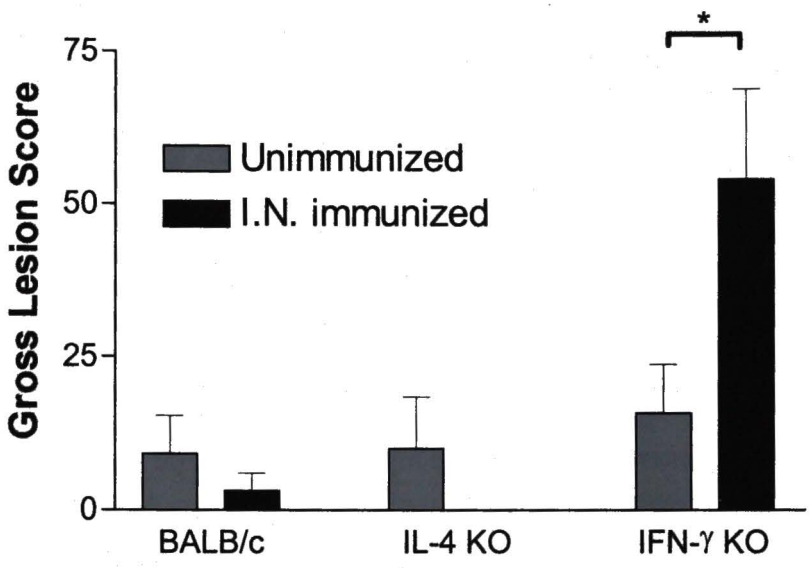

Figure 2. Gross lesion of lung in intranasal immunized BALB/c, IL-4 KO and IFN-γ KO mice. Mice were intranasally immunized with mycoplasma antigen on day 0 and boosted on day 7 and challenged on day 1 4. 14 days post-infection, lungs were scored for percentage of gross lesion. Vertical bars represent mean ± SE (n=8). "*" denotes statistical difference ($p \leq 0.05$) from strain unimmunized.

Discussion

Adaptive immune responses are an important component of mycoplasma respiratory disease, as they are both protective and contribute to the pathogenesis of the disease. However, what components of the adaptive immune response are protective and which are detrimental are still unknown. In particular, the activation of Th1 and Th2 cell subsets and their function has not been studied during mycoplasma disease, despite the fact that their impact on other diseases has been well characterized (52, 141-148). Th subsets are divided by the cytokines they secrete, IFN-γ being Th1 and IL-4 being Th2. Previous research has demonstrated that IFN-γ impacts disease pathogenesis during mycoplasma respiratory disease (Chapter II). However, the loss of IFN-γ impacts the generation of an effective innate immune response and this disruption can affect the development of adaptive immune responses. By utilizing immunization techniques in IFN-γ and IL-4 KO mice, we can determine the impact of Th1 and Th2 mediated responses on the development of mycoplasma disease pathogenesis.

There are differences in cytokine secretion of T cells isolated from cytokine KO mice. BALB/c stimulate splenocytes demonstrate a mixed Th1 Th2 response towards mycoplasma, as significant levels of IL-2, IL-4, IL-10, IFN-γ and TNF-α were increased. The lymphocytes isolated from the spleen of IFN-γ KO mice were strongly polarized Th2 as they have significant levels of Th2 cytokines evident, but lack the secretion of Th1 type cytokines. Demonstrating that systemically T cells of IFN-γ KO mice are Th2, while the T cells of IL-4 KO mice systemically are Th1 by nature. As IL-4 KO splenocytes do not secrete IL-4. Interestingly, the lung lymphocytes of BALB/c mice

secrete a slightly different cytokines, as there are significant increases in IL-4, IL-5, GM-CSF, IFN-γ and TNF-α secretion. Demonstrating that there are different immune responses systemically versus locally in BALB/c mice. Interestingly, lung lymphocytes of IFN-γ only differ in levels of IFN-γ, while IL-4 KO lymphocytes only lack IL-4 and a decrease in IFN-γ levels. IL-4 KO mice do not have less severe disease pathology than BALB/c mice (Chapter II). This cytokine data suggests that IL-4 KO mice do not have exaggerated IFN-γ levels; therefore do not offer any more protection than seen in control mice. This suggests that T cells of the cytokine KO mice only differ in their respective KO cytokines, and other functions of the T cells are not impaired. This being said, systemically it demonstrates that the T cells are skewed either Th2 in IFN-γ KO or Th1 in IL-4 KO, while locally there is only slight differences in cytokines secretion of T cells in cytokine KO mice.

Th2 mediated responses are detrimental during mycoplasma respiratory disease. Several respiratory disease models have demonstrated that strong Th2 responses are detrimental, and lead to chronic forms of the disease (149-151). Research has yet to determine the role of Th1 and Th2 responses during mycoplasma respiratory disease. The fact that a mixed T cell responses is noticed in immuno-competent mice, suggests one component of Th cell responses are beneficial and the other is detrimental. Immunized IFN-γ KO mice, which have activated Th2 responses prior to infection, have higher lesion severity than un-immunized IFN-γ mice. Suggesting that Th2 mediated responses is responsible for pathogenesis seen in BALB/c mice. Interestingly, IL-4 KO mice have the same amount of lesions as BALB/c mice, suggesting that Th1 mediated

responses are not necessarily beneficial, however they are not detrimental. Further studies to understand T cell involvement must be done. Regardless, this work does demonstrate that Th 2 cell responses can be detrimental, and this information can be used for better vaccine development. Arguably, if you can design a vaccine that does not promote Th2 mediated response; you would provide better protection against mycoplasma infection.

CHAPTER VIII

DISCUSSION

In conclusion, this work demonstrated several novel and unique functions of IFN-γ and IL-4 in respiratory tract immunity. I have demonstrated differences in upper and lower respiratory tract immunity, that IFN-γ counteracts NK dampening effects, IL-4 dampens mycoplasma-induced BHR, and Th2 type responses are detrimental during mycoplasma infection. These studies will change the way respiratory immunology is viewed. This information can be used for the development of vaccine strategies and to develop new techniques for control acute asthmatic attacks. Future work will be to determine the mechanisms behind NK and Th2 mediated functions and how to utilize them for beneficial use in vaccinations.

There are clearly differences in upper and lower respiratory tracts in their mechanisms of immune responses that control mycoplasma infections. Lower respiratory tract immune responses toward mycoplasma are dependent on IFN-γ mediated innate immune responses. The loss of these mechanisms leads to a significant increase in mycoplasma CFU burden and increased organism growth. This is all in direct contrast to the upper respiratory tract that does not require either IFN-γ or IL-4 to mount effective

immune responses towards mycoplasma infection. Thus, the mechanism behind the generation of immune responses toward mycoplasma infection is distinct between upper and lower respiratory tracts.

The differences in immune response between upper and lower respiratory immune responses in IL-4 and IFN-γ KO mice give valuable insight into the development of vaccines. Few studies have examined the difference in upper and lower respiratory tracts in any respiratory infection, let alone during mycoplasma infection. This will impact vaccine strategies as the new wave of vaccination is the intranasal delivery of the antigen to confer protection, in fact the newest line of nasal flu vaccines are all ready available (152). The type of immune responses needed to clear a pathogen from the lung, may not be effectively generated or be an incorrect response when immunity is first generated in the nasal passage. The wrong immune responses can be detrimental as seen in such respiratory diseases as tuberculosis and in the first vaccine against RSV (98, 151). On the other side, it may be possible to generate effective upper respiratory tract immunity to block the colonization of the nasal passages, even when impossible to develop a vaccine that confers protection in the lung. In fact, I believe we can utilize our model of mycoplasma infection to study this. We have previously demonstrated that a 10 μl innoculum preferentially deposits most of the antigen in the nasal passages. By utilizing this strategy, we can develop several studies. First, to determine if we preferentially infected the nasal passages of IL-4 and IFN-γ KO mice, is there dissemination of the organism to the lung, or is the mycoplasma localized to the upper respiratory tract? Followed by immunization of the upper respiratory tract only in these KO mice, this will

allow us to determine if we can confer protection from mycoplasma infection in these mice. These studies would help to determine if it is possible to skew the immune responses of the upper respiratory tract and confer protection to the lung. This obviously would be important in vaccine strategies, in that it could be possible to influence the type of immune responses generated during vaccination with appropriate use of adjuvants. Thus, further studies to understand differences in immune responses of the upper and lower respiratory tracts would be beneficial for vaccination and therapeutic strategies.

NK cells of the lung have unique anti-inflammatory properties that IFN-γ counteracts. As mentioned earlier, I have demonstrated that the IFN-γ is critical for the development of an effective innate immune response towards mycoplasma infection. The next logical assumption was that NK cell-derived IFN-γ was necessary for activation of macrophages to clear mycoplasma from the lung, as this had been previously suggested in earlier papers (71, 72). However, we clearly show this is not the case, as NK cell-depletion does not affect the ability of BALB/c mice to clear mycoplasma from the lung, but NK cell-depletion in IFN-γ KO mice conferred protection from mycoplasma organisms. Though the mechanism behind NK mediated activity in IFN-γ KO mice is still unclear, it is evident that NK cells can influence the type of inflammatory response in both cellular infiltrate and cytokine responses. Though depletion of NK cells in BALB/c had no affect, I believe these NK cell anti-inflammatory mechanisms are present in these mice. In fact, in preliminary studies, depletion of NK cells before immunization in BALB/c mice, then allowing the NK cells to recover before infection, resulted in better protection than immunization alone. This suggests that in some cases NK cells can be

detrimental even in BALB/c mice. I have shown that immunization of IFN-γ KO mice leads to increased disease severity; thus, Th2 responses are more likely pathogenic during mycoplasma infection. Anti-inflammatory processes tend to influence adaptive immune responses towards Th2 phenotypic development (52). However, further research must be done to identify and understand the role of these NK cells. It may be possible that these lung NK cells are unique, much like alveolar macrophages, and therefore play important roles in protecting the lung from damages by inflammatory processes. Once levels of IFN-γ are increased, it is capable of counteracting these NK cell-mediated events. Thus, NK cells of the lung dampen innate immune mechanisms, and impair clearance of mycoplasma from the lung in IFN-γ deficient environment.

IL-4 is important in dampening allergic bronchial hyperresponsiveness (BHR) during mycoplasma infection. The dogma of asthma and asthma induced BHR is that IL-4 mediated responses are responsible (65, 66). It has long been known that respiratory infections, including *Mycoplasma pneumoniae*, can exacerbate acute asthma attacks (58). Also childhood *M. pneumoniae* infections are implicated as a cause for the development in asthma later in life (58). To date, the mechanisms by which mycoplasma infections exacerbate asthmatic attacks is unclear. My research has demonstrated that allergic BHR during mycoplasma infection is not IL-4 mediated. Furthermore, IL-4 dampens this mycoplasma-induced BHR. This is in complete contradiction to the current state of asthma research. Though, Th1 mediated cytokines have been detected in the BAL of asthmatic patients, and some research suggests that Th1 mediated responses may contribute to asthma (66), no research to date has suggested that IL-4 could actually play

a beneficial role during BHR. Further research needs to be done to understand the mechanism behind the IL-4-mediated responses that protect the lung during BHR. Several possibilities exist, other Th2 mediated cytokines, such as IL-5 or IL-12, could be elevated to compensate for the loss of IL-4. IL-5 has been isolated from the BAL of asthma patients experiencing wheezing. This may be the case in our model except that in antigen-specific stimulation of T cells isolated from the lungs of infected IL-4 KO mice there is no increase in levels of IL-5. Suggesting that IL-5 is not the most likely candidate for BHR activity in IL-4 KO mice. IL-13 has similar functions to that of IL-4, and could induce allergic BHR. To date, I have not examined the levels of IL-13 in either mRNA or protein levels in IL-4 KO mice, and this would be the next logical step in the study. The other possibility is that IL-4 is dampening pro-inflammatory or Th1-mediated events, and in the absence of IL-4, these events exacerbate mycoplasma-induced BHR. This would correlate with human studies that suggest the involvement of Th1 mediated responses contribute to exacerbated BHR (66). This would be one of the first papers to demonstrate this in an animal model. Regardless of mechanism, this work clearly shows that IL-4 dampens BHR during mycoplasma respiratory infection.

The purpose of these studies was to identify the roles of IFN-γ and IL-4 during mycoplasma respiratory infection. I found there were distinct differences in the cytokine requirements of IL-4 and IFN-γ during the development of immune responses of the upper and lower respiratory tracts during mycoplasma infection. Interestingly, we discovered that IFN-γ is important in pulmonary tract immunity against mycoplasma, and influences NK cell-mediated functions, while IL-4 aids in dampening mycoplasma

induced bronchial hyperresponsiveness. Though diverse, this research begins to uncover the contribution of IL-4 and IFN-γ during mycoplasma disease. I feel this work will not only impact the research into immune responses towards mycoplasma respiratory infection, but will give insight into the basic nature of immune development towards respiratory infectious agents. Which will possibly change the development of therapeutic and vaccine strategies for respiratory disease.

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