Dobbs, Nicole A., Setting Us Up to Fail: Pulmonary Dendritic Cells Promote Immunopathology During Mycoplasma Respiratory Disease. Doctor of Philosophy (Molecular Biology and Immunology), August 2010, 113 pp., 1 table, 18 illustrations, bibliography, 154 titles.

The purpose of these studies was to define the contributions of T helper 2 cells and dendritic cells toward the development of immunopathology during mycoplasma respiratory disease. IFN-γ^+ $CD8^+$ T cells, IFN-γ^+ Th1 cells and IL-13^+ Th2 cells developed over the course of mycoplasma infection. By day 14 post-infection, the results demonstrated a significant and preferential increase of an IL-13^+ Th2 cell sub-population in the LRNs. Additional studies using $STAT4^{-/-}$ animals, which have a Th2 polarized environment, demonstrated no difference in disease compared to the wild-type animals. Absence of STAT6, which strongly contributes to a Th1 polarized environment, conveyed significantly more protection from mycoplasma disease in immunized mice compared to $STAT4^{-/-}$ and WT mice. By day 14 post-infection, all mice had significantly more IL-13^+ Th2 cells than IFN-γ^+ Th1 in the LRN compared to $STAT6^{-/-}$ immunized mice, thus suggesting that the reduction in the IL-13^+ Th2 population leads to protection, while an increase in Th2 is pathogenic.

Additional studies demonstrated that pulmonary dendritic cells support the mycoplasma-specific $CD4^+$ and $CD8^+$ T cell activation when stimulated with mycoplasma antigen. Knowing that T cells and DCs have an intimate relationship during mycoplasma disease, sub-classes of cytokine differentiated BMDCs were created to

attempt to skew to the protective arm of immunity against mycoplasma disease. However, *in vivo* adoptive transfer studies demonstrated antigen pulsed DCs accelerated and exacerbated the pathological effects of mycoplasma disease. The exacerbation was antigen-specific and lymphocyte dependent. Mice that received antigen pulsed DCs demonstrated a significant increase in IL-13^{+} Th2 cell sub-population in the LRNs with a similar trend found in the lungs prior to infection. The same exacerbation was seen when antigen pulsed pulmonary DCs were adoptively transferred into mice, but not with antigen pulsed splenic DCs. Prior to infection, mice that received antigen-pulsed pulmonary DC, not splenic DC, had a significant increase in a IL-13^{+} Th2 population in the LRNs. Taken collectively, these studies demonstrate two key players in the development of the detrimental response against mycoplasma disease. This knowledge will assist in the development of targeted vaccines that will promote protection over pathology.

SETTING US UP TO FAIL: PULMONARY DENDRITIC CELLS PROMOTE IMMUNOPATHOLOGY DURING MYCOPLASMA RESPIRATORY DISEASE

DISSERTATION

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LIST OF ABBREVIATIONS

AAMs	Alternatively Activated Macrophages
AHR	Airway Hypersensitivity Reaction
ANOVA	Analysis of Variance
Ag	Antigen
Ab	Antibody
APC	Antigen Presenting Cells
APC.Cy7	Allophycocyanin-Cy.7
BMDC	bone marrow derived dendritic cells
BALF	Bronchoaleveolar lavage fluid
CD	Cluster of Differentiation
CFSE	Carboxyfluorescein succinimidyl ester
CFU	Colony Forming Unit
DC	Dendritic Cells
ELISA	Enzyme-linked Immunosorbent Assay
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
Ig	Immunoglobulin

IFN-γ	Interferon gamma
IL-	Interleukin
IP	Intraperitoneal
KO	Knock out
LRN	Lower Respiratory Node
MHC	Major Histocompatibility Complex
NK cells	Natural Killer cells
PerCP	Peridinin Chlorophyll-*a* Protein
PMA	Phorbol 12-Myristate 13- Acetate
PBS	Phosphate buffered saline
PE	Phycoerythrin
PE.Cy-	Phycoerythrin-cyanine
PPLO	Pleuropneumonia like organism
RSV	Respiratory syncytial virus
STAT	Signal Transducers and Activators of Transcription
Th cells	T helper cells
CTLs	Cytotoxic T Lymphocytes
TLRs	Toll-like receptors
TGF-β	Transforming Growth Factor-beta
TNF-α	Tumor necrosis factor- alpha
SCID	Severe combined immune deficiency
WT	Wild type

CHAPTER I

INTRODUCTION TO THE STUDY

Mycoplasmas are probably the most under recognized pathogens known today. These bacteria have unusually small genomes and are known to infect and colonize mucosal surfaces such as those along the respiratory and urogenital tracts. Belonging to the class *Mollicutes*, meaning "soft skin," they differ from other bacteria in that they lack a cell wall, rendering them resistant to certain antibiotics. First isolated in 1898, *Mycoplasma mycoides* subsp. *mycoides* was reported as the causative agent of contagious bovine pleuropneumonia in cattle (1). Since then, mycoplasmas are found to be the etiological agents of a wide range of diseases in both animals and humans (2, 3).

Mycoplasmas are excellent examples of respiratory pathogens that infect a wide variety of hosts including reptiles, cattle, pigs, chickens, dogs and rodents, as well as humans. The most recognized human pathogen is *Mycoplasma pneumoniae*, the leading cause of respiratory related illness worldwide, which is responsible for the respiratory disease "walking pneumonia." This disease is a community-acquired pneumonia commonly affecting students who inhabit dormitories, military personnel in military barracks and attendees at summer camps (4-6). The Center for Disease Control estimates that *M. pneumoniae* causes 2 million cases of pneumonia per year and about 100,000 of those results in hospitalization. To complicate matters, *M. pneumoniae* is also implicated in the exacerbation of other respiratory conditions such as

asthmatic airway hypersensitivity reactions, chronic obstructive pulmonary disease (COPD) and cystic fibrosis (7-13). While many of these cases of pneumonia are acute, some result in chronic infections, which can lead to the development of immunopathology. Because of their prevalence, mycoplasmas have a profound economic impact not only on health care and biomedical research but also on agriculture as an infectious agent of cattle, swine, sheep, poultry and other livestock (2). A hallmark of these infections is the persistence of the organism despite the presence of immune response.

The reasons that mycoplasma infections can persist are not entirely clear. It is clear that host immune responses against mycoplasma are complex and ultimately determine resistance to infection, severity of disease and spread of infection in the body. Although immunity is known to prevent or control infections, immune responses can also promote the development of inflammatory lesions associated with mycoplasma disease. T cells are critical in the regulation of immunity, and have a critical impact on the progression of mycoplasma disease (14).

Antibiotic therapy can be problematic; therefore the next obvious move to combat mycoplasma respiratory disease is vaccination. Current vaccination strategies have had limited success, and in many cases, vaccination has exacerbated mycoplasma disease. In order to improve vaccine design, the immune responses generated against mycoplasma must be understood. Current immunologic research on mycoplasma respiratory disease has slowly defined the roles of all the individual cell types and their influence during the generation of immune response to mycoplasmas. The findings in this thesis demonstrate that DCs along with Th2 cells are the major contributors to the immunopathology generated during mycoplasma respiratory disease.

The Immune System

The immune system is a diffuse network of organs, tissues, cells and cell products in the body known to defend the host from a variety of pathogens and other foreign substances, as well as destroy infected or malignant cells and remove cellular debris. Components of the immune system include the thymus, spleen, lymph nodes, stem cells, white blood cells, antibodies and cytokines (15). It identifies molecular patterns that distinguish the class of invader and has the ability to determine the difference between a pathogen versus self molecules. Then, this recognition event is converted into a variety of effector responses, each uniquely suited for eliminating this particular type of pathogen. Certain exposures induce a memory response, characterized by a more rapid and heightened immune response upon secondary attack. These responses are known as immunological memory and are the fundamental foundation for vaccination. However, it should be understood that to accomplish these fundamental tasks, the immune system has to divide up the responsibilities into two different types of immune responses known as the innate and adaptive (or acquired) immunity.

Innate Immunity

Innate immunity is the first line of defense in response to pathogens and recognizes a broad spectrum of pathogenic invaders. Most of the molecular and cellular components of innate immunity are pre-deployed before an infection takes place and usually prevents and eliminates most infections within hours of the encounter. Innate immunity is a combination of physical barriers, chemical barriers and cellular lines of defense (15). Physical barriers include the skin and mucous membranes. Chemical barriers consist components, such as acidity of the stomach, lysozyme in tears and antimicrobial peptides on the skin. The cellular line of defense includes several white blood cells, such as neutrophils, macrophages, dendritic cells, and natural killer

cells. Neutrophils function in the innate response through phagocytosis, expression of reactive oxygen and nitrogen species and antimicrobial peptides. Macrophages are known for phagocytosis and reactive oxygen and nitrogen species, as well as expression of inflammatory mediators, cytokines, chemokines and antigen presentation. Natural killer cells (NK cells) are most important for their expression of IFN-γ during the initial stages of infection and lysis of virally infected cells. Dendritic cells function similar to macrophages, but they are primarily known for their potent antigen presenting capabilities. When pathogens breach the anatomical and chemical barriers, an inflammatory response is generated usually within minutes. Inflammation leads to the recruitment of additional leukocytes that adhere to the endothelial cell walls and pass through the walls of capillaries into the damaged tissue space in a process known as extravasation (15). These recruited leukocytes phagocytose pathogens and release molecular mediators to perpetuate the inflammatory response and recruit additional effector cells. In most situations, the inflammatory response is acute, but in other cases inflammation can be chronic, as seen in such conditions as arthritis or in mycoplasma disease. If the infection persists, the activation of the innate immune response leads to the development of a more specific adaptive immune response.

Adaptive Immunity

While innate immunity is ideal to immediately eliminate a wide range of potential pathogens or other irritants, the adaptive immune response takes time to develop and is specific for the pathogen. B and T lymphocytes are characteristic of adaptive immunity, and they are responsible for the development of antibody (humoral) and cell-mediated responses (15). The adaptive immune response either enhances phagocytic function or adds to the arsenal unleashed

against the infection. During these steps, if unable to clear the infection, the immune system localizes the infection to prevent dissemination to other tissues and more severe disease.

While innate and adaptive immunity may seem independent, they are actually interdependent. If the pathogen avoids clearance by the innate immune system the adaptive immune response is critical to combat the infection. Therefore, recognition of the pathogens by the innate immune response sets the stage for an effective adaptive response.

Pulmonary Immunity

The basic function of the lungs is respiration, the act of taking up oxygen in exchange for the excretion of carbon dioxide. While performing this necessary function, the lungs are constantly exposed to a variety of inert particles as well as potential pathogens. The lungs have developed several novel pathways to process and expel foreign antigens, while not interfering with normal function (16). Much needs to be understood about how the lungs maintain this homeostasis, keep the inflammatory response in check to minimize tissue damages, but are still capable of responding to pathogens or disease-causing material.

The Components of Innate Pulmonary Immunity

Alveolar Macrophages

Alveolar macrophages are different from other tissue specific macrophage, in that they are the only macrophage that comes in contact with air. This tissue specific class of macrophage is located in the alveolar space, within the alveolar surfactant film produced by the type II alveolar lining cells (17). They are identified by the expression of surface markers, $CD11c^{+}$, typically a dendritic cell marker and the pan macrophage marker, F4/80 (18). In a normal resting animal, without infection, this cell population makes up over 90% of the cell population recovered in bronchoalveolar lavage (BAL) (19-21). The lung maintains these alveolar

macrophages in a quiescent state in order to avoid damage to the cells involved in gas exchange. This quiescent state reduces their ability to produce inflammatory cytokines and decreases their ability to phagocytose materials (22). Alveolar macrophages also suppress the activity of interstitial and alveolar DCs through the secretion of nitric oxide, prostaglandins, interleukin-10 (IL-10) and transforming growth factor-β (TGF-β), which in turn suppresses overall T cell activation and the development of the adaptive response (23-25).

Pulmonary Dendritic Cells

Dendritic cells (DCs) are one of the most potent antigen presenting cells present in all tissues. In the macroenvironment such as the skin, lungs or liver, DCs remain in an immature state defined by low expression of co-stimulatory molecules and increased plasticity allowing the DCs to endocytose the surrounding environment in search for antigens (26). When DCs detect antigen, the antigen is digested and presented by with the Major Histocompatibility Class molecules (MHC), which allows the DC to present the digested antigen to a corresponding T cell via the T cell receptor complex (TCR). In order to activate naïve T cells, the DC has to mature by decreasing plasticity and increasing the expression of co-stimulatory molecules. The maturation of DCs occurs during the translocation from the site of infection to the nearest lymph node to activate naïve T cells.

Respiratory DCs are found throughout the respiratory tract and within and below the airway epithelium, in the nasal mucosa, lung pleura and connective tissues and in the alveolar air spaces. All known respiratory DCs are positive at some level for $CD11c^+$ and $F4/80^-$ (18, 27). Alveolar macrophages are both $CD11c^+$ and $F4/80^+$. During steady state conditions, respiratory DCs within the airways provide immunologic surveillance of all particulate matter inhaled. These airway DCs are present in an immature state, and a large majority of the particulate matter

is inert. When the inert particles are phagocytosed they do not elicit a strong maturation response, therefore co-stimulatory molecule expression remains low. These immature DCs return to the nearest draining lymph nodes (in this case the mediastinal lymph nodes) and DC create tolerance among T cells to the inert antigen (27). This demonstrates that along with pathogen detection responsibilities, these DC also perform a specialized function of generating immune tolerance to inert particles.

While these DCs are very important regarding lung defense against pathogens, these cells also have been implicated in adverse reactions such as asthma and allergic reactions (28, 29). Both asthma and allergic reactions are overreaction to inert particulates in the air. An asthma attack presents as constriction of the airways due to inflammation caused by an influx of immune cells, particularly eosinophils, ultimately limiting the amount of oxygen exchange. Similar to some of our ideas, the asthma and allergy responses seemed to be caused by a deregulation in the immune response. A polarized Th2 response has been strongly implicated as a part of this deregulated immune response (30, 31).

The Components of Adaptive Immunity

T cells

The T cell, in many cases, is likely to determine the nature of host responses generated against mycoplasma, and these responses subsequently determine the outcome of infection. T cells are critical in the resistance to almost every other infectious agent, but T cells can also contribute to host immune-mediated inflammatory responses that are important in disease pathogenesis (15). Interaction with both the innate and adaptive arms of the immune system demonstrates T cell capabilities to be either regulatory or effector cells in response to infection. Activation of T cells begins with antigen presenting cells (e.g. macrophages, dendritic cells, B

cells) displaying peptides from pathogens or their products to T cells, which express receptors specific for these antigens. Once activated, T cells influence all arms of immunity. The release of T cell factors initiate and modulate the activity of inflammatory cell populations and participate in inducing and maintaining acquired immunity and generating memory lymphocyte populations (15). T cells can fine-tune humoral immunity by determining the class of and influencing the specificity of antibody responses. Overall, the tremendous potential in T cell activity makes delineation of the role of this lymphocyte population in disease very complex, but of fundamental importance. T cells are divided into two major basic groups differentiated by the expression of cell surface receptors and their effector functions. T helper cells (Th) express the co-receptor CD4, while CD8 is found on the surface of cytotoxic T lymphocytes (CTLs).

$CD4^+$ T helper Cells

T helper cells are further divided into more complex groups defined mostly by the cytokines produced. The current classifications of these $CD4^+$ Th cell groups include Th1, Th2, Th17 and T regulatory (T_{reg}) cells. Each of these Th cell subsets has defined but somewhat overlapping roles in host immunity. Th1 cells secrete interferon-gamma (IFN-γ), interleukin-2 (IL-2), tumor necrosis factor – beta (TNF-β also known as lymphotoxin) and granulocyte macrophage-colony stimulating factor (GM-CSF) (32, 33). The secretion of these cytokines amplifies the host immune response to intracellular bacteria and viruses making this immune response better at mediating cellular immunity. Th1 cells can also contribute to the development of inflammatory responses and can shape antibody responses to foster killing of some extracellular pathogens. Th2 cells are most effective as mediators of the humoral immune responses and are characterized by the secretion of IL-4, IL-5, IL-9, and IL-13 (32-34). These cytokines promote antibody class switching by B cells as well as differentiation of B cells into

antibody secreting plasma cells. IL-4, for example, can help initiate B cell activation and can also promote immunoglobulin class switching to immunoglobulin E (IgE). IgE, along with IL-5, is most effective against extracellular parasites and assists in eosinophil and mast cell degranulation. Th17 cells secrete IL-17, IL-17F, IL-6 and IL-22 (35). Secretion of these cytokines assists in the induction of inflammation and activation of neutrophils to combat extracellular bacteria. T_{reg} cells are a special group of effector T cells that have the ability to suppress the inflammatory response through either contact dependent mechanisms such as Fas/FasL interactions or through secretion of cytokines, such as IL-10 and transforming growth factor-beta (TGF-β), known to suppress T cell functions and induce tolerance to antigens (34). T_{reg} cells can also help dampen inflammatory responses by inhibiting the maturation of dendritic cells, which in turn leads to reduced T cell activation.

The cytokines secreted and surface molecules expressed by these effector Th cell subsets define their functional capabilities, and sometimes these responses can be beneficial to the host. However, in some situations, these Th cell subsets may exacerbate autoimmune reactions, allergies, asthma, and infectious disease states, such as inflammatory reactions or may promote chronic infections. For example, Th1 responses are beneficial to control many intracellular pathogens and fungal infections, but they could also exacerbate autoimmune diseases such as psoriasis, multiple sclerosis and rheumatoid arthritis (36-40). Similarly, Th2 cell activation can control or eliminate extracellular pathogens such as *Borrelia burgdorferi* and helminthes, but these responses can worsen some viral infections, allergic responses, asthma and airway hypersensitivity reactions (41-48). Th17 effector cells are important for an effective immune defense of infections caused by *Klebsiella pneumoniae, Bacteroides fragilis, Candida albicans*, as well as *Mycobacterium tuberculosis* (49-51). However, this subset of T cells is also implicated

in the exacerbation of autoimmune diseases such as rheumatoid arthritis (52, 53). IL-10, a product of T_{reg} and other cells types, can contribute to persistence of some infections through the dampening of the host responses capable of combating infection (54-60). Thus, the control of Th cell subset differentiation and their subsequent activation can ultimately determine the outcome of infections.

$CD8^+$ T cells

$CD8^+$ T lymphocytes also contribute to host immune responses against infections. Their major functions include the killing of bacterially- or virally-infected cells, as well as cancer cells. Cytolytic activity is mediated through the release of perforin, granzyme B and other factors or the interaction between FasL and Fas on the surface of CTL and target cells (61). In both cases, apoptosis of the target cell occurs. Although killing of cells is a major function of CTL, $CD8^+$ T cells can also produce substances, e.g. granulysin, that directly kill intracellular bacteria (62), suggesting $CD8^+$ T cells may also mediate killing of intracellular pathogens. In addition, $CD8^+$ T cells can secrete IFN-γ and other cytokines that can also modulate immune and inflammatory responses against infectious agents, such as activation of NK cells or macrophages (63). Interestingly, $CD8^+$ T cells were once referred to as T suppressor cells, because of their ability to dampen immune responses, particularly those mediated by Th cells (64). Thus, $CD8^+$ T cells could play a number of roles in the generation of immunity and pathogenesis of infectious disease.

Cytokines

Cytokines are low molecular weight regulatory glycoproteins usually considered molecular mediators of the immune response. These soluble proteins are secreted by white blood cells and a variety of other cells in the body in response to stimuli. Cytokines are used for cell-to-

cell communication and influence the development and behavior of effector cells (15). These glycoproteins bind to specific receptors on the membrane of target cells, triggering signal transduction pathways that will ultimately influence the gene expression of the target cells. Susceptibility to cytokines depends on the receptor expression, and only picomolar quantities of cytokines are required to generate a response (15). Cytokine influence is elicited in an autocrine, paracrine or endocrine fashion, and regulates the intensity and duration of response by stimulating or inhibiting the activation, proliferation or differentiation of various cells.

Interferon-gamma

Interferon-gamma (IFN-γ) is a pleiotrophic cytokine considered pro-inflammatory by nature. Its effects are found in both innate and adaptive immunity. The major cell types known to secrete IFN-γ are the Th1, $CD8^+$ T cells and natural killer cells (NK) (15). This cytokine acts as a master regulator of many inflammatory pathways because it is involved in the upregulation of additional pro-inflammatory cytokines, such as interleukin-12 (IL-12) and tumor necrosis factor-α (TNF-α) and chemokines (65). Unlike IFN-α and IFN-β, IFN-γ is not upregulated in the earlier stages of a viral infection. However later during a viral infection, secretion of IFN-γ affects B cells by influencing an isotype switch to IgG2a (66). IFN-γ improves the function of antigen presenting cells by activating both macrophages and dendritic cells to upregulate both MHC I and II molecules and co-stimulatory molecules used to activate T cells (65). Additionally, these IFN-γ activated macrophages also produce reactive nitrogen intermediates and improved phagocytosis over antigens. As far as innate immunity is concerned, secretion of IFN-γ recruits neutrophils, stimulates them to produce chemokines and adhesion molecules and triggers superoxide production and respiratory burst (67). While NK cells are known producers of IFN-γ

especially in the early phases of host infection, IFN-γ activates the NK cells enhancing cytotoxicity and cell mediated immune response.

Interleukin-13

Interleukin-13 (IL-13) is most notably secreted by Th2 cells, but it has been also shown to be produced by Th1 cells, $CD8^+$ T cells, as well as NK cells (68). IL-13 is located 25 Kb upstream from the IL-4 gene, clustered among other notable Th2 cytokines. IL-13 shares 25% protein homology to the IL-4, but it shares many similar structural and functional properties, including sharing an identical receptor (69). For B cells, IL-13 induces an isotype switch to IgE. While IL-4 is required for the induction of Th2 cells, IL-13 has no affect on T cells (70). IL-13 (and IL-4) have a wide variety of effects on macrophages, in terms of generating alternatively activated macrophages. IL-13 enhances macrophage capacity for fluid phase pinocytosis and endocytosis, promotes macrophage fusion into giant cell formation and increases MHC II and co-stimulatory molecule expression increasing antigen presentation capabilities (71). At the same time macrophages exposed to IL-13 show a reduction in the functions of autophagy, nitric oxide production, and macrophage-mediated killing of pathogens. The development of the Th2 response and expression of IL-13 is important for the clearance of parasites, but this arm of the immune response has also been implicated in allergies and asthma (68, 72). IL-13 is expressed at higher levels at steady state in the lungs of asthmatics compared to normal patients (73-75). Although IL-4 has been identified as the major cytokine known to exacerbate asthma, IL-13 has been found at the same sites and tends last longer than IL-4 (76).

Deciphering the Immune Response to Mycoplasma Respiratory Disease

Mycoplasma pulmonis is a pathogen that causes respiratory disease in rodents and the immune response elicited by this pathogen mimics the human immune response to *M.*

pneumoniae. Therefore, *M. pulmonis* serve as an excellent model for studying the host inflammatory response during acute and chronic mycoplasma respiratory disease. Similar to humans, not all strains of mice have the same immune response to a respiratory mycoplasma challenge. C57BL/6 mice are more resistant to mycoplasma infections. Mycoplasma disease in C57BL/6 mice results in an acute infection with little to no lesion damage and quick clearance of the microorganism (77, 78). However, C3H/HeN and BALB/c mice are susceptible to mycoplasma infections resulting in a chronic disease with increased tissue damage (77-79). The chronic disease state can be characterized by the increase in clinical disease demonstrated by weight loss, matted or ruffled fur, and malaise. The pulmonary immunopathology involves gross lesions with infiltrating granulocytes and lymphocytes, perivascular and peribronchial lymphoid hyperplasia and alveolitis resulting in damage to the alveolar epithelium. The immune mechanisms leading to the development of immunopathology are unknown, but currently under investigation.

Several early studies using severe combined immunodeficient mice (SCID) mice (animals deficient in B and T cells) or athymic mice (animals deficient in T cells only) revealed the importance of the adaptive immune response in anti-mycoplasma defense (80, 81). *M. pulmonis* infected SCID and athymic mice demonstrated reduced pulmonary lesions when compared to immunocompetent counterparts (80, 81). However, unlike their immunocompetent counterparts, the immunodeficient mice were unable to control dissemination of the microorganism from the lungs into the rest of the body. This leads to colonization of adjacent organs, such as the spleen and liver, as well as invasion into the joints, creating arthritis (80). Ultimately, left untreated these mice would be unable to sufficiently clear the pathogens and death would result. The susceptible immunocompetent mice, while suffering from an

exacerbated inflammatory response resulting in lesion damage, are able to contain the microorganisms at the site of infection, the lungs. Reconstitution of SCID and athymic mice with functional T cells or splenocytes from immunocompetent mice restores the immunopathology (80).

T cells and their subsets are implicated as a part of the protective and pathologic immune response to mycoplasma respiratory infections. In order to explore the specific roles of $CD4^+$ and $CD8^+$ T cell subsets play in the exacerbation of the pulmonary inflammatory response, Jones *et al*. performed a series of depletion studies on BALB/c mice (82). Undepleted mice demonstrated $CD4^+$ T cells as the major population of T cells in the lungs of mice 14 days after infection. Depletion of the $CD8^+$ T cell subsets in mice revealed increased lesion severity and signs of clinical illness, including a significant increase in weight loss. Depletion of $CD4^+$ T cells decreased lesion severity without any signs of clinical illness. However, the numbers of mycoplasma colony forming units (CFU) recovered from the lungs of the control mice and all depleted mice did not differ, signifying that depletion of the T cell subsets did not impact the clearance of the microorganism from the lungs. This implicates $CD4^+$ T cells as possible contributors to the immunopathology, but the question remains as to which subset of $CD4^+$ T cells, T helper 1 (Th1 = IFN-γ producing cells) or T helper 2 (Th2 = IL-4 producing cells), were responsible for the pathology.

To begin answering this question, Woodlard *et al*. performed a series of studies using IFN-$\gamma^{-/-}$ mice or IL-$4^{-/-}$ mice (83). IFN-$\gamma^{-/-}$ mice, in theory, should have a more Th2 polarized response. IL-$4^{-/-}$ mice should have a more Th1 polarized response. The purpose of this study was to demonstrate the roles of these specific cytokines in the upper and lower respiratory tract during mycoplasma infection. Loss of either IFN-γ or IL-4 has no effect on the upper respiratory

tract in terms of disease pathogenesis or clearance of the microorganism from the nasal passages. However, the loss of IFN-γ in the lower respiratory tract (lungs) results in more severe disease and increased lesion scores as well as increases in the number of mycoplasma CFU. Loss of IL-4 in the upper and lower respiratory tract did not differ from the control wild type (WT) BALB/c infected mice in terms of disease severity and clearance of the microorganisms. These studies implicate that a Th2 biased environment similar to that of the IFN-γ $^{-/-}$ mice increases immunopathology and severity of mycoplasma disease.

Th2 immune responses in the lung have been linked to IL-4 dependent inflammatory reactions that occur during allergic asthma airway hypersensitivity reactions (84). Infections of *M. pneumoniae* have been implicated in the exacerbation of asthma, but the mechanism behind exacerbated airway hypersensitivity reaction (AHR) caused by respiratory infections is unknown (8, 85, 86). Using IL-4 $^{-/-}$ mice, Woolard *et al*. demonstrated that IL-4 independent pathways were responsible for the exacerbation of methacholine-induced airway hyperreactivity (87) . *M. pulmonis* infected IL-4 $^{-/-}$ mice infected mice showed little difference in AHR, lesion scores and histopathology compared to infected WT BALB/c mice. This indicates other pro-inflammatory cytokines such as IL-13 or IL-9 may be responsible for AHR. Recently intracellular cytokine staining of $CD3^{+}CD4^{+}$ T cells from the lungs of mice 14 days after mycoplasma infection demonstrated that $CD4^{+}$ T cells were producing large amounts of IL-13 (a Th2 cytokine), more so than IL-4 or IFN-γ (our unpublished data).

T cell activation requires the assistance of antigen presenting cells (APCs) in order to become activated into an effector T cell. This prompted an inspection into which APCs are influential during mycoplasma disease. $CD11c^{+}$ dendritic cells (DCs) are the major APC in the lung during mycoplasma infections, and support mycoplasma specific T cell activation better

than that of pulmonary F4/80^{+} macrophages (88). There is a significant increase in the number of CD11c^{+} DCs in the lung at 14 days post-infection. Multicolor fluorescent staining revealed CD11c^{+} DCs are co-localized with CD4^{+} T cells at the lesion site in the lungs of *M. pulmonis* infected mice.

It is clear that the immune response against mycoplasma respiratory disease is both protective and pathologic. The work summarized in this dissertation addresses the cellular components that contribute to the immunopathologic responses. The IL-13^{+} Th2 sub-population becomes dominant in the lower respiratory nodes and lung during mycoplasma disease and reduction of this population in immunized transgenic mice resulted in greater protection against mycoplasma. Additionally, DCs, the major APCs in mycoplasma disease, supported the activation of both CD4^{+} and CD8^{+} mycoplasma-specific T cells, both of which are known to have contrasting effects on the immune response generated against mycoplasma disease. Finally, manipulation of bone marrow derived DCs exacerbated the immunopathology of mycoplasma disease through the activation of IL-13^{+} Th2 cells. Similar to BMDCs, pulmonary DCs promote the same Th2 response *in vivo*, which ultimately leads to increased pathology during infection. Understanding these components can assist in the development of a better-targeted vaccine.

CHAPTER II

T helper 2 Responses Dominate in Mycoplasma Respiratory Disease and Impair Protection

The purpose of these studies was to identify the dominant T cell subsets that develop during mycoplasma respiratory disease. We hypothesized Th2 responses generated after mycoplasma infections are immunopathologic, and Th1 responses are protective. To explore these ideas we took advantage of the availability of signal transducer knockout mice, $STAT^{-/-}$, that have demonstrated T cell polarization in other disease models. Expansion activated T cell populations were monitored at the site of infection, the lungs, lower respiratory nodes (LRN), and the spleen. Intracellular cytokine staining of IFN-γ and IL-13 was used to identify these specific Th1 and Th2 cells. The results in WT mice demonstrated a significant increase in $IL\text{-}13^{+}$ Th2 cells found in the LRNs of mice 14 days post-infection, and immunized $STAT6^{-/-}$ mice had increased protection against mycoplasma associated with a reduction in the amount of $IL\text{-}13^{+}$ Th2 cells found in their LRNs after infection.

Introduction

The kind of T cells generated by the host during a disease state has a strong impact on the outcome of the disease. $CD4^{+}$ T helper 1 (Th1) cells are identified through their secretion of interferon-gamma (IFN-γ) and are important in assisting in a cell-mediated immune response

against ex tracellular pathogens. However, these Th1 cells have also been linked the exacerbation of chronic inflammatory diseases and autoimmunity (36-40). $CD4^+$ T helper 2 (Th2) cells, identified by their secretion of cytokines IL-4, IL-5, IL-9 and IL-13, are necessary for effective clearance of parasites from the host, but there is substantial evidence that the Th2 subsets are also responsible for mediating asthmatic airway hypersensitivity reactions (41-48). T cells responses in mycoplasma disease are important to disease outcome as well.

Mycoplasma respiratory disease is chronic by nature and the immune response against it is both protective and pathologic. Gross lesions develop after 7 days of infection corresponding with an influx of lymphocytes (89). Several studies using lymphocyte-deficient animals demonstrated that during mycoplasma infection, these animals did not develop any immunopathology, but could not control dissemination of the bacteria (80, 81, 90). However, when reconstituted with functional T cells the immunopathology returns as well as control over dissemination of the bacteria. Depletion of $CD4^+$ T cells during infection demonstrated a reduction in immunopathology and clinical disease, but depletion of $CD8^+$ T cells had the reverse effect indicating that $CD8^+$ T cells assist in the dampening of the inflammatory response to mycoplasma (91). Recent immunization studies using cytokine deficient mice demonstrated IL-$4^{-/-}$ mice had the greatest protection and least pathology (92). However, IFN-$\gamma^{-/-}$ animals demonstrated exacerbation of the immune response resulting in increased pathology and decreased protection during mycoplasma respiratory disease. IFN-$\gamma^{-/-}$ mice generate a more Th2 skewed immune response, and IL-$4^{-/-}$ animals will generate a more Th1 skewed response. Therefore, we hypothesized that the Th2 responses generated after mycoplasma infection are immunopathologic, and Th1 responses are protective.

To explore this hypothesis, studies were designed to identify the dominant T cell subsets that develop during mycoplasma respiratory disease, hypothesized to be the Th2 subset, and demonstrate that the Th2 polarized environments exacerbate disease while Th1 polarized environments generate more protection. For these later studies, we took advantage of the availability of signal transducer knockout mice, STAT$^{-/-}$s, that have demonstrated T cell polarization in other disease models. Expansion activated T cell populations were monitored at the site of infection, the lungs, as well as the lung draining lymph nodes, lower respiratory nodes (LRN), as well as the spleen. Intracellular cytokine staining of IFN-γ and IL-13 was used to identify these specific Th1 and Th2 cells. Indeed, these data indicate that Th2 cells dominate at day 14 post-infection and immunization of STAT6$^{-/-}$ mice offered greater protection from mycoplasma disease.

Materials and Methods

Mice. Female BALB/cAnNHsd wild-type mice, tested to be virus- and mycoplasma free, were obtained from Harlan Sprague-Dawley (Indianapolis, IN). Female age-matched BALB/cJ wild type mice, homozygous STAT 6$^{-/-}$ (C.129S2-*Stat6*^{tm1Gru}/J on a BALB/c background) and homozygous STAT4$^{-/-}$ (C.129S2-*Stat4*^{tm1Gru}/J on a BALB/c background) mice were purchased and tested to be virus- and mycoplasma free from Jackson Laboratories (Ben Harbor, ME). Mice were housed in sterile microisolator cages supplied with sterile bedding, with food and water provided *ad libitum*. Mice used in the study were between 6 and 10 wks of age. Female mice were used in all studies. Before experimental infection, mice were anesthetized with an i.p. injection of diluted ketamine-xylazine. The animal studies were reviewed and approved by the

University of North Texas Health Science Center Institutional Animal Care and Use Committee.

Mycoplasma. The UAB CT strain of *M. pulmonis* was used in all experiments. Stock cultures were grown, as previously described (93) in mycoplasma PPLO medium (Acumedia) and frozen in 1-ml aliquots at -80°C. For inoculation, thawed aliquots were diluted to 2 x 10^5 CFU/20 µl. Nasal-pulmonary inoculations of 20 µl of diluted mycoplasma were given for experimental infections.

UV irradiated mycoplasma preparation. *M. pulmonis* was cultured at 37°C in mycoplasma broth medium and harvested at pH 7. Cells were then centrifuged at 10,000 rpm for 20 min, and pellets were resuspended in 10 ml sterile PBS and placed into a sterile T-25 cell culture flask. The mycoplasma was UV irradiated on a UV transilluminator (Fotodyne Inc., Hartland WI) for 20 minutes. A sample of this preparation was spot plated onto mycoplasma plates and incubated for 7 days to ensure that all mycoplasma was killed. Protein concentration was determined using Bradford protein assay (Bio-Rad, Hercules, CA). Aliquots were stored frozen at -80°C.

Lymphocyte Isolation. Mononuclear cells were isolated from lungs, as previously described but with some minor alterations to improve cell recovery (94). Lung lobes were dissected and placed individually in GentleMACs (Miltenyi Biotec, Auburn, CA) tube with 5 ml digest medium containing RPMI 1640 medium (HyClone Laboratories, Logan, UT), 300 U/ml *Clostridium histolyticum* type I collagenase (Worthington Biochemical, Freehold, NJ), 50 U/mL DNase (Sigma-Aldrich, St. Louis, MO), 5% FBS (HyClone Laboratories), 10 mM HEPES (Fisher Scientific, Pittsburgh, PA), and antibiotic/antimycotic solution (Life Technologies, Grand Island, NY). The lung samples were homogenized using a GentleMACs machine (Miltenyi Biotec) on the setting Lung version 2.0. Subsequently the homogenates were incubated at 37°C

while mixing on a Nutator (Fisher Scientific) for 15 - 20 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 (Cedarlane Laboratories, Burlington, NC).

Spleen and lower respiratory lymph node (LRN) cells were isolated after centrifugation of all suspensions, followed by red cell removal using ammonium chloride potassium carbonate lysis buffer.

Intracellular cytokine stimulation and staining. Lymphocytes from the lower respiratory nodes, lung and spleen were purified and stimulated overnight with UV irradiated mycoplasma (5μg/ml). The next day 50 ng/ml Phorbol 12-Myristate 13- Acetate (PMA; Sigma-Aldrich, St. Louis, MO), and 500 ng/ml Ionomycin (EMD, Gibbstown, NJ) was added for the last 5 hrs of the culture before staining.

To accomplish intracellular cytokine staining, GolgiPlug, containing Brefeldin A (BFA) (BD PharMingen, San Jose, CA), was added 4 hrs prior to the harvest of the cell cultures. Staining of the cells involved incubation at 4°C for 15 min in staining buffer (PBS $^{+}$ 2% FBS $^{+}$ 2 mM EDTA) with saturating amounts of the cell-surface antibodies and anti-CD16/CD32 to block Fc receptors. Additional cell surface markers used to identify cells included PE-Cy7-labeled anti-CD3 mAb, Alexa Fluor 488-labeled anti-CD4 mAb, peridinin chlorophyll-*a* protein (PerCP)-labeled anti-CD8 mAb (all from BD PharMingen) and APC-Cy7 labeled anti-CD44 mAb (BioLegend, San Diego, CA). To measure intracellular cytokines, cells were fixed and permeabilized at 4°C for 20 min using the intracellular cytokine staining kit from BD PharMingen. After washing in permeabilization wash buffer, the cells were incubated in saturating amounts of allophycocyanin (APC)-labeled anti-IFN-γ mAb and PE-labeled anti-IL-

13 mAb (both from eBioscience, San Diego, CA) at 4°C for 20 min. The cells were washed in staining buffer (PBS, 2% FBS, 2mM EDTA) resuspended in BD Stabilizing Fixative (BD PharMingen, San Diego, CA). Data were acquired and analyzed using a BD LSR II Flow Cytometer System and BD FACSDiva v5 Software (Fullerton, CA). Lymphocyte gates and detector voltages were set using unstained spleen cells. The flow cytometry data were further analyzed using FlowJo flow cytometry analysis software (Tree Star, Ashland, OR).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 lymphocyte population, the total number of lymphocytes isolated from each tissue was multiplied by the percentage.

Statistical Analysis. Data were evaluated by ANOVA, followed by Fisher protected least square differences multigroup comparison. These analyses were performed using the StatView version 5.0.1 (SAS Institute, Cary, NC) computer program. A p value ≤ 0.05 was considered statistically significant.

Results

There is a preferential increase of IL-13^{+}Th2 cells in the lower respiratory nodes and lungs by day 14 post-infection.

Previous studies have shown that the CD4^{+} Th cells are associated with immunopathology and these cells are found in large numbers at the lesion site in the lungs of mycoplasma-infected mice 14 days post-infection (91, 95). It is unknown which subsets of Th cells are predominant during infection. To determine which Th cell populations develop and where these cells develop over the course of a mycoplasma respiratory disease, lymphocytes

were isolated from the LRN, lungs and spleen on days 3, 5, 7 and 14. Cells from uninfected animals (day 0) were also isolated and functioned as controls. These lymphocytes were stimulated overnight with mycoplasma antigen and subsequently stained for cell surface markers to identify T cells and intracellular staining to identify specific Th sub-populations. T helper cell populations were identified $CD3^{+}CD4^{+}CD8^{-}CD44^{high}$. Th1 cells were defined as IFN-γ^{+}IL-13^{-}, and Th2 cells were defined IFN-γ^{-} IL-13^{+}.

At day 5 post-infection, a significant increase in Th1 and Th2 cells occurred in both the lower respiratory nodes (LRN) as well as in the spleen, but there was no preference in Th1 versus Th2 population development at this time (Figure 1a and 1c). The lungs did not develop a significant increase in the T helper cell populations until day 7 post-infection (Figure 1b). At this time, there was no preference in Th1 or Th2 populations in the lungs as well as the LRN and spleen. However, by day 14 post-infection there was a significant increase IL-13^{+} Th2 cells in both the LRNs with a similar trend found in the lungs. These findings are further supported by a comparison of the changes in percentages of these Th1 and Th2 cells types from the LRN, lungs and spleen as seen in Table I. The ratio of Th1 versus Th2 in the LRN and lungs skew towards a Th2 response especially by day 14 post-infection, while the spleen ratio is balanced between Th1 and Th2 cells.

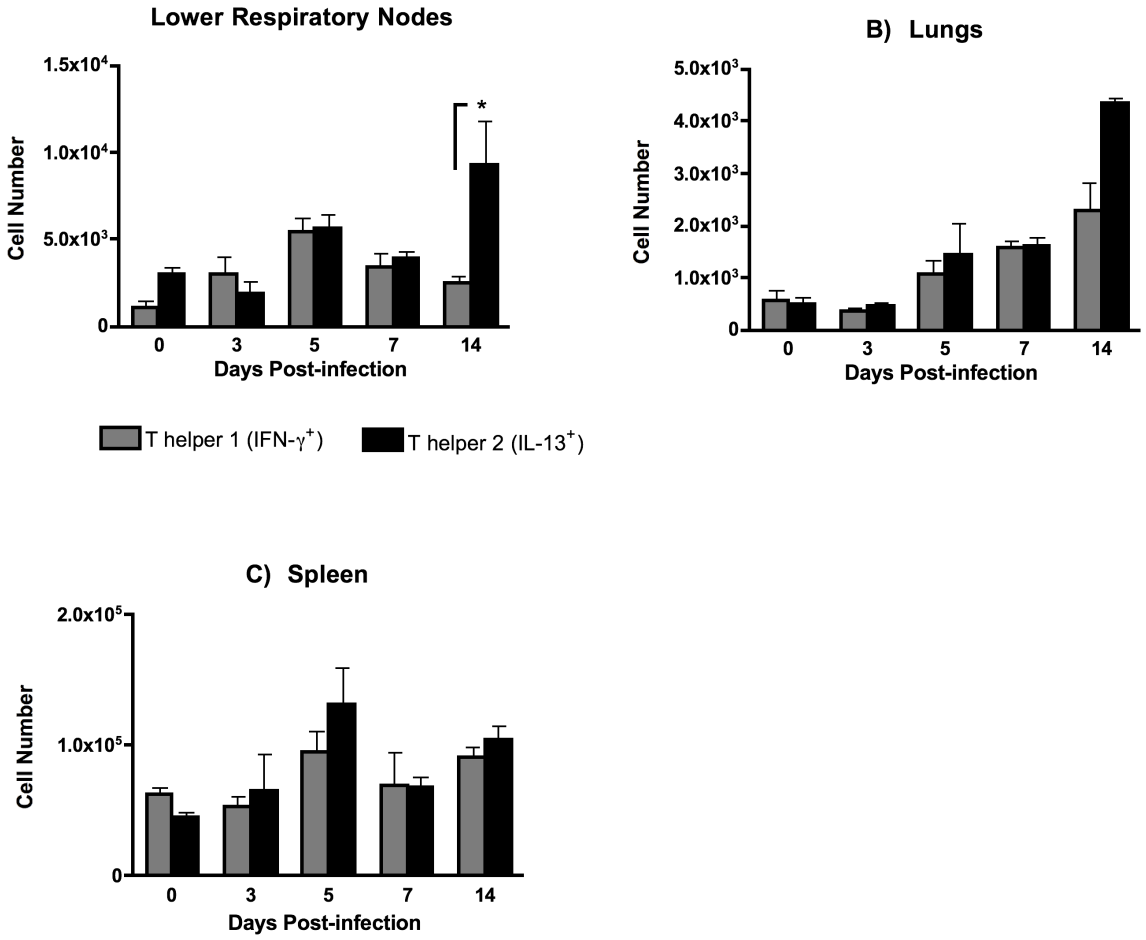


Figure 1. Development of T helper subsets generated in mycoplasma-infected mice. Mice were infected with *M. pulmonis* and were sacrificed on days 0, 3, 5, 7 and 14 post-infection. Lymphocytes were isolated from the lower respiratory nodes (LRN) (a) and the lungs (b) and the spleen (c). These lymphocytes were stimulated overnight with UV irradiated mycoplasma. The next day cells were subjected to intracellular staining to identify Th subsets. Th1= $CD3^+CD4^+CD8^-CD44^{high}IL\text{-}13^-IFN\text{-}\gamma^+$ (grey bars) and Th2 = $CD3^+CD4^+CD8^-CD44^{high}IL\text{-}13^+IFN\text{-}\gamma^-$ (black bars). An asterisk (*) indicates a significant difference ($p \leq 0.05$) in Th2 cell numbers from the Th1 cell numbers. Vertical bars and error bars represent the mean +/- SE (Day 0: n=3, Day 3: n= 6, Day 5 n= 6, Day7 n=3 and Day 14: n=10).

Table I. Comparison of Th1 versus Th2 Flow Data Percentages[a].

		Cell Type Percentages (SD)		
ORGAN[b]	**DAY[c]**	**Th 1[d]**	**Th2[d]**	**Ratio (Th1 : Th2)**
LRN	**0**	4.66 (1.6)[e]	9.49 (1.1)	1 : 2
	3	4.48 (2.1)	2.8 (0.8)	1.6 : 1
	5	4.19 (0.7)	4.3 (0.6)	1 : 1
	7	4.71 (0.9)	5.6 (0.8)	1 : 1.2
	14	2.32 (0.8)	5.46 (2.7)	1 : 2.4
LUNG	**0**	7.52 (3.8)	7.29 (1.0)	1 : 1
	3	4.84 (1.7)	6.32 (2.8)	1 : 1.3
	5	10.08 (2.9)	10.7 (2.4)	1 : 1.1
	7	11.37 (2.1)	17.63 (2.4)	1 : 1.6
	14	4.78 (1.7)	9.36 (2.2)	1 : 2
SPLEEN	**0**	8.00 (0.4)	5.84 (0.3)	1.4 : 1
	3	6.21 (1.3)	6.86 (5.7)	1 : 1.1
	5	8.37 (1.8)	11.31 (3.2)	1 : 1.4
	7	6.70 (3.0)	7.53 (1.8)	1 : 1.1
	14	7.91 (1.5)	9.03 (1.6)	1 : 1.1

[a] BALB/c mice were intranasally infected with *M. pulmonis*, and sacrificed at days
3, 5, 7 and 14. Lymphocytes were isolated, stimulated overnight, and intracellularly
stained to identify Th cell population. Day 0 are uninfected mice.
[b]Source of lymphocytes
[c]days post-infection when lymphocytes were collected
[d]Th1 cells are identfied as IFN-γ^+ and Th2 cells are IL-13^+.
[e]Mean percentages of each cell type and standard deviation.

Activated IFN-γ^+ $CD8^+$ T cells increase in the lower respiratory nodes, lungs and spleen during mycoplasma respiratory disease.

The $CD8^+$ T cells are important in dampening the immune response to mycoplasma respiratory disease, but do not change the numbers of mycoplasma during infection. $CD8^+$ T cells are important to combat intracellular pathogens, but *M. pulmonis* is an exogenous pathogen. It is thought that $CD8^+$ T cells may assist in regulation of the immune response to mycoplasma through their secretion of IFN-γ (14). The numbers of activated of $CD8^+$ T cells were determined in the LRN, lungs and spleen of mycoplasma-infected mice throughout the course of infection. These $CD8^+$ T cells were identified as $CD3^+CD4^-CD8^+CD44^+$ and intracellular staining identified IFN-γ^+ cells.

An expansion of IFN-γ^+ activated $CD8^+$ T cell population was detected in the LRN, lungs and spleen during the course of mycoplasma respiratory infection. Similar to what was found with the T helper populations, there was an increase in the activated IFN-γ^+ $CD8^+$ T cells at day 5 post-infection compared to uninfected mice in both the LRN and spleen (Figure 2a and 2c). At day 7 post-infection, there was an increase in IFN-γ^+ $CD8^+$ T cells compared to uninfected mice in the lungs (Figure 2b). IL-13 was not detected in these $CD8^+$ T cells during the infection.

Immunized STAT $6^{-/-}$ mice have increased resistance to mycoplasma.

To further explore the hypothesis that the Th2 responses lead to pathology and Th1 responses are more protective during mycoplasma respiratory disease, we used animals deficient in the STAT, signal transducers and activators of transcription, signaling pathways. STATs are a family of transcription factors that activate gene transcription in response to a number of different cytokine/cytokine receptor activation pathways. Two of these STAT molecules, STAT4 and STAT6, are involved in T cell polarization. IL-12 uses the STAT4 signaling pathway to

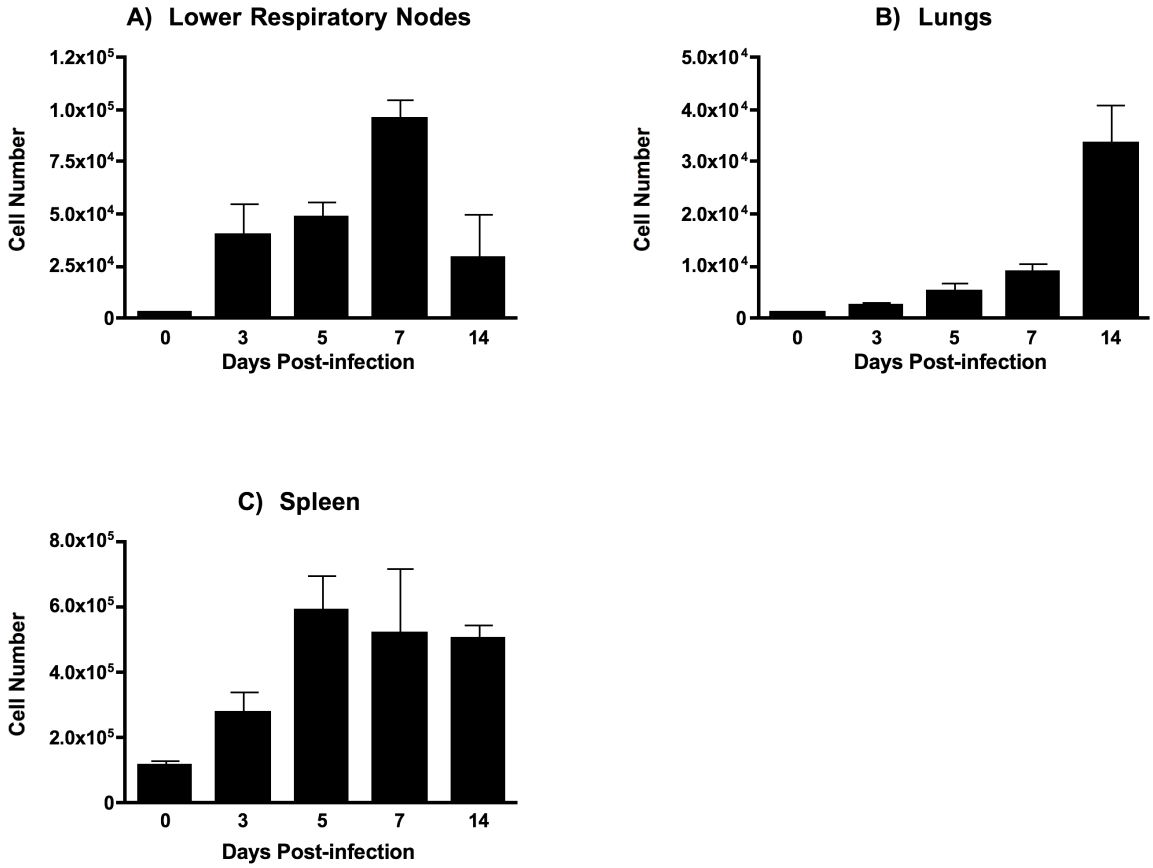


Figure 2. Development of IFN-γ^+ $CD8^+$ T cells generated in mycoplasma-infected mice. Mice were infected with *M. pulmonis* and were sacrificed on days 0, 3, 5, 7 and 14 post-infection. Lymphocytes were isolated from the lower respiratory nodes (LRN) (a) and the lungs (b) and the spleen (c). These lymphocytes were stimulated overnight with UV irradiated mycoplasma. The next day cells were subjected to intracellular staining to identify activated $CD8^+$ T cells subsets: $CD3^+CD4^-CD8^+CD44^+$IFN-$\gamma^+$$IL\text{-}13^-$.Vertical bars and error bars represent the mean +/- SE (Day 0: n=3, Day 3: n= 6, Day 5 n= 6, Day7 n=3 and Day 14: n=10).

polarize T helper cells to a Th1 phenotype and Th2 polarization requires IL-4 signaling through the STAT6 pathway. Therefore, mice deficient in STAT4 should have a Th2 skewed immunologic response to antigen and STAT6 deficient animals should have a Th1 skewed immunologic response. Prior to infection a group of $STAT4^{-/-}$, $STAT6^{-/-}$ and age-matched WT mice were immunized intranasally using mycoplasma membrane antigen once a week for two weeks. Unimmunized animals received only intranasal PBS. Animals were infected with *M. pulmonis* and the infection was monitored for 14 days. Mycoplasma numbers were determined in the lungs after infection as a measure of protection.

At day 14 post-infection, there was no difference in the CFU recovered from the lungs of any of the unimmunized animals regardless of their strain (Fig. 3). Immunization induced protection regardless of the strain as demonstrated by a reduction CFU recovered from the lungs compared to the unimmunized counterparts (Fig. 3). However, the $STAT6^{-/-}$ mice had significantly less CFU recovered from the lungs when compared to the immunized WT or immunized $STAT4^{-/-}$ mice. There was no difference in CFU recovery between $STAT4^{-/-}$ mice and WT mice.

T cell subsets were identified in the LRNs of the respective mice. Similar to the previous studies results, $IL\text{-}13^{+}$ Th2 subsets dominated the immune responses in LRN (Fig. 4a). As expected, $STAT6^{-/-}$ mice had the lowest numbers of Th2 cells compared to all other mice. Despite the interruption of the IL-12 pathway in $STAT4^{-/-}$ mice, there was no difference in the IFN-γ^{+} Th1 response or the IFN-γ production from $CD8^{+}$ T cells (Fig. 4b).

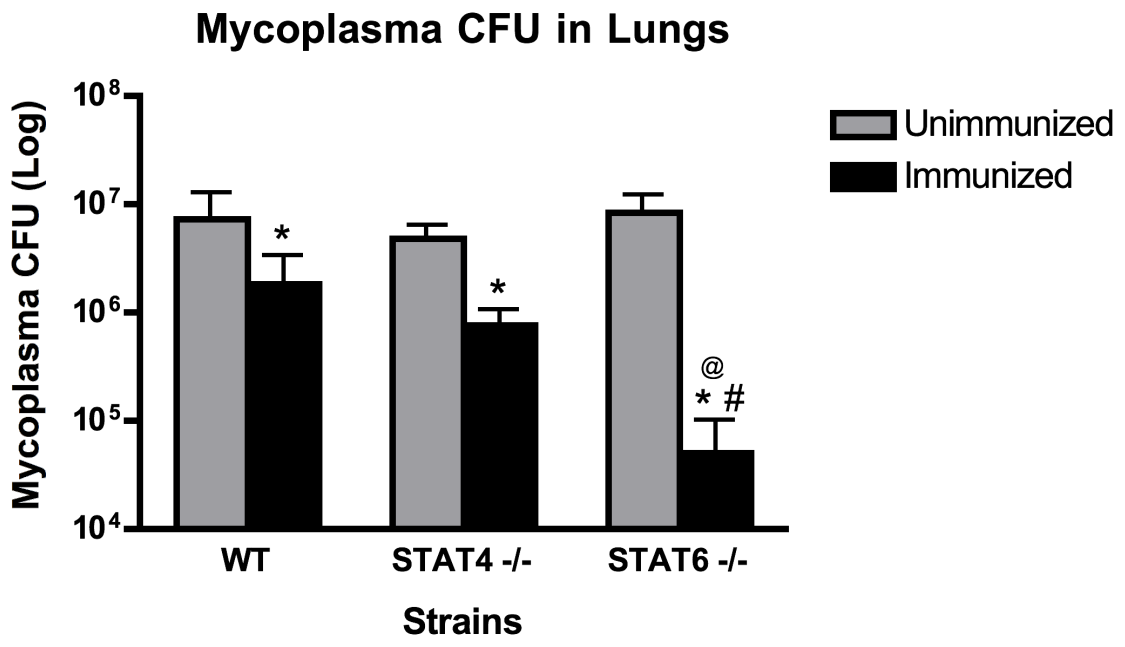


Figure 3. Mycoplasma CFU in the lungs of unimmunized and immunized WT, STAT4$^{-/-}$, and STAT6$^{-/-}$ mice fourteen days post-infection. Mice were immunized with mycoplasma antigen or PBS once a week for two weeks prior to infection. Mice were infected and illness was monitored for two weeks. At day 14, mice were sacrificed and CFU were recorded. The grey bars represent unimmunized mice, and black bars represent immunized mice. A numeral sign (#) indicates a significant difference ($p \leq 0.05$) in CFU number from immunized Stat4$^{-/-}$ mice. A "@" sign indicates a significant difference ($p \leq 0.05$) in CFU number from immunized WT mice. An asterisk (*) indicates a significant difference ($p \leq 0.05$) in CFU number from unimmunized mice. Vertical bars and error bars represent the mean +/- SE (unimm WT: n=10 & imm. WT: n=12, unim. STAT4: n=12, imm. STAT4 n=11, unim. STAT6: n=11, imm. STAT6: n=12).

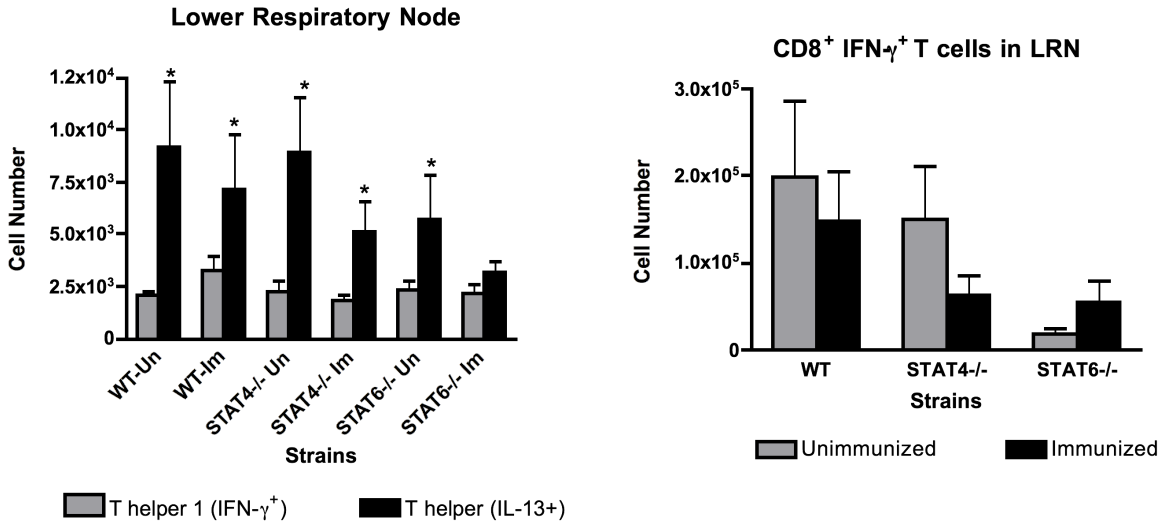


Figure 4. Numbers of activated T cell subsets in the LRN of unimmunized and immunized WT, STAT4$^{-/-}$, and STAT6$^{-/-}$ mice fourteen days post-infection. Mice were immunized with mycoplasma antigen or PBS once a week for two weeks prior to infection. Mice were infected and illness was monitored for two weeks. Lymphocytes were isolated from the lower respiratory nodes (LRN). These lymphocytes were stimulated overnight with UV irradiated mycoplasmas. The next day cells were subjected to intracellular staining to identify activated Th cells (a) and CD8$^+$ T cells subsets (b). Th1= CD3$^+$CD4$^+$CD8$^-$CD44^{high}IL-13$^-$IFN-γ$^+$ (grey bars) and Th2 = CD3$^+$CD4$^+$CD8$^-$CD44^{high}IL-13$^+$IFN-γ$^-$ (black bars) and activated CD8$^+$ T cells subsets: CD3$^+$CD4-CD8$^+$CD44$^+$IFN-γ$^+$IL-13$^-$. An asterisk (*) indicates a significant difference ($p \leq 0.05$) in Th2 cell numbers from Th1 cell numbers. Vertical bars and error bars represent the mean +/- SE (unimm WT: n=10 & imm. WT: n=12, unim. STAT4: n=12, imm. STAT4 n=11, unim. STAT6: n=11, imm. STAT6: n=12).

Discussion

The purpose of these studies was to identify the dominant T cell subsets that develop during mycoplasma respiratory disease, hypothesized to be the Th2 subset, and demonstrate that the Th2 polarized environment exacerbate disease while Th1 polarized environment generate more protection. The first set of studies demonstrated IL-13^{+} Th2 cells dominated the immune response to mycoplasma in the LRNs and the lungs at 14 days post-infection. The rise in this Th2 population is thought to contribute to pathology; since this is the time where gross lesions are typically observed in mycoplasma infected mice (89). Similar findings have been seen in murine models of asthma or RSV infections, where Th2 cells are notorious for exacerbating the pathological side of the disease (30, 31, 96).

The most significant finding came from the increased protection demonstrated by the STAT6$^{-/-}$ animals. Similar to our findings with IL-4$^{-/-}$ animals, STAT6$^{-/-}$ animals demonstrated increased protection associated with immunization, as seen with the reduction in mycoplasma numbers recovered from the lung. Another profound finding was that these mice, that were the most protected from mycoplasmas, had a depression of their IL-13^{+} Th2 cells relative to Th1 in the LRN compared to STAT4$^{-/-}$ and WT animals. This data is consistent with what was seen in IL-4$^{-/-}$, indicating that production of IL-4 leads to increased Th2 response, which inhibits clearance of mycoplasmas and interferes with the generation of protective immunity.

STAT4$^{-/-}$ mice, contrary to our hypothesis, did not demonstrate any difference in disease compared to the WT animals, but immunization did provide some protection as demonstrated by the decrease in mycoplasma organisms isolated from the lungs post-infection. This discrepancy could be explained because STAT4$^{-/-}$ mice still produce just as much IFN-γ as WT mice. This

indicates blockage of the STAT4 signaling pathway is not important to the generation of Th1 cells during mycoplasma disease. It was expected that the blockage of the STAT4 signaling pathway would lead to an increase in the Th2 phenotype because it interferes with the IL-12/1L-23 signaling necessary for the production of IFN-γ leading to a Th1 phenotype, but this was not the case. Indeed other authors indicated alternative pathways around STAT4 that up-regulate IFN-γ and that $CD8^+$ T cells do not need STAT4 for IFN-γ production (97).

Overall, these results support the idea that Th2 cells are associated with immunopathology of mycoplasma disease and that Th2 cells interfere with the development of protective immunity. Th1 cells may be more protective, but it is unclear if it is just the balance between the Th populations that lends to protection. Perhaps use of an adjuvant or antibody that could skew the immune response away from Th2 and more towards Th1 would be one vaccination strategy that can be taken away from these results.

These studies demonstrated the generation of Th2 immune responses correspond to disease pathology and impaired generation of protective immunity after mycoplasma infection. During these studies, both $CD4^+$ and $CD8^+$ T cells were activated and increased in number during disease progression. While it is clear that T cell development influences the outcome of disease, T cells rely on activation from antigen presenting cells (APCs). Sun *et al*. demonstrated pulmonary $CD11c^+$ $F4/80^-$ dendritic cells (DCs) are the major APC that support mycoplasma specific T cell activation compared to $CD11c^-$ $F4/80^+$ macrophages, especially after infection (95). Interestingly, $CD11c^+$ DCs and $CD4^+$ T cells were found co-localized in the lesion site of mycoplasma-infected lungs, 14 days post-infection. However, it was never identified which sub-populations of T cells these pulmonary DCs activated. I hypothesized that pulmonary DCs could activate both $CD4^+$ and $CD8^+$ T cells, and this is the focus of the next set of studies.

CHAPTER III

Pulmonary Dendritic Cells Support Both $CD4^+$ and $CD8^+$ T cell Activation Against Mycoplasma.

The purpose of this study was to determine whether pulmonary $CD11c^+$ DCs support the activation of both $CD4^+$ T helper and $CD8^+$ T cells against mycoplasma antigen. We hypothesized that pulmonary DCs could support the activation of both sub-populations, and indeed, these results indicate that pulmonary DCs do support the activation of mycoplasma-specific $CD4^+$ and $CD8^+$ T cells. However, DCs from mycoplasma-infected mice show a preference for the activation of $CD4^+$ Th cells after infection, indicating that post-infection DCs may have a preference of this population of T cells. Because $CD4^+$ and $CD8^+$ T cells have different roles in mycoplasma disease, it is important to determine whether DCs have the capacity to influence the activation of these lymphocyte populations. This knowledge would make the DC population an excellent target for vaccine development, particularly if these cells could be influenced to promote a more protective immune response.

Introduction

Chronic mycoplasma respiratory disease typically results in the development of immunopathology, and T cells are a part of this immune response (14). 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 (81). 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 results in similar disease compared to wild-type (WT) mice. Work in T cell deficient hamsters, also demonstrate that these hamster have less severe disease than hamsters with a full immune arsenal (98). Furthermore, pulmonary T cell responses are central to the outcome of disease. The depletion of T helper cells (Th) results in less severe lung disease, demonstrating that a Th cell response contributes to disease pathology in the lung (82). Further studies indicate that a population of Th cells can convey resistance to infection (99). To the contrary, depletion of $CD8^+$ T cell from mice, leads to an exacerbation of mycoplasma pulmonary disease (82). These results suggest that $CD8^+$ T cells play a unique regulatory role within mycoplasma disease in the lower respiratory tract. Thus, pulmonary T cell activation, and the mechanisms that regulate these responses, is instrumental in the pathogenesis of mycoplasma respiratory disease of the lower respiratory tract.

Because of their central role in developing T cell responses, antigen-presenting cells (APC) should be influential in determining immune-mediated pathology or protection during mycoplasma respiratory disease. Recent studies by Sun *et al*. demonstrated, after mycoplasma infection, there were increasing numbers of $F4/80^+$ cells and more mature/activated $CD11c^+$ $CD11b^+$ cells in lungs of mice (95). $CD11c^-$ $F4/80^+$ cells and $CD11c^+$ $F4/80^-$ cells populations showed different patterns of cytokine mRNA expression, supporting the idea that these cells have different impacts on immune responses generated in response to infection. In fact, $CD11c^+$ $F4/80^-$ cells from the lungs of infected mice were most capable of stimulating mycoplasma-

specific T cell responses *in vitro*, and $CD11c^+$ cells localized and interacted with $CD4^+$ Th cells in inflammatory infiltrates in the lungs of infected mice. Thus, $CD11c^+$ $F4/80^-$ cells, most likely DC, appear to be major APC population responsible for T cell stimulation in the lungs of mycoplasma-infected mice, and these *in vivo* interactions likely contribute to the immune responses that impact disease pathogenesis.

The purpose of this study was to determine whether pulmonary $CD11c^+$ DCs support the activation of both $CD4^+$ T helper and $CD8^+$ T cells against mycoplasma antigen. As both T cell populations influence the outcome of mycoplasma infection, it is important to determine whether DCs have the capacity to influence the activation of these lymphocyte populations. This knowledge would make the DC population an excellent target for vaccine development, particularly if these cells could be influenced to promote a more protective immune response.

Material and Methods

Mice. Female BALB/cAnNHsd WT mice, tested to be virus- and mycoplasma free, were obtained from Harlan Sprague-Dawley (Indianapolis, IN). Mice were housed in sterile microisolator cages supplied with sterile bedding, with food and water provided *ad libitum*. Mice used in the study were between 6 and 10 wks of age. Female mice were used in all studies. Before experimental infection, mice were anesthetized with an i.p. injection of diluted ketamine-xylazine. The animal studies were reviewed and approved by the University of North Texas Health Science Center Institutional Animal Care and Use Committee.

Mycoplasma. The UAB CT strain of *M. pulmonis* was used in all experiments. Stock cultures were grown, as previously described (93) in mycoplasma PPLO medium (Acumedia)

and frozen in 1-ml aliquots at -80°C. For inoculation, thawed aliquots were diluted to 2 x 10^5 CFU/20 µl. Nasal-pulmonary inoculations of 20 µl of diluted mycoplasma were given for experimental infections.

Mycoplasma membrane antigen preparation. Crude preparations of *M. pulmonis* membrane were used for immunization and *in vitro* stimulation and prepared as previously described (100). Briefly, *M. pulmonis* was cultured at 37°C in mycoplasma broth medium and harvested at pH 7. Cells were then centrifuged at 10,000 rpm for 20 min, and pellets were resuspended in 5 ml sterile 0.25 M NaCl. Following a second centrifugation at 9,000 rpm for 20 min, pellets were resuspended in a total of 4 ml of 2M glycerol at 37°C. Cells were then sonicated at the highest setting for 15 seconds using Vibra cell sonicator (Sonics & Materials/Vibrio Cell, Newtown, CT), followed by incubation at 37°C for 10 min. For cell lysis, 0.5 ml of the cell preparations 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 h. Membranes were resuspended in 5 ml sterile PBS (HyClone Laboratories, Logan, UT) and stored at -80°C. All centrifugations were done at 4°C. Protein concentration was determined using Bradford protein assay (Bio-Rad, Hercules, CA).

Pulmonary mononuclear cell isolation. Lungs were perfused with PBS without magnesium or calcium to minimize contamination of the final lung cell population with those from blood. The lungs were separated into individual lobes and finely minced. The tissues were suspended in RPMI 1640 (HyClone Laboratories, Logan, UT) medium 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°C while mixing on a Nutator (Fisher Scientific, Pittsburgh, PA) for 60 min. During the incubation period, the tissue was vigorously pipetted every 20 min. After incubation, the digestion mixture was passed through a 250 μm nylon mesh to remove undigested tissue. RBCs were lysed with ammonium chloride-potassium carbonate lysis buffer. Mononuclear cells were purified from cell suspensions by density gradient centrifuge on Lympholyte M (Cedarlane Laboratories, Burlington, NC). The mononuclear cells were further purified using magnetic bead isolation.

Cell sub-population purification. $CD11c^+$, $F4/80^+$ or T cells were purified using paramagnetic bead-conjugated Ab and an AutoMACs (Miltenyi Biotec, Auburn, CA) following manufacturer's instructions. $CD11c^+$ cells were collected by positive selection using CD11c (N418) MicroBeads (Miltenyi Biotec), $F4/80^+$ cells were collected by two binding steps: cells were reacted with biotin-anti-mouse F4/80 (1:40 dilution) (Invitrogen, Caltag laboratories, Carlsbad, CA 92008) followed by Anti-Biotin MicroBeads (Miltenyi Biotec). In order to get pure $CD11c^+$ or $F4/80^+$ without $CD11c^+$ $F4/80^+$ double positive cells, pulmonary mononuclear cells from uninfected mice and *M. pulmonis* infected mice were divided into two parts, one for $CD11c^+$ cells isolation and one part for $F4/80^+$ cells isolation. For $CD11c^+$ $F480^-$ cell purification, mononuclear cells were first depleted of $F4/80^+$ cells using magnetic beads and $CD11c^+$ cell were subsequently isolated. Similar procedure was applied to obtain $F4/80^+$ $CD11c^-$ cells. After depletion of $CD11c^+$and $F4/80^+$ cells, pulmonary T cells were isolated by Pan T cell Isolation Kit (Miltenyi Biotec), which depleted non-T cells using a cocktail biotin-conjugated Ab followed by Anti-Biotin MicroBeads. These untouched T cells were further fractionated into

$CD4^+$ and $CD8^+$ T cell sub-classes using CD8a (Ly-2) microbeads (Miltenyi Biotec) to positively select out the $CD8^+$ T cells, leaving the $CD4^+$ untouched.

Antigen-specific in vitro stimulation of T cells. T cells (2×10^5/well) purified from lungs of mycoplasma-infected or uninfected mice were cultured in the presence or absence of APC populations (5×10^4/well) in Microtest™ U-bottom 96-well culture plates (Becton Dickinson Labware, Franklin Lake, NJ 07417) in complete RPMI-1640 media (Hyclone Laboratories) supplemented with 10% FBS (HyClone Laboratories), 10mM HEPES, 10mM L-glutamine, antibiotic/antimycotic solution (Life Technologies), and 50 μM 2-mercaptoethanol (Life Technologies). Lymphoid cells were stimulated at 37°C and 5% CO_2. Cells were stimulated with or without 5 μg/ml of prepared mycoplasma Ag in a final volume of 200 μl/well of culture medium. Supernatants were collected four days later and stored at -80˚C until assayed for cytokine levels.

Cytokine measurements. Due to the small quantities of supernatant from the co-cultures, IFN-γ levels were measured with a Bio-Plex (Bio-Rad, Hercules, CA). Microtiter 96-well filter bottom plates were used. To each well, 50 μl of anti-cytokine beads in assay diluent was added. After each step, plates were washed with Bio-Plex washing buffer. A 50 μl of sample or standard were added per well. Plates were incubated at room temperature while being shaken in the dark for 2hr. 25 μl of biotinylated secondary antibodies were added to each well. Plates were incubated at room temperature while shaken in the dark for 1 hour. 50 μl of streptavidin-PE were added to each well. Plates were incubated at room temperature, while being shaken in the dark for 30 min. Samples were read using a Bio-Plex 100 system (Bio-Rad). Cytokine levels were determined by comparison with standard curves generated from murine recombinant cytokines and analyzed using Bio-Plex Manager software (Bio-Rad).

Statistical Analysis. Data were evaluated by ANOVA, followed by Fisher protected least square differences multigroup comparison. These analyses were performed using the StatView version 5.0.1 (SAS Institute, Cary, NC) computer program. A p value ≤ 0.05 was considered statistically significant.

Results

$CD11c^+$ pulmonary DCs from both naïve and mycoplasma infected animals support the activation of mycoplasma-specific $CD4^+$ and $CD8^+$ T cells when stimulated with mycoplasma antigen.

Previous studies demonstrated that $CD11c^+$ F4/80- pulmonary DCs are the major antigen presenting cell population in mycoplasma disease, supporting the activation of mycoplasma-specific T cells isolated from the lungs of mycoplasma-infected mice better than $CD11c^+$ $F4/80^+$ alveolar macrophages (95). $CD11c^+$ DCs were found co-localized with $CD4^+$ T cells in the lesion sites in the lungs of mycoplasma-infected mice. However, these T cell activation studies were performed using total T cells isolated from the lung. DCs are known for their cross presentation capabilities, meaning these cells can present exogenous antigen via MHC class I (major histocompatibility complex I) to $CD8^+$ T cells as well as present antigen via MHC class II to $CD4^+$ T cells. We hypothesized that $CD11c^+$ pulmonary DCs could activate both $CD4^+$ and $CD8^+$ T cells when co-cultured with mycoplasma membrane antigen.

To determine whether pulmonary DCs have the capacity to promote activation of mycoplasma-specific $CD8^+$ T cells and Th cells, dendritic cells, $CD4^+$ T cells and $CD8^+$ T cells were isolated from the lungs naïve and mycoplasma-infected mice. DCs and naïve and T cells

were co-cultured with or without mycoplasma membrane antigen for four days. T cell activation was measured by the increase of IFN-γ in the supernatants. When co-cultures were stimulated both mycoplasma-specific $CD4^+$ and $CD8^+$ T cells produced significantly more IFN-γ compared to unstimulated co-cultures (Figure 1a and b). There was no difference in the levels of IFN-γ secreted in from $CD8^+$ T cells stimulated by DCs from naïve or mycoplasma infected mice. However, mycoplasma-specific $CD4^+$ Th cells secreted significantly more IFN-γ when co-cultured with DCs from mycoplasma-infected animals than when co-cultured with naïve DCs. Co-cultures with naïve T cells did not show any increase in the IFN-γ with or without antigen (data not shown).

Discussion

T cell responses during mycoplasma disease are both protective and pathologic. This suggests that different sub-populations have different influences on the immune response against mycoplasma respiratory disease. $CD4^+$ Th cells can promote either immunopathology in mycoplasma disease or resistance to infection (82). Depletion of $CD8^+$ T cells during mycoplasma respiratory disease demonstrated and significant increase in disease severity. Each of these T cells subsets have significant roles during the immune response to mycoplasma disease, but T cells require activation by antigen presenting cells to elicit their effect on the immune system.

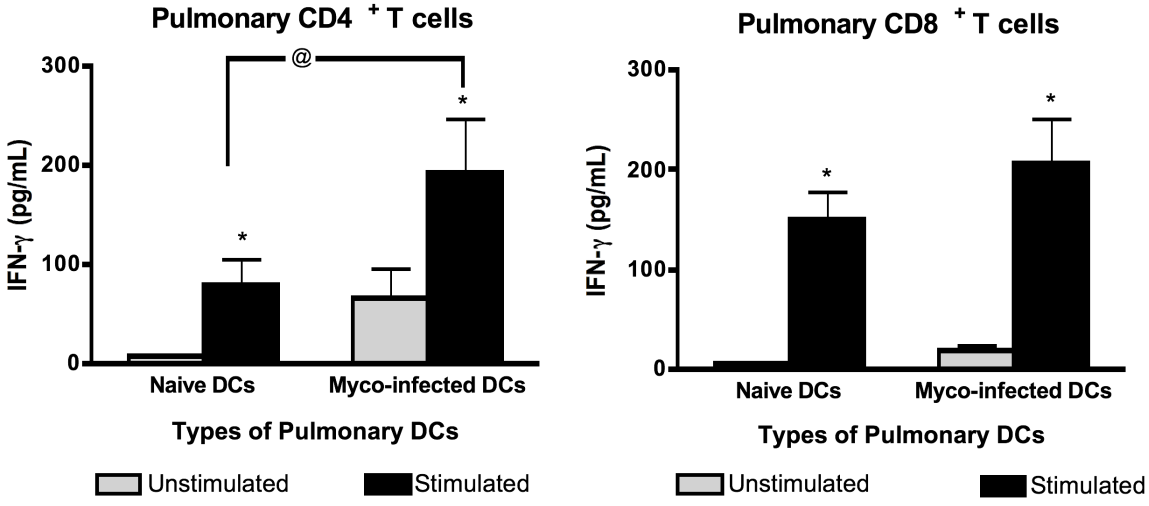


Figure 1. Mycoplasma-specific pulmonary CD4$^+$ and CD8$^+$ T cells co-cultured with pulmonary DCs. Uninfected and mycoplasma-infected mice were sacrificed and pulmonary CD11c$^+$ F4/80$^-$ DCs, CD4$^+$ and CD8$^+$ T cells were purified. Co-cultures were established and cells were stimulated with mycoplasma antigen for 4 days. Supernatants were assessed for IFN-γ production with cytometric bead assays. An asterisk (*) indicates a significant difference ($p \leq 0.05$) in IFN-γ detected from unstimulated co-cultures. A "@" sign indicates a significant difference ($p \leq 0.05$) in the IFN-γ detected from naïve DC co-cultures. Vertical bars and error bars represent the mean +/- SE (CD4 expts: naïve and myco DC unstim: n=13 each, CD4 naïve DC stim: n=15 and CD4 myco DC stim: n=13; CD8 expts: naïve and myco DC, stim and unstim: n=13).

Recent studies from our lab demonstrated that $CD11c^+$ $F4/80^-$ pulmonary dendritic cells are the major APC to activate T cells during mycoplasma disease (95). DCs demonstrated superior ability to support mycoplasma-specific T cells activation as compared to pulmonary macrophages. Confocal microscopy revealed these $CD11c^+$ DCs were found co-localized with $CD4^+$ T cells at the lesion sites in mycoplasma infected mice were these cells were more than likely interacting, and the $F4/80^+$ cells were found to be on the periphery and not interacting with the T cells (95). It has not been demonstrated whether or not DCs can actually support the activation of either $CD4^+$ or $CD8^+$ T cells.

We hypothesized that the $CD11c^+$ F4/80- pulmonary DCs could support both the T cell activation of mycoplasma-specific $CD4^+$ and $CD8^+$ T cells when stimulated with mycoplasma antigen. Indeed, when compared to unstimulated co-cultures, pulmonary dendritic cells support the activation of $CD4^+$ and $CD8^+$ T cell IFN-γ responses when stimulated with antigen. Interestingly, DCs from the lungs of infected mice have the greatest effect on $CD4^+$ T cell activation. This can be seen by comparing levels of IFN-γ produced by stimulated $CD4^+$ T cells which are significantly increased when co-cultured with DCs isolated from the lungs of mycoplasma-infected mice rather than stimulated naive pulmonary DCs. Whereas, there is no difference in the amount of IFN-γ produced by $CD8^+$ T cells co-cultured with DCs from the lungs of either naïve or mycoplasma infected mice. This indicates that during mycoplasma infections, DCs may be geared to preferentially support $CD4^+$ T cell responses over $CD8^+$ T cells. This had been observed also during confocal microscopy where the $CD8^+$ T cells were observed on the periphery of the lesions, while the $CD4^+$ T cells are inside the lesioned area (personal observation) in contact with DCs. Overall, the results from these studies indicate that T

cell/DC interactions are critical to the development of adaptive immunity in mycoplasma disease. Thus, DCs may be a key cell that determines the outcome of infection and understanding the specific roles of antigen presenting cells during mycoplasma disease will provide insights for designing a better targeted vaccine.

Dendritic cells are potent activators of effector T cells, and more importantly, these cells assist in the polarization of T helper cell type responses (15). This makes the DCs an obvious target for manipulation to skew the adaptive immunity. Recent research has developed new techniques using polarizing cytokines to generate different types of bone marrow derived DCs (BMDCs) that can skew their function (101-103). These techniques have been successfully used in cancer therapy and other infection studies (104 Otsu 2006, 105-107). I hypothesized that cytokine differentiated BMDCs sub-classes could be generated to skew the adaptive immune response to a more protective immune response against mycoplasmas respiratory disease. While these studies may seem like a novel approach to immunological manipulation, these studies will also provide clues to the role pulmonary DCs during the pathogenesis of mycoplasma disease, especially when compared to other tissue specific DCs.

CHAPTER IV

Dendritic Cell Sub-Populations Contribute to Immunopathology and Accelerated Disease Progression in *Mycoplasma pulmonis* Infected Mice

The purpose of these studies was to evaluate the capacity of cytokine differentiated DC populations to influence the generation of protective and/or pathologic immune responses during mycoplasma respiratory disease. We hypothesized that adoptive transfer of mycoplasma antigen-pulsed BMDCs could influence the host to generate protective T cell responses during mycoplasma infection. However, adoptive transfer of any antigen-pulsed DCs enhanced pathology in the recipient mice when challenged with mycoplasma disease. Similar results were observed with the adoptive transfer of antigen-pulsed pulmonary, but not splenic, DCs. Lymphocytes generated in uninfected mice after the transfer of either antigen-pulsed BMDCs or pulmonary DCs were shown to be Th2 cells, known to be associated with immunopathology. Although, protective responses could not be achieved through this novel approach of manipulating BMDCs, the discovery of the role of pulmonary DCs and their contribution pathogenesis during mycoplasma infection was far more important. This knowledge will contribute to an improved vaccination strategy.

Introduction

As mucosal pathogens, mycoplasmas infect a wide variety of hosts causing respiratory and urogenital diseases having a profound impact on the agricultural industry, due to loss of livestock, as well as human disease (14) *M. pneumoniae* is the common human respiratory pathogen known to cause the community-acquired pneumonia known as "walking pneumonia," afflicting students who inhabit dormitories, military personnel in military barracks and attendees at summer camps (4-6). *M. pneumoniae* causes 30% of all cases of pneumonia in the United States, and is also implicated in the exacerbation of other respiratory conditions such as asthmatic airway hypersensitivity reactions, chronic obstructive pulmonary disease (COPD) and cystic fibrosis (3, 7-13, 108, 109). *Mycoplasma pulmonis*, a natural rodent pathogen, is an excellent animal of *M. pneumoniae*, allowing the characterization of immune responses during the pathogenesis of mycoplasma respiratory disease. Histopathology revealed that both diseases are characterized by the accumulation of mononuclear cells such as macrophages and lymphocytes along the airways (79, 109-112). This suggests that the activation and recruitment of macrophages and lymphocytes is important to the development of both acute and chronic states of disease. To date, there are limited vaccines for mycoplasma and the vaccines that have been generated are not optimal and sometimes are implicated in exacerbation the disease rather than generating protection (77, 80, 90, 113).

Immunity to mycoplasma respiratory pathogens is a complex topic, because it is clear that the adaptive immunes system contributes to the pathology, as well as generates protection against *M. pulmonis* infection. Early studies on SCID mice, whose genetic mutation renders them incapable of producing functional B and T cells, demonstrated that these mice do not develop the

severe pathology found in their immunocompetent counterparts (77, 80, 81). As the infection progresses in these animals, the SCID mice are incapable of controlling dissemination of mycoplasma from the site of infection to the rest of the body. Ultimately, this results in the eventual development of arthritis and higher mortality rate. Reconstitution of SCID mice with functional lymphocytes restored the same pathology seen in wild-type (WT) mice, but controlled the dissemination of the organisms (77). Additional studies in T cell deficient hamsters also demonstrate that these hamsters have less severe disease compared to their WT counterparts (90, 113).

Pulmonary T cells are pivotal to determining the outcome of the mycoplasma infection. Depletion of $CD8^+$ T cells, leaving only the $CD4^+$ T helper cells, resulted increased clinical disease and lesion severity during mycoplasma infection (91). Depletion of $CD4^+$ Th cells decreased clinical disease and reduced lesion severity. These studies indicated that $CD8^+$ T cells function to dampen the inflammatory response, while the Th cells can exacerbate the disease. However, one additional caveat found in a study done by Bodhankar *et al*. demonstrated when Th cells from immunized mice were adoptively transferred into naïve mice, these mice were more protected from mycoplasma disease as shown in the reduction of CFU numbers recovered from the lungs (99). Therefore, Th cells can have conflicting roles when it comes to protection and pathology of mycoplasma disease.

T cells are the source of the pathology/protection balance, but these cells are a part of the adaptive immune response and require the assistance of professional antigen presenting cells during activation. Dendritic cells (DCs) are potent antigen presenting cells known for their extraordinary ability to activate both naïve $CD4^+$ and $CD8^+$ T cells (114-120). DCs are critical in the generation of allergic and asthmatic responses (28, 121-123) and therefore can play a role in

inducing immune-mediated inflammatory disease. Importantly, Sun *et al*. studies showed an increase in the number of $CD11c^+$ DCs during mycoplasma disease, and that these pulmonary DCs from infected mice were more capable of supporting T cell activation than their $F4/80^+$ macrophage counterparts (95). Additionally, confocal microscopy revealed pulmonary $CD11c^+$ DCs co-localized with $CD4^+$ T helper cells at the lesion sites in the lungs of mycoplasma-infected mice. Therefore, DCs are the major antigen presenting cells during mycoplasma respiratory disease, and are likely to influence T cell responses critical to the outcome of infection.

The purpose of these studies was to evaluate the capacity of DC populations to influence the generation of protective and/or pathologic immune responses during mycoplasma respiratory disease. To do this several populations of bone marrow derived DCs (BMDCs) were generated in differing cytokine environments in an attempt to influence the DC functionality and affect the T cell populations generated. Initially, differing cytokine environments affected co-stimulatory molecule expression on BMDCs and cytokine responses after *in vitro* infection with mycoplasma. Contrary to our initial hypothesis that one or all of these sub-populations may influence a more protective host response; adoptive transfer of antigen-pulsed DCs into the lungs of recipient mice enhanced the disease severity after mycoplasma infection. This accelerated disease pathogenesis was lymphocyte dependent and associated with the increase of $IL\text{-}13^+$ Th2 responses. Furthermore, naïve pulmonary DC similarly enhanced Th2 responses to mycoplasma. Thus, these results demonstrate that DCs can promote immunopathologic lymphocytes responses, most likely through the generation of mycoplasma-specific Th2 cell responses.

Materials and Methods

Mice. Female BALB/cAnNHsd wild type (WT) and SCID (BALB/cJHan™Hsd-*Prkdc*^{scid}) mice, tested to be virus- and mycoplasma free, were obtained from Harlan Sprague-Dawley (Indianapolis, IN). Mice were housed in sterile microisolator cages supplied with sterile bedding, with food and water provided *ad libitum*. Mice used in the study were between 6 and 10 wks of age. Female mice were used in all studies. Before experimental infection, mice were anesthetized with an i.p. injection of diluted ketamine-xylazine. The animal studies were reviewed and approved by the University of North Texas Health Science Center Institutional Animal Care and Use Committee.

Mycoplasma. The UAB CT strain of *M. pulmonis* was used in all experiments. Stock cultures were grown, as previously described (93), in mycoplasma PPLO medium (Acumedia) and frozen in 1-ml aliquots at -80°C. For inoculation, thawed aliquots were diluted to 2 x 10^5 CFU/20 µl. Nasal-pulmonary inoculations of 20 µl of diluted mycoplasma were given for experimental infections.

Mycoplasma membrane antigen preparation. Crude preparations of *M. pulmonis* membrane were used for immunization and *in vitro* stimulation and prepared as previously described (100). Briefly, *M. pulmonis* was cultured at 37°C in mycoplasma broth medium and harvested at pH 7. Cells were then centrifuged at 10,000 rpm for 20 min, and pellets were resuspended in 5 ml sterile 0.25 M NaCl. Following a second centrifugation at 9,000 rpm for 20 min, pellets were resuspended in a total of 4 ml of 2M glycerol at 37°C. Cells were then sonicated at the highest setting for 15 seconds using Vibra cell sonicator (Sonics & Materials/Vibrio Cell, Newtown, CT), followed by incubation at 37°C for 10 min. For cell lysis,

0.5 ml of the cell preparations 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 h. Membranes were resuspended in 5 ml sterile PBS (HyClone Laboratories, Logan, UT) and stored at -80°C. All centrifugations were done at 4°C. Protein concentration was determined using Bradford protein assay (Bio-Rad, Hercules, CA).

UV irradiated mycoplasma preparation. *M. pulmonis* was cultured at 37°C in mycoplasma broth medium and harvested at pH 7. Cells were then centrifuged at 10,000 rpm for 20 min, and pellets were resuspended in 10 ml sterile phosphate buffered saline and placed into a sterile T-25 cell culture flask. The mycoplasma was UV irradiated on a UV transilluminator (Fotodyne Inc., Hartland WI) for 20 minutes. A sample of this preparation was spot plated onto mycoplasma plates and incubated for 7 days to ensure that all mycoplasma was UV irradiated resulting in no growth on the plates. Protein concentration was determined using Bradford protein assay (Bio-Rad, Hercules, CA). Aliquots were stored frozen at -80°C.

Bone marrow derived DC generation and purification. Bone marrow cells were isolated from tibiae and femora of 6 to 8 week old female BALB/c mice. Red blood cells were removed using ammonium chloride potassium carbonate lysis buffer (124). Ten million cells were seeded into a T-75 culture flask in 20 ml of complete RPMI-1640 media (Hyclone Laboratories) supplemented with 10% FBS (HyClone Laboratories), 10mM HEPES, 10mM L-glutamine, antibiotic/antimycotic solution (Life Technologies), and 50 μM 2-mercaptoethanol (Life Technologies). All dendritic cell groups were grown for 6 – 9 days using 20 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) (Invitrogen, Carlsbad, CA.) added to the media. However, the G4DC group was generated using the addition of interleukin-4 (IL-

4) (Peprotech, Rockhill, NJ) to the media at 20 ng/mL, and the T10DC group was generated by adding 20 ng/mL transforming growth factor – beta 1 (TGF-β1) and 20 ng/mL interleukin-10 (IL-10) (Peprotech, Rockhill, NJ). The cells were collected from the flasks, labeled with paramagnetic bead-conjugated with anti-$CD11c^+$ mAb (N418) MicroBeads (Miltenyi Biotec, Auburn, CA) and sorted using the positive selection and magnetic columns on the AutoMACs (Miltenyi Biotec) per manufacturer's instructions.

***In vitro* stimulation of BMDC groups**. $CD11c^+$ purified from bone marrow cultures were cultured in 96-well round-bottom microtiter plates in RPMI 1640 (HyClone Laboratories) supplemented with 10% FBS (HyClone Laboratories), 10mM HEPES, 10mM L-glutamine, antibiotic/antimycotic solution (Life Technologies), and 50 μM 2-mercaptoethanol (Life Technologies). Cells were cultured at 37°C and 5% CO_2. Cells were stimulated with or without 5 μg/ml mycoplasma membrane Ag or 5 μg/ml of UV irradiated mycoplasma in a final volume of 200 μl/well of culture medium at a cell concentration of 2×10^6 cells/ml for 24 hours. For cytokine profile, these cells were also stimulated with ultrapure lipopolysaccharide (LPS) (InvivoGen, San Diego, CA) at a final concentration of 10 μg/ml, fibroblast stimulating lipopeptide-1 (FSL-1) (InvivoGen, San Diego, CA), a synthetic homologue of the MALP-2 agonist from *M. fermentans* at a final concentration of 1 μg/mL or live M. pulmonis with an average concentration of 1.25×10^7 CFU/ml. These cells were stimulated overnight at 37°C and 5% CO_2 and supernatants were collected for cytokine detection.

Cytokine Assays. The levels of cytokine (IL-10 and IL-12p40) were measured by capture ELISAs. All ELISA performed using OptEIA™ mouse ELISA kit from BDPharMingen Co. Briefly, Probind 96-well flat-bottom microtiter plates (BD Biosciences, Bedford, MA) were coated overnight at 4°C with capture Ab. Plates were washed with PBS/ 0.01% Tween 20 and

blocked with PBS/Tween 20 supplemented with 10% FBS solution overnight at 4°C. Following washing, supernatants of cell culture were placed into the appropriate wells and incubated overnight at 4°C. After washing, a mixture of diluted biotinylated Ab and Avidin-HRP Ab was added to each well and incubated for two hours at room temperature. 3,3'5,5'-tetramethylbenzidine substrate (TMB, Moss, Pasadena, MD) was used to reveal the reaction. Plates were read using Synergy HT Multi-Mode Microplate reader (Biotek, Winooski, VT) at an absorbance of 630 nm. Cytokine levels were determined by comparison with standard curves generated from murine recombinant cytokines after log/log quadratic linear regression analysis using Gen5™ Data Analysis Software (Biotek, Winooski, VT).

Flow Cytometry. Two-color immunofluorescent staining was performed to identify co-stimulatory molecule on unstimulated BMDC populations using a combination of phycoerythrin (PE)-labeled anti-murine CD11c mAb with fluorescein isothyocyanate (FITC)-labeled Ab specific for CD40, CD80 (B7.1), CD86 (B7.2) or MHC II (BD PharMingen), respectively. Briefly, 5 x 10^5 cells per tube were incubated with purified 2.4G2 mAb (CD16/CD32; BD PharMingen) for 5 min at 4°C to reduce nonspecific binding of FcII/IIIRs before fluorescent Ab staining. The cells were incubated for 30 min at 4°C with 200 μl fluorescent Ab (2 μg/ml). The cells were washed in staining buffer (PBS, 2% FBS, 2mM EDTA) and fixed with 2% paraformaldehyde solution for 30 min, cells were then resuspended in staining buffer and data was acquired using a Beckman Coulter Cytomics FC500. Data analysis was performed using the CXP Analysis software provided by Beckman Coulter (Fullerton, CA). Lymphocyte gates and detector voltages were set using unstained (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 lymphocyte population, the total number of lymphocytes isolated from each tissue was multiplied their percentage.

Adoptive Transfer of BMDCs. After overnight stimulation with or without mycoplasma antigen, the cells were washed 3 times to remove any unbound antigen using culture media (RPMI 1640 (HyClone Laboratories) supplemented with 10% FBS (HyClone Laboratories), 10mM HEPES, 10mM L-glutamine, antibiotic/antimycotic solution (Life Technologies), and 50 μM 2-mercaptoethanol (Life Technologies)). The cells were finally resuspended in cold phosphate buffered saline at a concentration of $1x10^7$ cells/ml. Before adoptive transfers, mice were anesthetized with an i.p. Injection of diluted ketamine-xylazine. Using a 1 ml syringe and 22-gauge, 1-inch dosing needle with stainless steel ball at the end (Braintree Scientific, Inc., Braintree, MA), 50 μl (5×10^5 cells) were deposited in the lungs of anesthetized mice. Control mice received 50 μl of PBS only. Animals were infected 10 days post-adoptive transfer.

MouseOx Pulse Oximeter. The MouseOx® Pulse Oximeter (Starr Life Sciences Corp., Oakmont, PA) was used for non-invasive measurement of oxygen saturation. The CollarClip™ Sensor was placed around the necks of conscious mice to measure the flow through the carotid artery, which provides strong breathing and cardiac signals. Each animal was measured using the Quick Averaging Diagnostic Window, set to take an average of 30 seconds of good readings on all variables, which takes on average 2-5 minutes for a healthy mouse and up to 20 – 30 minutes for a very sick mouse. Every mouse was read individually every two days during the fourteen-day infection.

Assessment of gross lesions. Lungs were removed, and each lobe was examined 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 (125).

Intracellular cytokine stimulation and staining. Lymphocytes from the lower respiratory nodes, lung and spleen were purified and stimulated overnight with UV irradiated mycoplasma (5μg/ml). The next day 50 ng/ml Phorbol 12-Myristate 13- Acetate (PMA; Sigma-Aldrich, St. Louis, MO), and 500 ng/ml Ionomycin (EMD, Gibbstown, NJ) was added for the last 5 hrs of the culture before staining.

To accomplish intracellular cytokine staining, GolgiPlug, containing Brefeldin A (BFA) (BD PharMingen, San Jose, CA), was added 4 hrs prior to the harvest of the cell cultures. Staining of the cells involved incubation at 4°C for 15 min in staining buffer (PBS^+ 2% FBS^+ 2 mM EDTA) with saturating amounts of the cell-surface antibodies and anti-CD16/CD32 to block Fc receptors. Additional cell surface markers used to identify cells included PE-Cy7-labeled anti-CD3 mAb, Alexa Fluor 488-labeled anti-CD4 mAb, peridinin chlorophyll-*a* protein (PerCP)-labeled anti-CD8 mAb (all from BD PharMingen) and APC-Cy7 labeled anti-CD44 mAb (BioLegend, San Diego, CA). To measure intracellular cytokines, cells were fixed and permeabilized at 4°C for 20 min using the intracellular cytokine staining kit from BD PharMingen. After washing in permeabilization wash buffer, the cells were incubated in saturating amounts of allophycocyanin (APC)-labeled anti-IFN-γ mAb and PE-labeled anti-IL-13 mAb (both from eBioscience, San Diego, CA) at 4°C for 20 min. The cells were washed in staining buffer (PBS, 2% FBS, 2mM EDTA) resuspended in BD Stabilizing Fixative (BD PharMingen). Data were acquired and analyzed using a BD LSR II Flow Cytometer System and BD FACSDiva v5 Software (Fullerton, CA). Lymphocyte gates and detector voltages were set using unstained spleen cells. The flow cytometry data were further analyzed using FlowJo flow

cytometry analysis software (Tree Star, Ashland, OR).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 lymphocyte population, the total number of lymphocytes isolated from each tissue was multiplied their percentage.

Lymphocyte Isolation. Mononuclear cells were isolated from lungs, as previously described but with some minor alterations to improve cell recovery (94). Lung lobes were dissected and placed individually in GentleMACs (Miltenyi Biotec, Auburn, CA) tube with 5 ml digest medium containing RPMI 1640 medium (HyClone Laboratories, Logan, UT), 300 U/ml *Clostridium histolyticum* type I collagenase (Worthington Biochemical, Freehold, NJ), 50 U/mL DNase (Sigma-Aldrich, St. Louis, MO), 5% FBS (HyClone Laboratories), 10 mM HEPES (Fisher Scientific, Pittsburgh, PA), and antibiotic/antimycotic solution (Life Technologies, Grand Island, NY). The lung samples were homogenized using a GentleMACs machine (Miltenyi Biotec) on the setting Lung version 2.0. Subsequently the homogenates were incubated at 37°C while mixing on a Nutator (Fisher Scientific) for 15 - 20 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 (Cedarlane Laboratories, Burlington, NC).

Spleen and lower respiratory lymph node (LRN) cells were isolated after centrifugation of all suspensions, followed by red cell removal using ammonium chloride potassium carbonate lysis buffer.

Mycoplasma CFU counts. The number of mycoplasma colony forming units (CFU) in the lungs and nasal passages were determined as previously described but with a few modifications (126). Briefly, lung lobes were dissected and put in a GentleMACs (Miltenyi

Biotec) tube with 5 ml digest media containing RPMI 1640 medium (HyClone Laboratories, Logan, UT), 300 U/ml *Clostridium histolyticum* type I collagenase (Worthington Biochemical, Freehold, NJ), 50 U/mL DNase (Sigma-Aldrich, St. Louis, MO), 5% FBS (HyClone Laboratories), 10 mM HEPES (Fisher Scientific, Pittsburgh, PA), and antibiotic/antimycotic solution (Life Technologies, Grand Island, NY). The lung sample was homogenized using a GentleMACs machine (Miltenyi Biotec) on the setting Lung version 2.0. After the tissue was homogenized, a 250µl sample of lung homogenate was removed for further processing. The samples were sonicated (Vibra cell sonicator; Sonics & Materials/Vibro Cell) for 1 minute 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°C, colonies were counted, and the number of CFU recovered from each tissue was calculated.

Statistical Analysis. Data were evaluated by ANOVA, followed by Fisher protected least square differences multigroup comparison. These analyses were performed using the StatView version 5.0.1 (SAS Institute, Cary, NC) computer program. A p value ≤ 0.05 was considered statistically significant.

Results

Differing cytokine environments impact the phenotype of BMDCs generated and their response to stimuli.

In an attempt to alter BMDC functions, BMDCs were differentiated in differing cytokine environments as previously described (101-103, 127). Granulocyte macrophage-colony stimulating factor (GM-CSF) is the major cytokine used to stimulate the growth of dendritic cells

from bone marrow. To establish an unaltered set of BMDCs, a group was generated using only this cytokine, which are denoted in these data as GMDC. In attempt to generate a set of BMDCs with T helper (Th) 2 polarizing capabilities, bone marrow was cultured with GM-CSF and interleukin-4 (IL-4), a Th2 promoting cytokine, and are referred to as G4DC. The final group of BMDCs was cultured using transforming growth factor-beta 1 (TGF-β1) and IL-10 in an attempt to generate a group of BMDCs with anti-inflammatory capabilities. This group of dendritic cells is called T10DC.

To establish whether these culture conditions did influence the differentiation of BMDCs, unstimulated dendritic cells were sorted, and $CD11c^+$ cells were collected and stained for the expression of co-stimulatory molecules. The G4DC group showed the greatest expression of co-stimulatory molecules CD40, CD80 (B7.1) and a significant increase in the molecule CD86 (B7.2) (Figure. 1a), while the T10DC group showed the lowest expression of these molecules. The GMDC group had median expression of CD40, CD80 and CD86 molecules, but similar expression of MHC II as G4DC. This data indicate that the cytokine environment seems to affect the phenotype of these BMDCs.

To distinguish whether these different phenotypes had an effect of the BMDC cytokine secretion in response to mycoplasma infection, these BMDCs were challenged with viable mycoplasma organisms, ultra-pure LPS [a toll-like receptor (TLR) 4 agonist] and FSL-1 [a TLR 2,6 agonist] in culture media without the addition any cytokines. The GMDC group secreted significantly more IL-12p40 in response to live mycoplasma (Fig.1b) than G4DC and T10DC. A similar pattern was observed between the groups with ultra-pure LPS and FSL-1. The T10DC group produced significantly more IL-10 than G4DC when challenged with mycoplasma and LPS (Fig. 1c). The GMDC group produced a median amount of IL-10 under the same

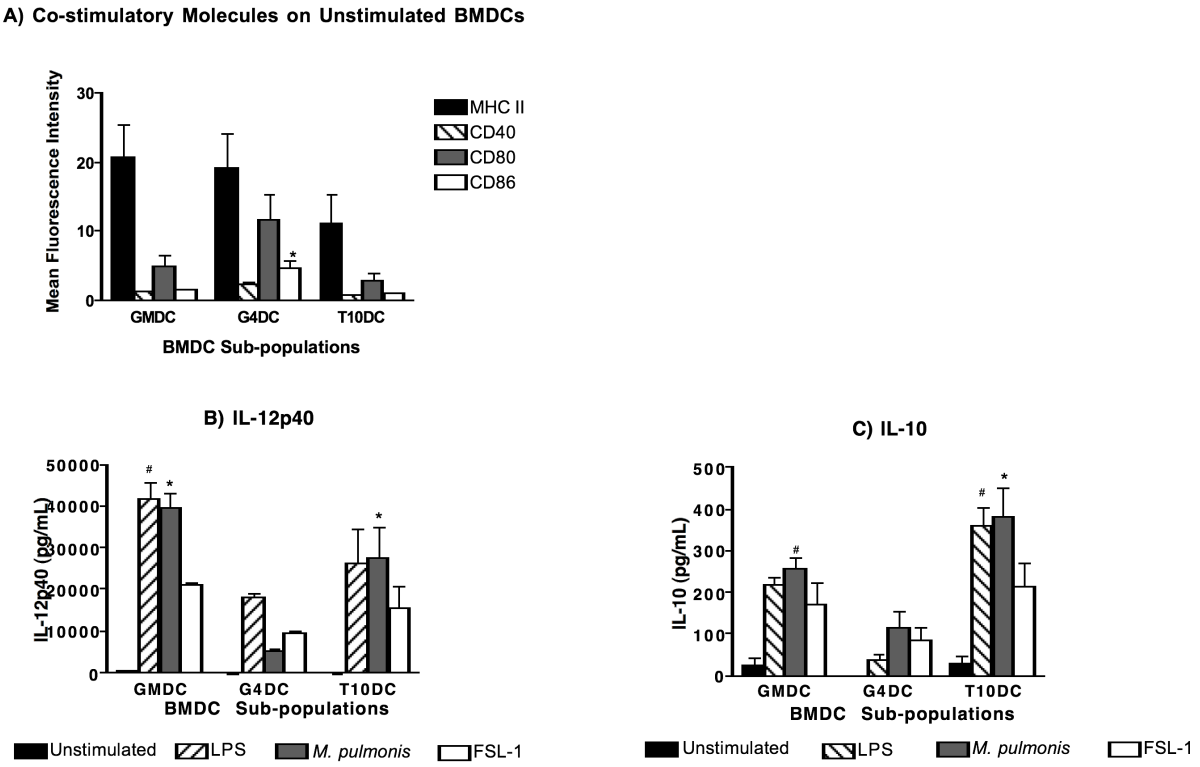


Figure 1. ***In vitro*** **studies of BMDC co-stimulatory molecules and cytokine profiles**. BMDCs were grown out for 6 days in the following culture conditions: GMDC = GM-CSF only, G4DC= GM-CSF and IL-4, and T10DC= GM-CSF, TGF-β1 and IL-10. (a) Unstimulated $CD11c^+$ DCs were isolated and co-stimulatory molecules MHC II (black bar), CD40 (hatched bar), CD80 (grey bar), and CD86 (white bar) were stained. Vertical bars and error bars represent the mean +/- SE (n=9). An asterisk (*) indicates a significant difference ($p \leq 0.05$) from GMDC and T10DC groups. Cytokine levels after overnight stimulation with antigen: ultra-pure LPS (hatched bars), viable *M. pulmonis* (grey bars) and FSL-1 (white bar). Supernatants were collected and run on ELISA to detect IL-12p40 (b) and IL-10 (c) cytokine expression levels. A numeral sign (#) indicates a significant difference ($p \leq 0.05$) from G4DC group with regard to LPS stimulation. An asterisk (*) indicates a significant difference ($p \leq 0.05$) from the G4DC group in both figures with regard to mycoplasma stimulation.

stimulation conditions. Overall, these results suggest that while the G4DC group have greater co-stimulatory molecule expression, which may increase the ability to activate T cells, they do not respond as strongly to stimuli as that of the GMDC and T10DC. The T10DC group has the ability to secrete more IL-10 in response suggesting that this group may have more anti-inflammatory properties. This would suggest that differing culture conditions did have an effect on the BMDCs and thereby may affect their functionality *in vivo*.

Adoptive transfer of antigen-pulsed BMDCs accelerates mycoplasma disease.

To examine whether BMDC populations influenced mycoplasma disease progression $CD11c^+$ BMDCs were isolated and pulsed overnight with mycoplasma antigen. These cells were washed to remove any unbound antigen and deposited intratracheally into the lungs of recipient mice. Ten days post-adoptive transfer, mice were infected with *M. pulmonis* intranasally and monitored for 14 days (Fig. 2).

Mouse weights were recorded on days 0, 4, 7, 10, 12 and 14. During the early days following the infection (days 0 – 5), there was no difference between any of the adoptive transfer groups or the PBS control group, and all the mice were active and exhibited no signs of illness (Fig. 3a). The first sign that inoculation of antigen-pulsed BMDC populations had an impact on recipient mice was at day 7 post-infection (Fig. 3b). T10DC inoculated mice lost significantly more weight on day 7 then GMDC, G4DC and the PBS control group. Mice in the T10DC adoptive transfer groups were clinically ill with matted fur, hunched posture and general malaise as pictured and 68% of these animals had already lost at minimum 10% of their original body weight (Fig. 3c). At the same time the GMDC group of mice demonstrated increased clinical illness, but not to the same degree the T10DC mice demonstrated. The GMDC mice lost significantly more weight than the G4DC, but only 25% of these animals lost a minimum of 10%

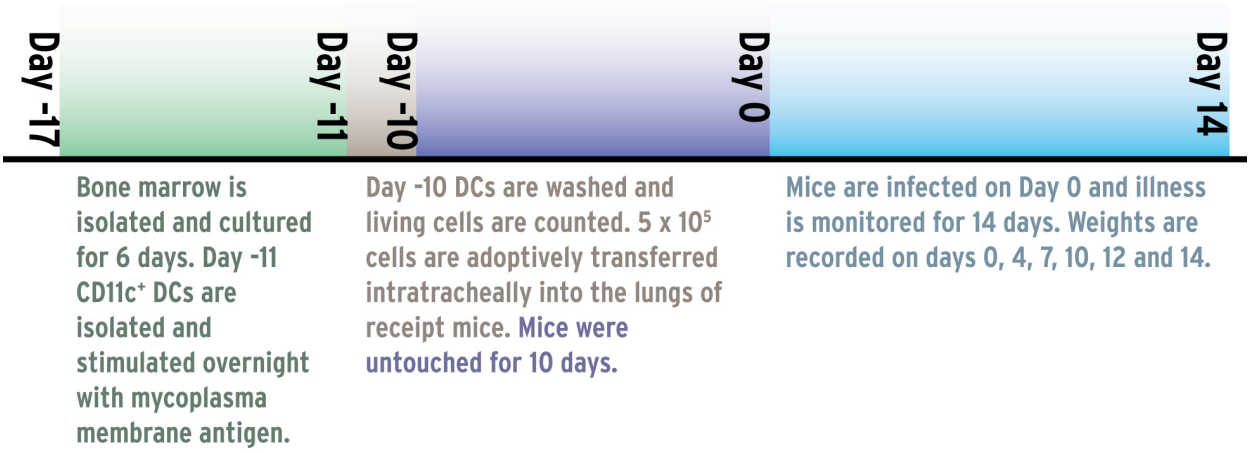
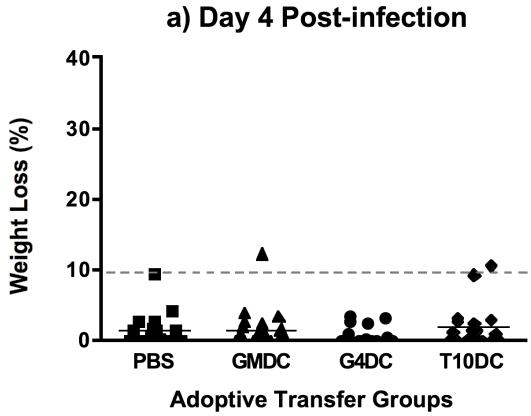


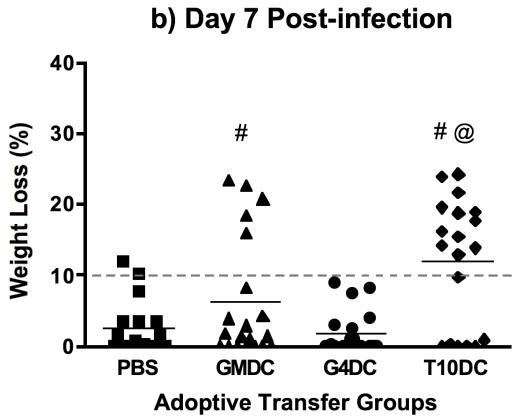
Figure 2. Time line of *in vivo* experimental design.

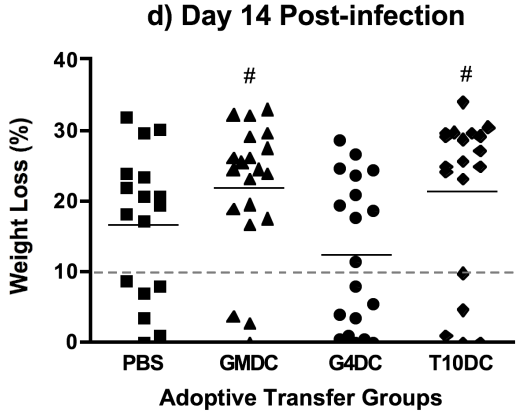
BALB/c donor mice were used to generate BMDCs at day 17 pre-infection. BMDCs were grown over 6 days and $CD11c^+$ DCs were sorted out and pulsed overnight with mycoplasma antigen. Any unbound antigen was washed off and antigen-pulsed $CD11c^+$ DCs were intratracheally deposited into the lungs of recipient mice at 5 x 10^5 cells/50µl per mouse. Ten days post-adoptive transfer animals were infected with *M. pulmonis* monitored for 14 days.

Figure 3. Weight loss of BMDC recipient mice during mycoplasma infection. Mycoplasma antigen-pulsed $CD11c^+$ DCs were intratracheally deposited into the lungs of recipient mice at 5 x 10^5 cells/50µl per mouse. Ten days post-adoptive transfer animals were infected with *M. pulmonis* monitored for 14 days. There was no difference in weight loss at day 4 post-infection (a). At day 7 post-infection, T10DC mice lost 10% or greater of their original start weight (b). This is illustrated in the picture comparing a PBS mouse to a T10DC recipient mouse at day 7 post-infection (c). At day 14 post-infection, weight loss was similar among all the groups. (d). A numeral sign (#) indicates a significant difference ($p \leq 0.05$) in weight loss from PBS and G4DC group in both figures. An @ indicates a significant difference ($p \leq 0.05$) in weight loss from the

GMDC group. Vertical bars and error bars represent the mean +/- SE (GMDC: n=17, G4DC: n=20, G4DC: n=19, T10DC: n=19).







of their weight. The PBS control group and the G4DC group did not demonstrate clinical disease severity at this time point as in T10DC and GMDC groups. Twelve percent of the PBS group of mice had lost 10% of their weight and none of the G4DC group had lost that more than 10% of the starting body weights. However by day 14 post-infection, all the groups of mice lost significant body weight (Fig. 3d). Both the T10DC and GMDC groups had still lost significantly more weight than the G4DC group, but the same was not true for the PBS control group. These data indicate that adoptive transfer of antigen-pulsed accelerate clinical disease particularly in the T10DC group first with the GMDC group following shortly behind. The G4DC group showed little difference in clinical disease and weight loss when compared to the PBS control.

A hallmark of chronic mycoplasma respiratory disease is the development of lesions in the lungs, which is tightly associated with the activation of the adaptive response, particularly the activation of T cells. At day 14 post-infection, mice were sacrificed, and gross lesions were scored. Consistent with the development of clinical disease, the GMDC and T10DC recipient mice had significantly higher gross lesion scores than that of the G4DC and PBS control mice (Fig. 4a) as shown in the comparison of PBS lungs next to T10DC lungs (Fig 4b). Despite demonstrating increased immunopathology and clinical disease, there was no difference in the number of mycoplasma CFU in the lungs 14 days post-infection (Fig. 5). These data demonstrate mice receiving antigen-pulsed DCs had an increase in clinical disease and immunopathology, but this did not have any impact on the clearance of the microorganisms from the site of infection.

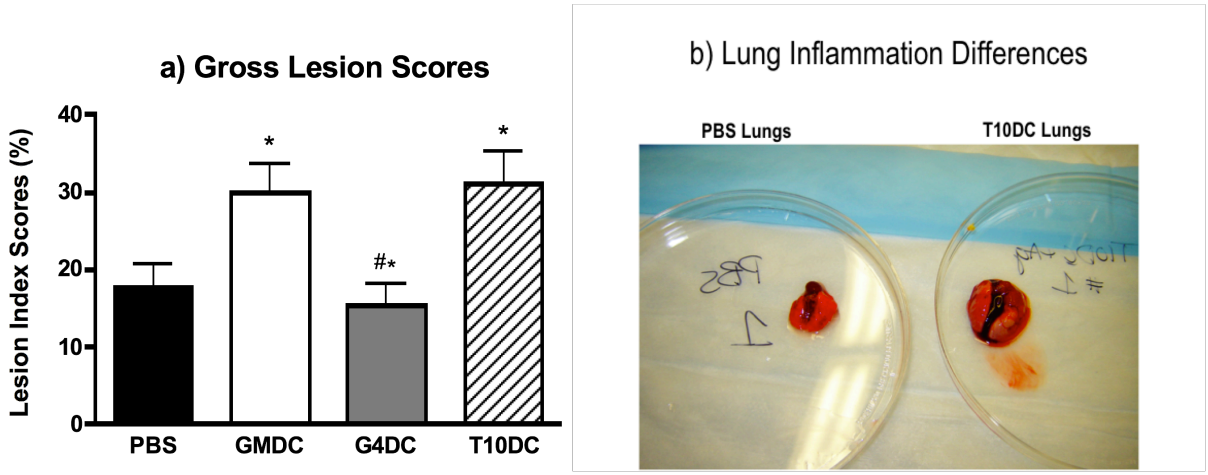


Figure 4. **Gross lesion index scores of BMDC recipient mice 14 days post-infection**. Mycoplasma antigen-pulsed $CD11c^{+}$ DCs were intratracheally deposited into the lungs of recipient mice at 5 x 10^{5} cells/50µl per mouse. Ten days post-adoptive transfer animals were infected with *M. pulmonis* monitored for 14 days. Lungs were scored for percentage of gross lesions. The GMDC group (white bar) and the T10DC group (hatched bar) had greater lesion severity compared to PBS (black bar) and G4DC (grey bar) (a). Differences in lesion scores are demonstrated by comparing lungs from PBS and T10DC mice at day 14 post-infection (b). An asterisk (*) indicates a significant difference ($p \leq 0.05$) from the PBS and GMDC groups. Vertical bars and error bars represent the mean +/- SE (GMDC: n=17, G4DC: n=20, G4DC: n=19, T10DC: n=19).

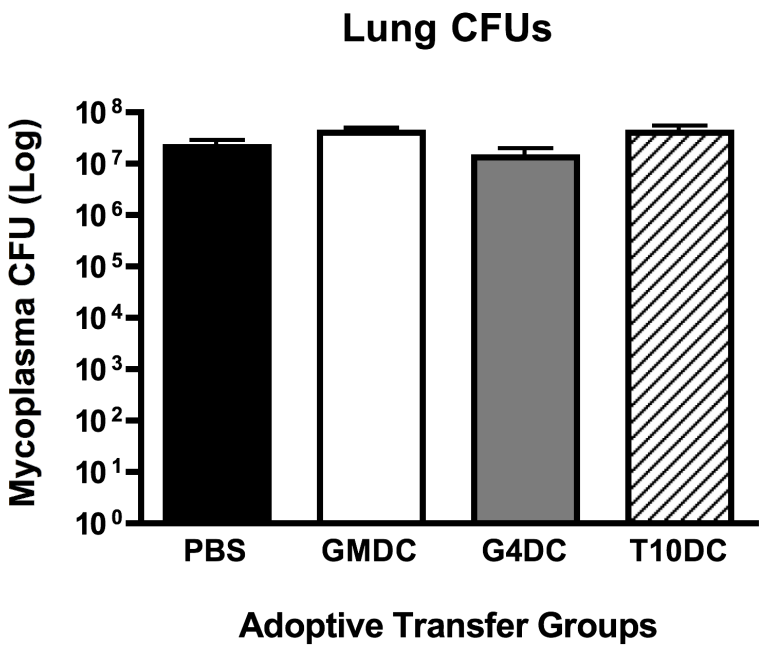


Figure 5. **Mycoplasma CFU from the lungs of BMDC recipient mice 14 days post-infection**. Mycoplasma antigen-pulsed $CD11c^+$ DCs were intratracheally deposited into the lungs of recipient mice at 5 x 10^5 cells/50μl per mouse. Ten days post-adoptive transfer animals were infected with *M. pulmonis* monitored for 14 days. There was no difference in CFU recovered from the lungs 14 days post-infection. PBS (black bar), GMDC (white bar), G4DC (grey bar) and T10DC (hatched bar). Vertical bars and error bars represent the mean +/- SE (GMDC: n=17, G4DC: n=20, G4DC: n=19, T10DC: n=19).

Exacerbation of mycoplasma disease due to the adoptive transfer of antigen-pulsed DCs is antigen specific.

To determine whether the increased clinical disease and pathology was antigen-specific to mycoplasma disease or due to some other non-specific event, mice were given BMDCs either pulsed with mycoplasma antigen or unpulsed. The control group of mice received PBS only. Since the T10DC group of BMDCs proved to most reliably affect the host's immune response, the following experiments only compared T10DC pulsed with mycoplasma antigen ($T10DC^{+}Ag$) or unpulsed T10DCs (T10DC-Ag). Ten days post-adoptive transfer, mice were intranasally infected with *M. pulmonis,* and weights were recorded along with physiologic measurements every two days during infection (day 2, 4, 6, 8, 10, 12 and 14).

Similar to what was seen with the initial studies, there was originally no difference in health or weight loss between the groups in the early stages of infection, such as day 4 (Fig. 6a). However, starting at day 6 post-infection the mice that received the $T10DC^{+}Ag$ lost significantly more weight than the other two groups of mice (Fig. 6b). By day 6 post-infection, 56% of the $T10DC^{+}Ag$ recipient mice had lost 10% of their weight and the T10DC-Ag and PBS mice had lost very little weight. These $T10DC^{+}Ag$ also demonstrated increased clinical disease though the matted fur and malaise, which was not observed in the other two groups. Thus, the exacerbation of disease was mycoplasma-antigen specific.

As the infection progressed, the T10DC-Ag recipients had increased clinical disease over the PBS control group, but never reaching the levels of the $T10DC^{+}Ag$ group. By day 14 post-infection, both groups of mice that received antigen-pulsed or unpulsed T10DCs lost significantly more weight than the PBS control group, but the $T10DC^{+}Ag$ inoculated mice lost significantly more weight than the T10DC-Ag (Fig. 6c). Ninety-four percent of the $T10DC^{+}Ag$

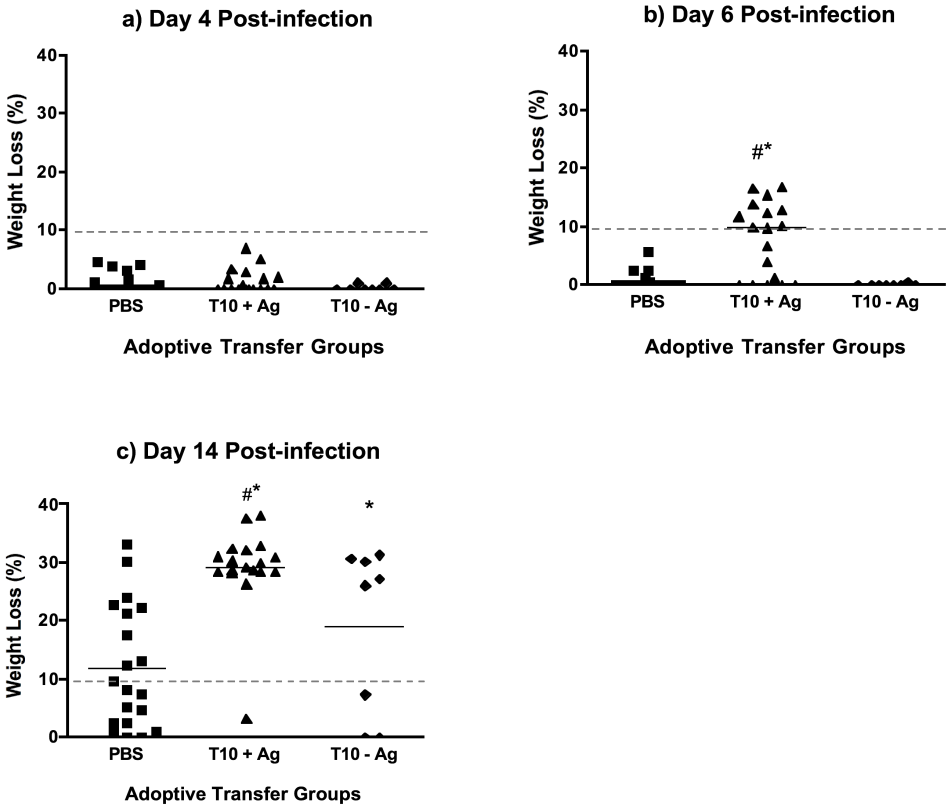


Figure 6. **Weight loss of pulsed and unpulsed T10DC recipient mice during mycoplasma infection.** Mycoplasma antigen-pulsed OR unpulsed $CD11c^+$ T10DCs were intratracheally deposited into the lungs of recipient mice at 5 x 10^5 cells/50μl per mouse. Ten days post-adoptive transfer animals were infected with *M. pulmonis* monitored for 14 days. There was no difference in weight loss at day 4 post-infection (a). At day 6 post-infection, there was a significant difference in the weight loss of $T10DC^+$Ag mice compared to the unpulsed and PBS control (b). At day 14 post-infection, a number of animals receiving unpulsed DCs had lost similar amounts of weight (c). A numeral sign (#) indicates a significant difference ($p \leq 0.05$) in weight loss from T10DC-Ag group in both figures. An asterisk (*) indicates a significant difference ($p \leq 0.05$) in weight loss from the PBS group. Vertical bars and error bars represent the mean +/- SE (PBS: n=20, $T10DC^+$Ag: n=18, T10DC-Ag: n=8).

recipient mice had lost 10% of their original body weight compared to only 63% of the T10DC-Ag mice and only 50% of the PBS mice. Although there is clearly a mycoplasma-specific effect, the later data shows that the unpulsed DC recipients catch up with the amount of weight loss a few days later during the infection demonstrating a slight non-specific affect from transfer of BMDCs alone.

Similar to the weight loss data, there was no difference in the percent reduction in oxygen saturation between the groups early in the infection (Fig. 7a). However, beginning at day 6 post-infection, T10DC^{+}Ag group shows a statistically significant reduction in arterial oxygen saturation compared to the PBS control mice (Fig. 7b), but the most significant reduction in arterial oxygen saturation occurred on day 14 post-infection (Fig. 7c). At day 14 post-infection, the T10DC^{+}Ag mice had a significant reduction in their arterial oxygen saturation compared to both the T10DC-Ag and the PBS control mice. These data further support the idea that the acceleration of clinical disease is antigen-specific.

Another indicator the acceleration of disease is antigen-specific, the gross lesion scores were observed. The T10DC^{+}Ag group of mice had significantly greater gross lesions than both the T10DC-Ag and PBS control mice at day 14 (Fig. 8).

The acceleration and exacerbation of mycoplasma respiratory disease is lymphocyte dependent.

The development of immunopathology is linked to the presence of T cells in the mycoplasma immune response, and since DCs are known for their ability to prime naïve T cells, it is obvious to ask whether or not these BMDCs require the presence of lymphocytes to accelerate the mycoplasma disease.

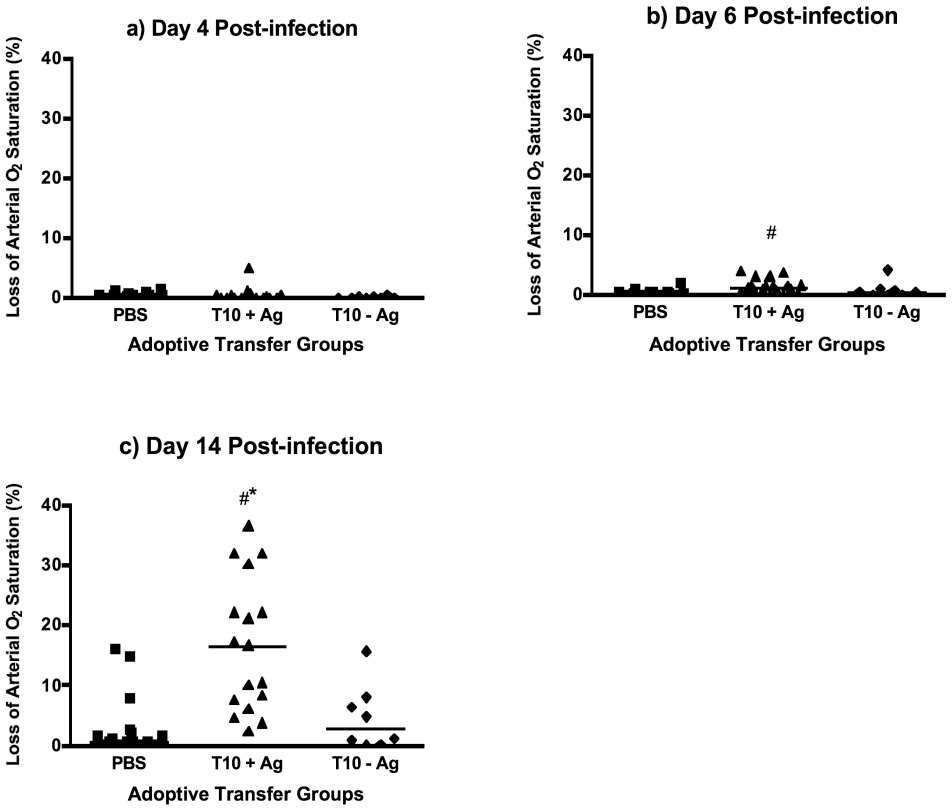


Figure 7. **Arterial oxygen saturation of pulsed and unpulsed T10DC recipient mice during mycoplasma infection.** Physiologic measurements were recorded using the Collar Clip™ Sensor on unanesthetized mice and the quick averaging diagnostic feature set to record a minimum of 30 seconds. There was no difference in arterial oxygen saturation at day 4 post-infection (a). At day 6 post-infection, there was a significant difference in the arterial oxygen saturation of $T10DC^{+}Ag$ mice compared to the PBS and T10DC-Ag groups (b). At day 14 post-infection, the reduction in arterial oxygen saturation was greater in $T10DC^{+}Ag$ compared to T10DC-Ag and PBS control mice (c). A numeral sign (#) indicates a significant difference ($p \leq 0.05$) in arterial oxygen saturation from PBS group in both figures. An asterisk (*) indicates a significant difference ($p \leq 0.05$) in arterial oxygen saturation from the T10DC-Ag group. Vertical bars and error bars represent the mean +/- SE (PBS: n=20, $T10DC^{+}Ag$: n=18, T10DC-Ag: n=8).

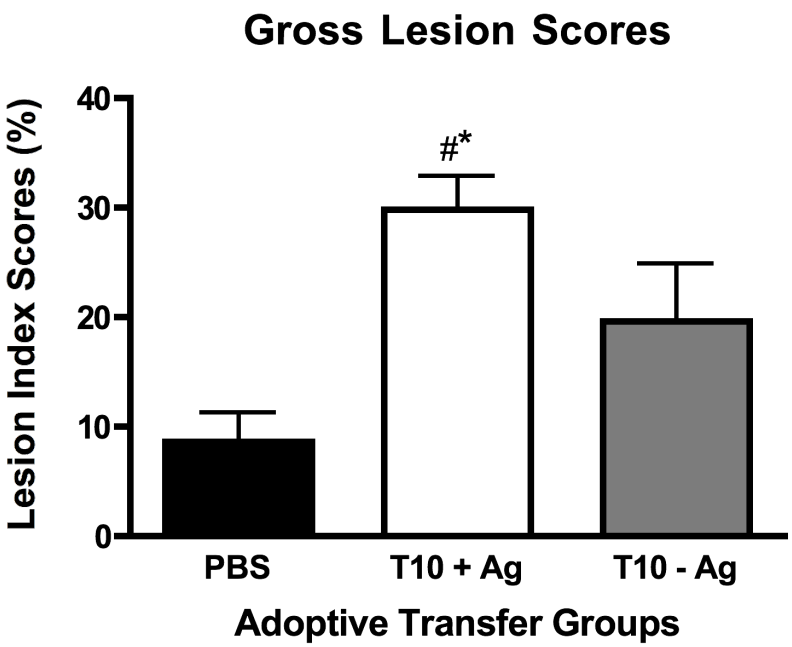


Figure 8. **Gross lesion index scores of pulsed and unpulsed T10DC recipient mice 14 days post-infection.** Mycoplasma antigen-pulsed OR unpulsed $CD11c^+$ T10DCs were intratracheally deposited into the lungs of recipient mice at 5 x 10^5 cells/50μl per mouse. Ten days post-adoptive transfer animals were infected with *M. pulmonis* monitored for 14 days. Lungs and scored for percentage of gross lesions. Lesion severity was significantly greater in animals that received $T10DC^+Ag$ (white bar) compared to T10DC-Ag (grey bar) or PBS controls (black bar). A numeral sign (#) indicates a significant difference ($p \leq 0.05$) in lesion severity from PBS group and an asterisk (*) indicates a significant difference ($p \leq 0.05$) in lesion severity from the T10DC-Ag group. Vertical bars and error bars represent the mean +/- SE (PBS: n=20, $T10DC^+Ag$: n=18, T10DC-Ag: n=8).

For these experiments, WT and SCID mice, lacking B and T cells, were recipients of antigen-pulsed or unpulsed T10DCs or PBS. Both SCID and WT mice were infected 10 days post-adoptive transfer and monitored for 7 days post-infection.

By day 7 post-infection, the WT mice that received $T10DC^{+}Ag$ lost significantly more weight than any of the other groups including WT: T10DC-Ag, WT: PBS, SCID: $T10DC^{+}Ag$, SCID: T10DC-Ag, and SCID: PBS (Fig. 9a). Along with the physical weight loss these WT: $T10DC^{+}Ag$ group was clinically ill, including the hunched posture, ruffled fur and general malaise. The WT: T10DC-Ag group was also beginning to look ill, but no weight loss was apparent. The majority of the SCID recipient mice did not show any clinical signs of illness or weight loss, and in fact, these mice continued to gain weight as the infection progressed.

The gross lesion scores were consistent with weight loss (Fig. 9b). The WT: $T10DC^{+}Ag$ group had significantly higher lesion scores than all other groups. Therefore, these data strongly reinforce the fact that the DC adoptive transferred into mice rely on the expression of lymphocytes for the acceleration and exacerbation of mycoplasma respiratory disease.

There is a significant increase in activated Th2 cells in uninfected mice that receive antigen-pulsed T10DC.

To determine if inoculation of T10DC activate T helper cells (Th), WT mice were given antigen-pulsed or unpulsed T10DCs or PBS as a control. After 10 days post-adoptive transfer, uninfected mice were sacrificed, and lymphocytes were isolated from the LRN and lung and stimulated overnight with UV irradiated mycoplasma. Intracellular cytokine staining was done to determine the number of Th cell subsets present in these mice. Th1 cells are defined as $CD3^{+}CD4^{+}CD44^{high}IFN\text{-}\gamma^{+}IL\text{-}13^{-}$ and Th2 cells are defined as $CD3^{+}CD4^{+}CD44^{high}IFN\text{-}\gamma^{-}IL\text{-}13^{+}$.

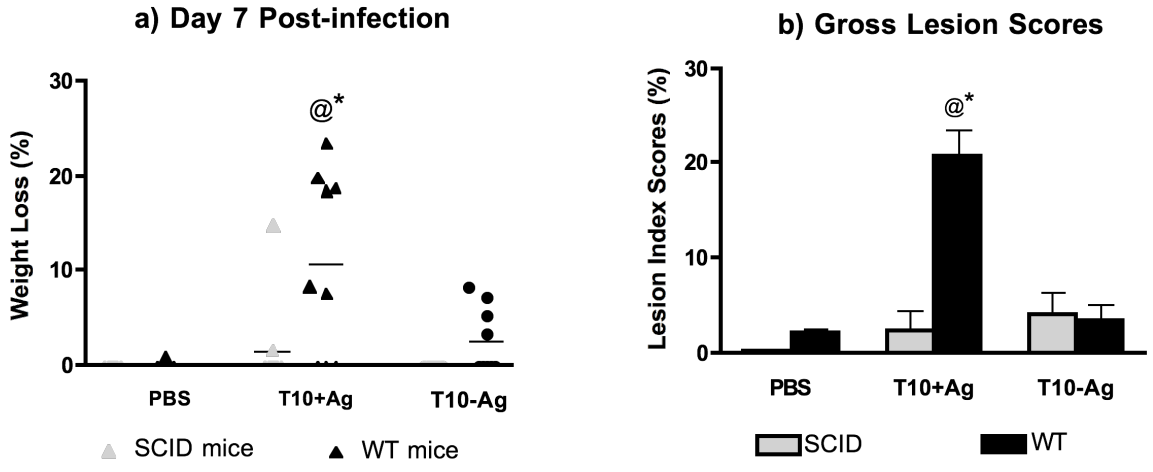


Figure 9. **Weight loss and gross lesion scores of SCID and WT T10DC recipient mice during mycoplasma infection**. Mycoplasma antigen-pulsed OR unpulsed CD11c$^+$ T10DCs were intratracheally deposited into the lungs of recipient mice at 5 x 10^5 cells/50µl per mouse. Ten days post-adoptive transfer animals were infected with *M. pulmonis* monitored for 7 days. At day 7 post-infection, there was a significant difference in the weight loss of WT T10DC$^+$Ag (black triangles) mice compared to the WT unpulsed (black circles) and PBS control (black squares), as well as all the SCID groups (grey squares, triangles and circles) (a). At day 7 post-infection, there was a significant difference in the lesion severity of WT T10DC$^+$Ag (center black bar) mice compared all other WT mice (black bars), as well as all the SCID groups (grey bars) (b). An "@" indicates a significant difference ($p \leq 0.05$) in weight loss from all SCID mice groups (T10DC$^+$Ag, T10DC-Ag, and PBS groups). An asterisk (*) indicates a significant difference ($p \leq 0.05$) in weight loss from the WT PBS and T10DC-Ag groups. Vertical bars and error bars represent the mean +/- SE (SCID PBS: n=10, SCID T10DC$^+$Ag: n=10, SCID T10DC-Ag: n=9, WT PBS: n=9, WT T10DC$^+$Ag: n=9, WT T10DC-Ag: n=9).

Mice that received mycoplasma antigen-pulsed T10DCs had a significant increase in the number of IL-13^{+} Th2 cells in the lower respiratory nodes (LRNs) when compared to both the PBS control mice and mice that received unpulsed T10DCs (Fig. 10a). It has been documented that upon intratracheal adoptive transfer of BMDCs the LRNs are the primary site of translocation (128), and the ability of the T10DCs to translocate to the LRN within 24 hours after intratracheal inoculation was confirmed using CFSE staining of cells (data not shown). In contrast to IL-13^{+} Th2 cells, Th1 cells are in the LRN is unchanged between all groups. There was a significant increase in the number of Th2 cells in the animals that received unpulsed DCs, but not to the extent seen with the T10DC^{+}Ag inoculated mice.

A similar trend was found in the lung lymphocytes (Fig. 10b), where there was an increase in the number of Th2 cells in the T10DC^{+}Ag mice as well as the T10DC-Ag and no change in the PBS mice, but these results were not statistically significant.

Adoptive transfer of antigen-pulsed naïve pulmonary DCs show the same exacerbation of mycoplasma disease in mice and flow cytometry reveals the generation of the same Th2 subset prior to infection.

All studies performed to this point demonstrated exacerbation of mycoplasma respiratory disease due to the adoptive transfer of bone marrow derived DCs, but could these results be indicative of the function of pulmonary dendritic cells during mycoplasma infection and is the a DC tissue specific result? To address this question, pulmonary and splenic CD11c^{+} F4/80^{-} DCs were isolated from each tissue compartment, pulsed overnight with mycoplasma antigen, and intratracheally transferred into recipient mice. Some mice were infected 10 days post-adoptive

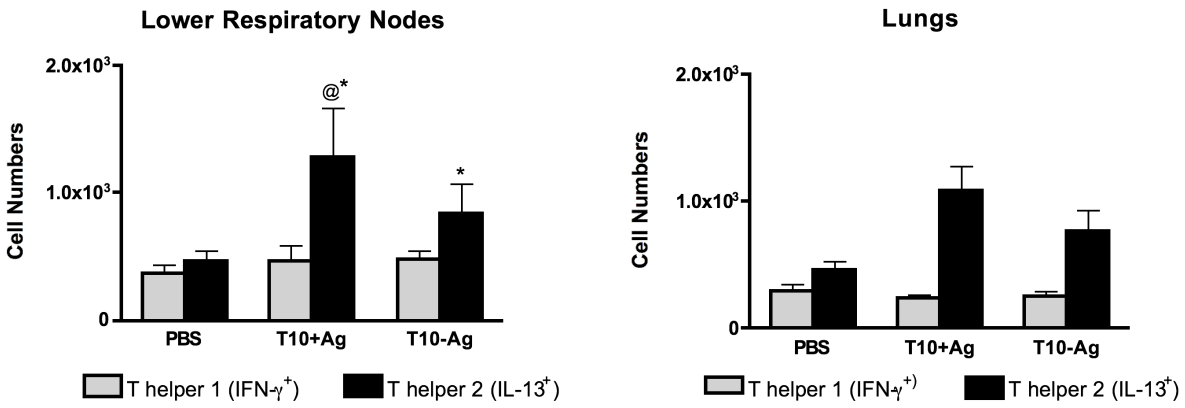


Figure 10. **Numbers of T helper cell subsets in the LRN and lungs of T10DC recipient mice prior to infection**. Mycoplasma antigen-pulsed OR unpulsed $CD11c^+$ T10DCs were intratracheally deposited into the lungs of recipient mice at 5 x 10^5 cells/50μl per mouse. Ten days post-adoptive transfer animals were sacrificed and lymphocytes were isolated from the lower respiratory nodes (LRN) (a) and the lungs (b). These lymphocytes were stimulated overnight with UV irradiated mycoplasma. The next day cells were subjected to intracellular staining to identify Th subsets. Th1= $CD3^+CD4^+CD8^-CD44^{high}IL\text{-}13^-IFN\text{-}\gamma^+$ (grey bars) and Th2 = $CD3^+CD4^+CD8^-CD44^{high}IL\text{-}13^+IFN\text{-}\gamma^-$ (black bars). A "@" sign indicates a significant difference ($p \leq 0.05$) in Th2 cell numbers from T10DC-Ag mice. An asterisk (*) indicates a significant difference ($p \leq 0.05$) in Th2 cell numbers from the PBS control mice. Vertical bars and error bars represent the mean +/- SE (LRN PBS n=6, LRN $^+$Ag n= 7, and LRN –Ag n=6; Lungs PBS n=6, Lungs n=5, Lungs –Ag n=6).

transfer, and the infection was monitored for 14 days. Gross lesions were observed post-infection as an indicator of disease severity. Uninfected mice given antigen-pulsed pulmonary or splenic DCs 10 days earlier were sacrificed and T helper cells sub-populations were detected by flow cytometry in the lower respiratory nodes, lungs and spleen.

Mice that received antigen-pulsed pulmonary DCs had greater disease severity as demonstrated with an increase in lesion index scores when compared to animals that received antigen-pulsed splenic DCs and PBS control animals (Fig. 11). Similar to what was seen in mice that had received antigen-pulsed T10DCs, antigen-pulsed pulmonary DCs had a statistically significant increase in the number of IL-13^{+} Th2 cells prior to infection, whereas the mice that had received antigen-pulsed splenic DCs or PBS had no difference in their Th1 or Th2 populations (Fig. 12).

Discussion

The purpose of these studies was to evaluate the capacity of BMDC populations, generated in polarizing cytokine environments, to influence the generation of protective immunity during mycoplasma respiratory disease. We hypothesized that at least one of these BMDC populations would enhance protection from mycoplasma disease, but intratracheal instillation of antigen-pulsed DCs into recipient mice enhanced the disease severity after infection. No benefit was observed to any mice that received BMDCs, regardless of the in vitro culture conditions. However, these novel techniques are effective in cancer therapy, where BMDCs are pulsed with tumor antigen in order to stimulate anti-tumor $CD8^{+}$ T cells when transferred back into the host (104). Additional infection

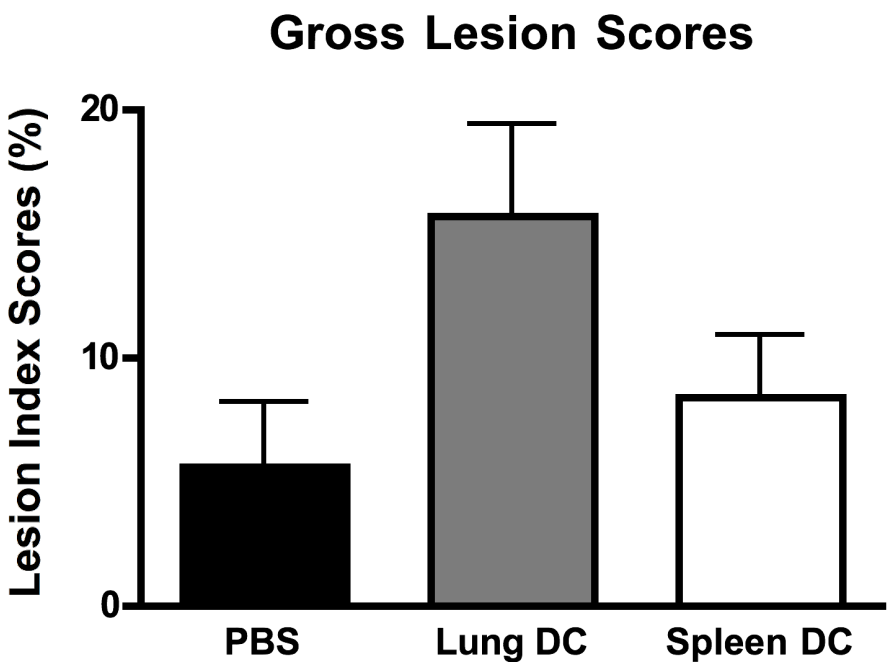


Figure 11. **Gross lesion index scores of pulsed and unpulsed T10DC recipient mice 14 days post-infection.** Mycoplasma antigen-pulsed splenic or pulmonary $CD11c^{+}F4/80^{-}$DCs were intratracheally deposited into the lungs of recipient mice at 5 x 10^5 cells/50µl per mouse. Ten days post-adoptive transfer animals were infected with *M. pulmonis* monitored for 14 days. Lungs and scored for percentage of gross lesions. Lesion severity was greater in animals that received lung DC (grey bar) compared to spleen DC (white bar) or PBS controls (black bar Vertical bars and error bars represent the mean +/- SE (PBS n=8, Lung n= 10, Spleen n=12).

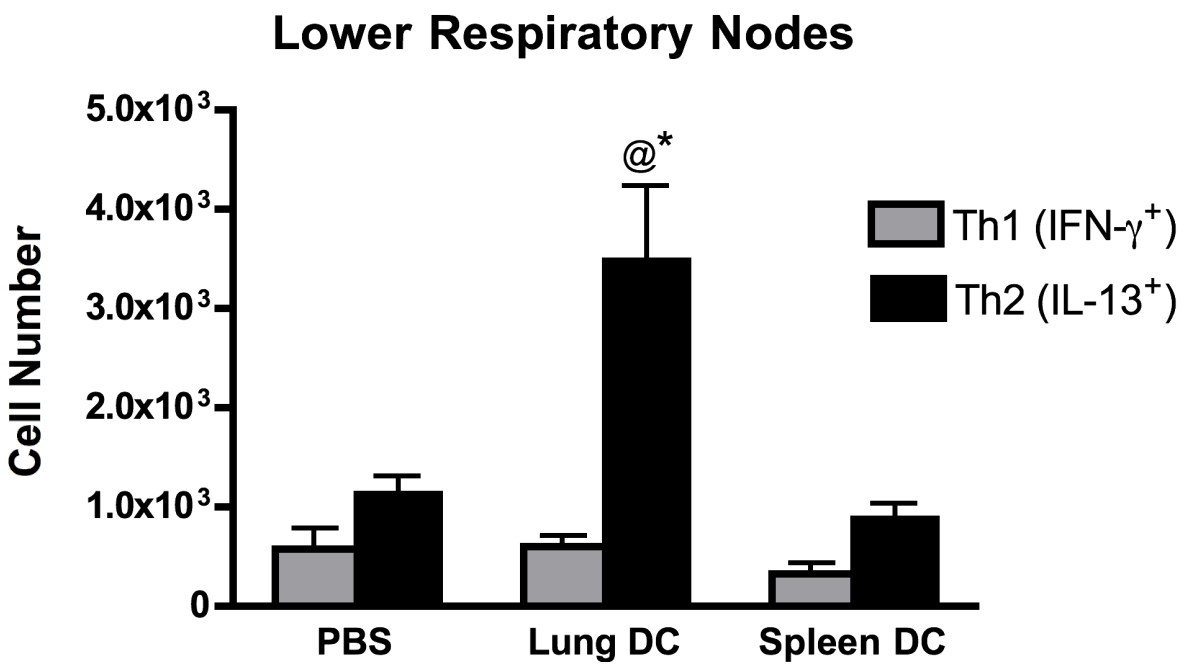


Figure 12. **Numbers of T helper cell subsets in the LRN and lungs of pulmonary and splenic DC recipient mice prior to infection.** Mycoplasma antigen-pulsed OR unpulsed $CD11c^+$ T10DCs were intratracheally deposited into the lungs of recipient mice at 5 x 10^5 cells/50μl per mouse. Ten days post-adoptive transfer animals were sacrificed and lymphocytes were isolated from the lower respiratory nodes (LRN) (a) and the lungs (b). These lymphocytes were stimulated overnight with UV irradiated mycoplasma. The next day cells were subjected to intracellular staining to identify Th subsets. Th1= $CD3^+CD4^+CD8^-CD44^{high}IL\text{-}13^-IFN\text{-}\gamma^+$ (grey bars) and Th2 = $CD3^+CD4^+CD8^-CD44^{high}IL\text{-}13^+IFN\text{-}\gamma^-$ (black bars). A "@" sign indicates a significant difference ($p \leq 0.05$) in Th2 cell numbers from T10DC-Ag mice. An asterisk (*) indicates a significant difference ($p \leq 0.05$) in Th2 cell numbers from the PBS control mice. Vertical bars and error bars represent the mean +/- SE (PBS n=11, $^+$Ag n= 10, –Ag n=10).

models (105 Anis 2007, Carrion 2007, 129-131) demonstrated that adoptive transfer of cytokine manipulated BMDCs had a beneficial effects, unlike our mycoplasma infection model.

Investigation into the mechanisms of how the BMDCs were eliciting their effects demonstrated the enhanced immunopathology was antigen specific and lymphocyte dependent. Mice that received antigen-pulsed BMDCs had increased disease pathogenesis compared to WT mice. Mice that received unpulsed BMDCs did have some accelerated disease compared to WT, but it was not to the degree that mice that received antigen-pulsed BMDCs. There is a slight non-specific effect. This may occur because these BMDCs are cultured in complex media in which they may pick up proteins from the media and present these as antigen after adoptive transfer, but even at this level these BMDCs did not provide any protection. However, SCID mice, deficient in B and T cells, did not display enhanced disease pathogenesis with either pulsed or unpulsed BMDCs indicating that lymphocytes are required. Thus antigen-pulsed DCs are likely stimulating T cells *in vivo* that contribute to the development of lesions.

This accelerated disease pathogenesis, after inoculation with antigen-pulsed DCs was associated with increases of Th2 cell numbers using intracellular cytokine staining IL-13^+ Th2 numbers were shown to increase 3 fold in the LRN, while there was no change in the numbers of IFN-γ^+ Th1 cells. Furthermore, naïve pulmonary DC, but not splenic DC, similarly enhanced Th2 responses to mycoplasma, as well as disease. Th2 cell responses are also associated with the development of immunopathology in a number of other respiratory disorders, such as asthma and respiratory syncytial virus (RSV) infections (30, 31, 96). For example, murine asthma models demonstrated Th2 responses exacerbate the airway hypersensitivity reactions and assist in the recruitment of eosinophils, which also exacerbate the condition (76). Other studies referenced similarly found an association of pulmonary DCs with the generation of asthmatic disease

pathogenesis. IL-13, used to identify our Th2 cells, is also known to exacerbate asthmatic conditions (76). Therefore, our findings in mycoplasma disease are consistent with and immunopathologic pathway, which is established by pulmonary DCs and Th2 cells.

Overall these studies describe the immunopathology network in which naive pulmonary DCs promote Th2 development after mycoplasma infection. These results are supported by our previous studies (92, 132) demonstrating that IL-4 interferes with the development of optimal protection, likely via Th2 cells, and increases severity of inflammatory lesions. Th2 cells are associated with the immunpathologic responses during mycoplasma infection. Thus, these results demonstrate that DCs can promote immunopathologic lymphocytes responses, most likely through the generation of mycoplasma-specific Th2 cell response.

CHAPTER V

DISCUSSION

Mycoplasmas are ubiquitous pathogens with the ability to infect a wide variety of hosts causing significant biomedical and economically important diseases. In general, these agents cause persistent infections with subclinical and clinical disease. Because of the chronic nature of these infections, it is likely that almost every component of the host immune system is involved. The ability of mycoplasma infections to persist, despite the intense immune and inflammatory responses, demonstrates that these organisms have very complex interactions with the host. While the immune responses of some hosts can resist mycoplasma infections, they can also contribute to disease pathology in other hosts, resulting in an apparently frustrated and ineffective response against the mycoplasma infections. T lymphocytes are a major component of the immune response against mycoplasma infection. Because T cells modulate most immune responses, the progression of mycoplasma respiratory disease is dependent on the balance between those T cell responses that may promote protection, and those that evoke immune-mediated pathogenesis. In this thesis, I demonstrated that 1) IL-13^{+} Th2 cells become dominate during mycoplasma infection and reduction in this population resulted in increased protection; 2) pulmonary DCs support both CD4^{+} and CD8^{+} mycoplasma-specific T cell responses and after infection DCs and may preferentially support Th activation; and 3) pulmonary DCs and BMDCs promote immunopathology during mycoplasma disease through the generation of IL-13^{+} Th2

cells. The information provided from these studies and compiled with existing research studies allows for the proposal of a model of mechanisms involved in the development of a frustrated immune response to mycoplasma disease (Fig. 1).

The initiation and type of the inflammatory responses resulting from mycoplasma infection is likely to be dosage dependent, and we believe that low levels of infections lead to generation of Th2-type responses. In support, Eisenbarth *et al*. demonstrated that DCs treated with low doses of LPS resulted in an immune response similar to what is seen in allergy/asthma studies (133). Recent studies using a murine *M. pneumoniae* model, demonstrated inoculation of a lower dose of mycoplasma into allergy-sensitized mice promoted an allergic inflammatory response, where as a higher dose of mycoplasma increased the production of prostaglandins, ultimately reducing this allergic inflammatory response (134). These studies, as well as the studies presented in this thesis, demonstrate that inoculation of mice at a low dose of mycoplasma that leads to persistent infections and chronic disease, generate an ineffective inflammatory response characterized by Th2 responses, similar that found in asthma/allergic diseases. Therefore, the generation of these apparently ineffective Th2 responses, along with mycoplasma's ability for antigenic variation, is beneficial to the pathogen and contributes to the persistent infection (135).

Based on our studies and those from the asthma/allergy realm, pulmonary DCs are critical to the generation and perpetuation of this ineffective immune response. Although macrophages are the major innate immune cells that control mycoplasma infection, DCs would also encounter the mycoplasma (136). In this case, pulmonary DCs would engulf the mycoplasma, and these cells would differentiate and mature as they translocate to the nearest lung draining lymph nodes, the mediastinal or lower respiratory nodes. It is this environment

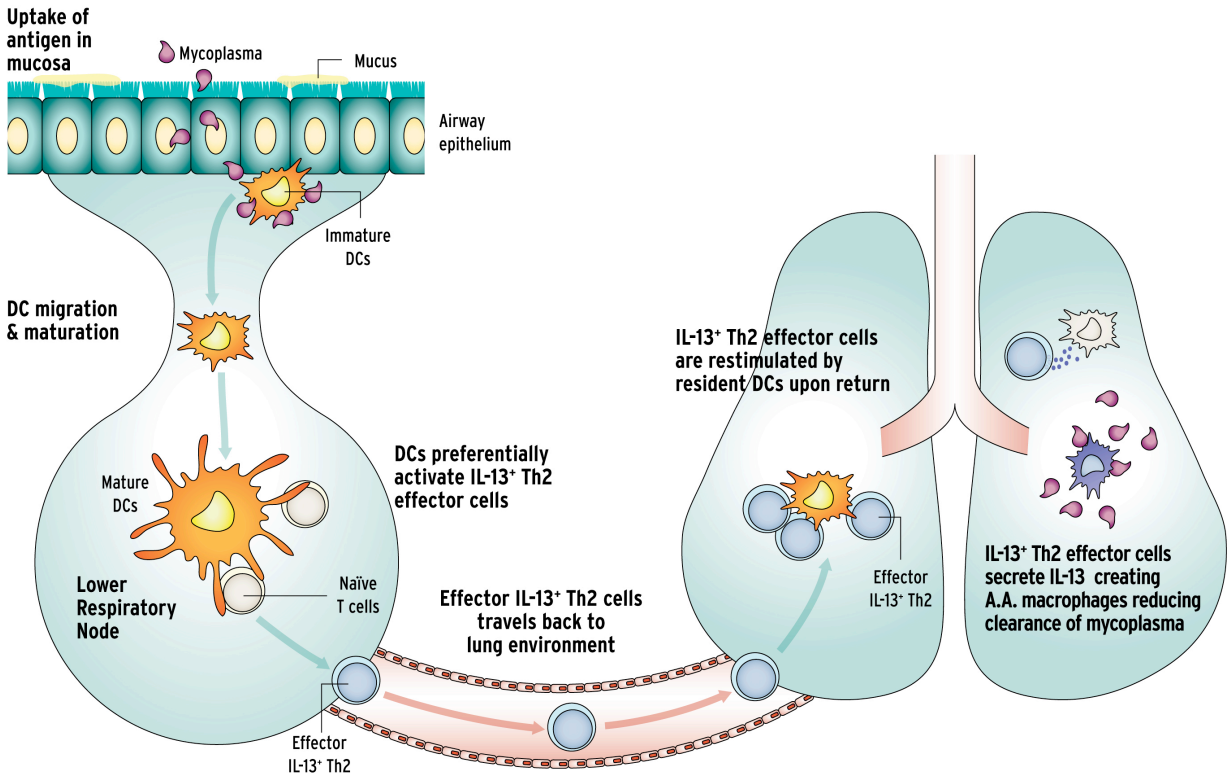


Figure 1. The proposed model of immunopathology development during mycoplasma respiratory disease. The knowledge gained from this thesis and previous research demonstrates that DCs will interact with mycoplasma in the airways early during a respiratory infection. DCs phagocytose and process mycoplasmas, while they mature and translocate to the lower respiratory nodes to present mycoplasma antigens for T cell activation. In the lower respiratory node, these mature DCs preferentially activate IL-13^{+} Th2 effector cells. These Th2 cells will translocate back to the site of infection, the lungs, where they are restimulated by the resident pulmonary DCs and secrete IL-13 into the environment. IL-13 acts upon macrophages generating an alternatively activated macrophage, which is ineffective for clearing the mycoplasma. This cycle would lead to an increase of mycoplasma numbers and immunopathology.

where pulmonary DC present antigen and activate the IL-13$^+$ Th2 cells, similarly seen in asthma models (84). Although my data indicate DCs contribute to the development of Th2 cells in the LRN, it is likely other innate cells in this lymphoid environment assist in the generation of Th2 cells, such as IL-4 or IL-13 producing NK T cells or basophils (137, 138). Regardless, pulmonary DCs generate IL-13$^+$ Th2 cells, which likely migrate into the lung as effector T cells, where they are restimulated with mycoplasma through antigen-presentation by the resident DCs, and induced to secrete IL-4, IL-13, and other Th2 cytokines.

The mechanisms through which Th2 cytokines contribute to immunopathology of mycoplasma respiratory disease are unknown. One possibility is that Th2 cytokines generate alternatively activated macrophages (AAMs), which are better prepared to clear parasitic infections rather than bacterial infections (71). In particular, expression of IL-4/IL-13 has profound effects on macrophages, in contrast to the effects of IFN-γ. IFN-γ enhances the phagocytic abilities of macrophages, whereas exposure of macrophages to IL-4/IL-13 decreases their phagocytic abilities (139). Additional modulatory effects of IL4/IL-13 on macrophages include inhibition of NO production, increasing arginase activity and inhibiting macrophage-mediated killing of pathogens (71). Additionally, murine models of asthma in which Th2 cytokines are highly expressed, demonstrated that alveolar macrophages express surface markers, Ym1, FIZZ1 and ARG1, indicative of AAMs (140, 141). Therefore, the IL-13$^+$ Th2 cells in mycoplasma infected mice may assist in the generation of AAMs. These AAMs could have a reduced capacity to eliminate mycoplasma from the pulmonary environment, contributing to the persistence of mycoplasma infection and increasing immunopathology associated with the perpetuation of a frustrated immune response (Fig. 1).

This model of the immunopathologic response provides the basis for future studies. For example, although previous studies from our lab demonstrated a role of IL-4 (92) (132), it is not clear what role IL-13 has in mycoplasma disease. IL-4 is a short-lived cytokine making it difficult to measure, but IL-13 has much more long lasting effects (76), which could lead to a greater impact on mycoplasma disease. IL-13 contributes to other respiratory diseases. For example, IL-13 steady state levels are higher in the lungs of asthmatic people versus normal people, and IL-13 has been strongly associated with the exacerbation of asthma and allergic airway hypersensitivity reactions (73-75). Investigations into the genetic components that influence the predisposition for the development of asthma in humans revealed that many asthmatic patients had polymorphisms in their IL-13 genes, indicating a role for IL-13 in this inflammatory disease of the respiratory tract (142-145). Based on our model of the events associated with lesion formation in mycoplasma disease, a major function of IL-13 could be the generation of AAMs. However, the presence of AAMs during mycoplasma infection is currently unknown. Initial research efforts should be spent studying alveolar and interstitial macrophages to determine if AAMs are found in the lungs after mycoplasma infection. Identification of the types of macrophages activated, or not, during disease would be beneficial particularly in light of new research indicating that interstitial macrophages, not alveolar macrophages, alter DC functions through the expression of IL-10 to down-regulate allergic responses (146). Once it is shown that alternatively activated macrophages are indeed present in mycoplasma pneumonia, several experimental approaches could be exploited to examine the role of IL-13 in generating AAMs, such as IL-13 KO mice or neutralizing antibodies to IL-13 {Kolodsick 2004}. If alternatively activated macrophages are the result of IL-13 during mycoplasma disease, we would predict by neutralizing the IL-13 effects, this would result in increased protection, e.g.

reduction of CFU, after mycoplasma infection. By blocking IL-13 activity, perhaps IFN-γ will have a greater impact by activating macrophages enabling more efficient killing of mycoplasma.

Overall our underlying assumption is that these IL-13$^+$ Th2 cells are immunopathologic. This idea is supported by the observations that Th2 cells become the dominant population during mycoplasma disease, and a depression in this population demonstrated increased protection. Th2 cells are the hallmark of other problematic respiratory diseases, such as asthma and RSV infection. However, there is also a need to demonstrate that Th2 cells indeed promote immunopathology and inhibit resistance to infection, and the IL-13 expression may not be the only effector mechanism contributing to the immunopathology. There are several ongoing studies that are attempting to directly address the role of Th1 and Th2 cells, in particular adoptive transfer of polarized Th cell lines specific for *M. pulmonis*.

One approach to evaluate the extent to which these IL-13$^+$ Th2 cells exacerbate disease would be to curb their source of activation, the pulmonary DCs. Studies in asthma, similar to that proposed for mycoplasma disease, found DCs are critical in the generation of pathogenic Th2 cells. Van Rijt *et al*. eliminated pulmonary DCs through the use of an diptheria toxin ablation system, and in doing so significantly reduced asthmatic hypersensitivity in OVA-sensitized mice (147). Similar to this approach, pulmonary CD11c$^+$ DCs in mice can be similarly eliminated and then determine what happens after mycoplasma infection. I hypothesize that removal of pulmonary DCs will probably reduce the number of Th2 cells, which will reduce the pathology and increase the clearance of the bacteria. In theory, these mice without DCs should be better protected from mycoplasma, since Th2 cells were preferentially activated by DCs. However, the DCs are also responsible for the activation of IFN-γ$^+$ CD8$^+$ T cells, and CD8$^+$ T cells dampen the inflammatory response during mycoplasma disease. Therefore, DC ablation studies may not have

such straightforward results, but should be an approach to further examine the role of DCs in generating and perpetuating the persistent inflammatory infection due to mycoplasma.

At this time, we have not explored the effects of different DC subsets during mycoplasma disease. It is difficult to discern whether the preferential activation of Th2 cells is due to the mycoplasma's interaction with the DCs or because of the predisposition of the pulmonary environment. A small study performed in our lab compared the expression of IL-4 from naïve pulmonary and splenic lymphocytes after non-specific activation of anti-CD3 antibody and CD28 from four different strains of mice, BALB/c, C3H, DBA, and C57 (148). The pulmonary environment from each of these four strains skewed to a Th2 type response, demonstrating that the environment DCs reside in may influence Th2 response regardless of the pathogen. While the BMDCs cultured in different cytokine environments demonstrated opposing effects during *in vitro* stimulation, the culture conditions had no effect on the outcome of the disease, other than enhancing the pathologic effects. However, in my studies, the adoptive transfer of antigen pulsed splenic DCs did not generate a Th2 response, indicating that DC sub-populations may still have different effects during mycoplasma disease. Recent publications continue to explore the different types of DCs that populate tissues. In the pulmonary environment alone, there are at least 5 sub-populations of DC described as resident DC, plasmacytoid DC, alveolar DC, inflammatory DC, and interferon-producing killer DC, and all with potential to have different effector functions (18). Plasmacytoid DCs, originally known for their pivotal to combat against viral infections, are now thought to be the key DC that induces tolerance to inert antigen in the pulmonary environment though activation of T regulatory cells known to suppress inflammatory responses (149). In fact, ongoing studies in our lab are indicating that T regulatory cells are beneficial in dampening the inflammatory response during mycoplasma disease. Thus, it is

possible that the ability to generate Th2 responses to mycoplasma infection could vary depending on the stage of disease, corresponding to the DC populations present in the lungs, and future studies could explore this possibility.

While DCs have been strongly implicated in the exacerbation of airway hypersensitivity reactions, another debate is taking form around the antigen presentation capabilities of basophils. Three compelling studies recently published demonstrate that basophils present antigen via the MHC II complex, express the co-stimulatory molecules CD80 and CD86, secrete IL-4 and generate Th2 cells in response to allergens or helminth infections (150). Transgenic animals, either totally ablated of $CD11c^+$ DCs or DCs with impaired MHC II expression, where still able generate Th2 effector cells against either allergens or helminthes. However, depletion of basophils in wild type C57Bl/6 mice using the MAR-1 antibody reactive to the FcεRI molecule, demonstrated a reduction in the Th2 cells resulting in impaired protection against helminthes (150). The results of all three studies lead researchers to assume that DCs are not necessary for the induction of Th2 differentiation However, Lambrecht *et al.* strongly refuted these claims by pointing out potential flaws and differences in experimental design, which could lead to opposing conclusions (29). The animal models used for these studies were intestinal helminthes infections and the allergen models used papain, a proteolytic enzyme, or injections of OVA antigens, whereas most allergen studies use an alum-induced response to OVA peptides via intranasal inoculation. The route of allergen inoculation may influence the outcome; especially since different DC subsets targeted during inoculation can have different effects on the immune response. As seen from the studies in this thesis, the tissue sources DCs originate from (pulmonary versus spleen versus BMDCs) influence the subsequent immune responses. One of these studies used both splenic DCs and BMDCs to generate results (151). Also, Th2 responses

in the gut against helminth infections many not represent how Th2 responses are generated in the lungs. Finally, the antibodies used for the depletion of basophils targeting FcεRI can cross-react and deplete some DC subsets as well, since some $CD11c^{+high}$ DC subsets express this FcεRI receptor after allergen exposure (29). As far as mycoplasma respiratory disease is concerned, it is unknown what kind of role, if any basophils play in the clearance or exacerbation of mycoplasma disease. Since basophils are a good source of IL-4 secretion, it is possible they assist in the development of Th2 cells in the LRN during T cell development in response to mycoplasma. Perhaps, futures studies should include monitoring the possible influence basophils have during mycoplasma disease.

Currently, the most beneficial vaccination strategy during mycoplasma would be to attempt to skew the T cell development to a more Th1 subset, and new research into T cell surface markers have exploited this idea. Taken the studies in this thesis into account, using DCs as a vaccination target would not be useful, since modulation of DCs could not skew the immune system to a more protective immune response. A novel approach to examining the potential role of Th1 cells would be to use antibody against TIM-3. TIM-3 is a cell surface marker expressed on Th1 type cells (152). Studies of experimental autoimmune encephalomyelitis (EAE), a murine disease model of multiple sclerosis, have shown that Th1-mediated responses are pathologic (38). This antibody was originally thought to deplete TIM-3 expressing Th1 cells, which was expected to reduce severity of EAE. However, administration of this anti-TIM-3 antibody exacerbated EAE responses, by enhancing the Th1 response *in vivo* (153). Similarly, researchers used this antibody on the OVA-sensitized mice in asthma studies and found that administration of this antibody did, indeed, shift the *in vivo* response to a more Th1 response, a more beneficial for these animals (154). Allergic airway hypersensitivity reactions thought to be mediated

through Th2 responses. Administration of anti-TIM-3 in asthma models shifted the *in vivo* response from Th2 to a more protective Th1. Therefore, using this anti-TIM-3 antibody could provide insights to the role of Th1 responses in mycoplasma disease and immunization, and if successful, this could support the exploration of using Th1 promoting adjuvants for mycoplasma vaccination.

While there is currently no effective vaccine against mycoplasma, investigations have begun to reveal key components of host immune response/mycoplasma interactions that participate in surveillance and disease pathogenesis. Utilizing approaches such as genetically engineered knock out animal models deficient in T cell cytokine production or adoptive transfer of lymphocytes, studies will continue to shed more light on the mechanisms that impact resistance to and progression of mycoplasma disease. Hopefully, such studies will elucidate methods to preferentially activate T cell mediated immune responses that confer protection while minimizing immune-mediated lung damage leading to the development of novel vaccines against mycoplasma diseases.

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