Odeh, Adam N., The Role of Regulatory T Cells in Mycoplasma Respiratory Infection. Doctor of Philosophy (Molecular Biology and Immunology), May 2011, 104 pp., 2 tables, 23 illustrations, bibliography, 137 titles.

The purpose of these studies was to examine the role of regulatory T cells (Tregs) in mycoplasma respiratory disease. Depletion of Tregs resulted in increased disease severity. Treg-depleted mice lost significantly more weight over the course of the experiment, and displayed a significantly higher incidence of both gross lung lesions and histological lung lesions at day 14 post-infection. Treg depletion resulted in increased cell infiltration into the lungs by day 14 post-infection, and significant increases in the serum levels of mycoplasma-specific antibodies. Treg depletion also led to an increase in the percentage of IL-13^+ T cells in the LRNs, meaning that the immune response was skewed towards a Th2 phenotype. There were no differences observed in lung CFU. These data demonstrate that Tregs in mycoplasma respiratory disease play a role in inflammation and disease severity, but have no effect on bacterial clearance. Importantly, depletion of Tregs causes a Th2-directed shift in the immune response.

Additional studies demonstrated that Tregs from mycoplasma-infected mice secreted IFN-γ or IL-17. IFN-γ^+ and IL-17^+ Treg populations both preferentially expanded in response to *M. pulmonis* infection. Depletion of Tregs resulted in decreased secretion of IFN-γ

and IL-17 by $CD4^+$ non-Treg cells. Cocultures of Tregs and T helper cells from mycoplasma-infected mice secreted large amounts of IFN-γ and IL-17 when stimulated with mycoplasma membrane antigen. Levels of IL-4, IL-10, and IL-13 did not significantly increase in response to antigen. Together these studies demonstrate that mycoplasma respiratory disease is influenced by Tregs. These data further suggest that mycoplasma-specific Tregs include two unique subpopulations that express either IFN-γ or IL-17, and that these Tregs may promote the secretion of IFN-γ and IL-17 by T helper cells. This may represent a novel mechanism of Treg-mediated immune suppression. This knowledge can assist in the development of treatments for mycoplasma respiratory disease and in the development of Treg-mediated therapies for a number of diseases.

THE ROLE OF REGULATORY T CELLS IN MYCOPLASMA RESPIRATORY INFECTION

DISSERTATION

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

University of North Texas

Health Science Center at Fort Worth

in Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

Adam N. Odeh, B.A.

Fort Worth, Texas

May 2011

ACKNOWLEDGEMENTS

I would like to thank the members of my committee, Drs. Rance Berg, Harlan Jones, Matthew Woolard, and John Schetz for their advice, encouragement, and patience throughout this process. I would especially like to thank my mentor and major professor Dr. Jerry Simecka for giving me this opportunity, and for believing my abilities as a scientist.

I would like to thank my lab mates who have helped over the course of my graduate career: Dr. Sheetal Bodhankar, Leslie Tabor, Mark Pulse, and Phung Nguyen. I would also like to thank Dr. Xiangle Sun for her assistance with flow cytometry. I would like to express my deep gratitude to Dr. Nicole Dobbs for her advice, her help with experiments, her ability to cheer me up when I was down, and her talent for keeping me sane all the times that I thought I was losing my mind.

Finally, I wish to thank my family for their support during these years. I could not have done this without all of you standing behind me.

TABLE OF CONTENTS

LIST OF TABLES

Table Page

LIST OF ILLUSTRATIONS

Figure Page

LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
Ag	Antigen
Ab	Antibody
APC	Antigen-Presenting Cell
APC/Cy7	Allophycocyanin/Cy7
CD	Cluster of Differentiation
CFSE	Carboxyfluorescein Succinimidyl Ester
CFU	Colony Forming Units
CTL	Cytotoxic T Lymphocyte
CTLA-4	Cytotoxic T Lymphocyte Antigen-4
DC	Dendritic Cell
ELISA	Enzyme-Linked Immunosorbent Assay
FITC	Fluorescein Isothiocyanate
GITR	Glucocorticoid-Induced TNF-Like Receptor
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
IFN-γ	Interferon-gamma
IL-	Interleukin-
LRN	Lower Respiratory Node

NK	Natural Killer Cell
PerCP	Peridinin Chlorophyll-α Protein
PMA	Phorbol 12-Myristate 13-Acetate
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
PE/Cy7	Phyroerythrin/Cy7
RSV	Respiratory Syncytial Virus
SCID	Severe Combined Immune Deficiency
STAT	Signal Transducer and Activator of Transcription
TGF-β	Transforming Growth Factor-β
Th	T Helper Cell
Treg	Regulatory T Cell
WT	Wild Type

CHAPTER I

INTRODUCTION TO THE STUDY

Regulatory T cells (Tregs) are a specialized subset of traditional $CD4^+$ T cells. Tregs are a small portion of the cell population, usually comprising 1-10% of all $CD4^+$ T cells in the thymus, blood, and lymph (1). Unlike traditional T helper (Th) cells, which act to direct and augment the immune response, Tregs act to suppress or dampen the immune response. They are believed to exist as controls on the body's immune system, preventing immune responses from causing damage to the body's own cells (known as immunopathology). The actual mechanisms of suppression by Tregs are still being defined, though cytokine secretion appears to be the main method of control. Several studies have demonstrated that Tregs secrete interleukin-10 (IL-10) and/or transforming growth factor-β (TGF-β) to suppress cell proliferation and activation (2). However, other methods of suppression, such as cytotoxicity or cytokine sequestration, may also play minor role (3-5).

In recent years, copious amounts of research have led to a better characterization and increased understanding of the mechanisms of Treg cells. Confirmation of the suppressive capabilities of Tregs led to theories that these cells could serve to limit damaging immune responses and prevent the induction of autoimmunity (6-9). In addition, it was postulated that

Tregs might have a deleterious effect on individual health by dampening necessary immune responses, thus contributing to the spread of diseases such as cancer (10-12). Much of the current research supports these theories, and there is now a rush to develop clinical applications that either augment or suppress Treg activity as a part of or in place of traditional disease therapies. Current theories of Tregs also suggest that these cells should have an effect on the immune response against pathogens, possibly dampening pathogen clearance and contributing to development of chronic infections (13, 14). However, surprisingly little research has been performed in the area of Tregs and infectious diseases.

Mycoplasma is a common respiratory pathogen that causes up to 30% of all community-acquired pneumonia cases each year, the majority being the result of infection with *Mycoplasma pneumoniae*. Respiratory infection with *M. pneumoniae* is commonly known as "walking pneumonia" (15). *M. pneumoniae* infection is characterized by high morbidity and low mortality, with many infections persisting for weeks, some requiring hospitalization (more than 100,000 people each year) (16, 17). Mycoplasma infections are also linked with exacerbation of a number of other diseases including increased severity of asthma and certain autoimmune conditions (16, 18, 19).

One of the characteristics of mycoplasma respiratory disease is the persistence of infection despite the presence of an immune response. Previous work examining mycoplasma infections has revealed that a large component of the immune response is immunopathologic (20, 21). Although the immune response does prevent the dissemination of the infection to other sites (21), there appears to be little correlation between the magnitude of the immune response and the burden of bacteria in the lungs, the primary site of infection, (20, 22, 23). It is the development

of the chronic inflammatory response that leads to the pathology characteristic of mycoplasma respiratory infections.

Given the presence of immunopathology in the lungs of mycoplasma infected animals, and the knowledge that Tregs may be very important in the control of such immune responses, it is important to examine the role of Tregs in mycoplasma respiratory disease. An understanding of the role of Tregs under such conditions could be useful in developing possible immune-mediated therapies for mycoplasma disease and contribute to a greater understanding of the behavior of Tregs, particularly regarding their role in infectious diseases. The findings of these studies will demonstrate that Tregs do exert an important measure of control over the immune response to mycoplasma respiratory infections, and that they do so through a novel mechanism.

The Immune System

The immune system relies on complex interactions between organs, tissues, and cells that have evolved to recognize and respond to foreign antigens (antigens that are not naturally expressed within the body itself). Foreign antigens are distinguished from self-antigens through the identification of specific molecular patterns associated with invading organisms. Recognition of these patterns initiates a complex reaction from the immune system designed to kill, clear, or contain the threat. The nature of each reaction depends on a number of factors, mainly the characteristics of the pathogen itself, but also genetic factors, route of entry or invasion, and level of infection. Furthermore, the immune response can be divided into two phases that differ in timing, level of specificity, and cell types involved (20, 24, 25).

The Innate Immune Response

The innate immune response occurs sooner than the adaptive immune response by virtue of the fact that most of its components are already in circulation or resident in specific tissues. Once foreign pathogens make it past physiological barriers (skin, mucous membranes, acidity of the stomach environment) and general antimicrobial defenses (enzymes, antimicrobial peptides), they are dealt with by cells such as natural killer (NK) cells, neutrophils, and macrophages (24-26). These cells can recognize a limited range of specific pathogen-associated molecular patterns through specialized receptors, such as Toll-like receptors and NOD receptors (27-29). In addition, NK cells are thought to recognize damaged host cells by the lack of expression of certain surface proteins, such as MHC class I (30). Through these mechanisms cells of the innate immune response can deal with a variety of different pathogens, but many organisms have evolved mechanisms to avoid this (24-26).

The Adaptive Immune Response

The adaptive immune response is characterized by it specificity, often tailored to the recognition of a few select proteins expressed by the invading organism. However, it takes longer to develop than innate immune responses, normally beginning 3-7 days after initial pathogen exposure (24, 25). The first step in the development of an adaptive immune response is antigen presentation, which is accomplished chiefly by dendritic cells, but also by macrophages, and, in certain cases, B cells (20). When presented with antigen, T cells work with B cells to initiate humoral immune responses (antibody-mediated) and/or cell-mediated immune responses. This allows the immune system to specifically target pathogenic organisms or infected host cells through mechanisms such as phagocytosis and direct killing (20, 24, 25).

In addition, T cells and B cells can become memory cells upon antigen exposure (31, 32). These cells retain specificity for a specific antigen and can react much more quickly upon secondary exposure. In the case of B cells, repeated exposures can increase the specificity of these memory populations as well as the magnitude of the resultant immune response (24, 25, 33).

It is important to note that both the innate and adaptive immune responses actually interact to initiate and enhance the effectiveness of the host response. The initial antigen presentation events that initiate the adaptive immune response occur during the innate immune phase, and innate immune cells continue to be active even once the adaptive immune response has begun (26). Furthermore, cytokines produced by cells of the innate immune phase can influence and direct adaptive immune responses by stimulating specific cell functions and creating polarizing environments that skew the immune response in one direction or another (20). Antibodies produced during the adaptive immune response can also enhance phagocytic functions of innate immune cells (24, 25).

Cytokines

Cytokines are specialized proteins that are secreted by immune cells as well as non-immune cells upon activation from a variety of stimuli (34). They generally are bound by cell-surface receptors, leading to the activation of signaling cascades. Through these signals, cytokines can direct cell development, alter protein expression, and initiate apoptosis. Cytokines can thus direct the immune response by activating or inhibiting cells, directing antibody production, inducing proliferation, and/or regulating protein secretion including the secretion of other cytokines (24, 25, 34).

Interferon-γ (IFN-γ) is the classic proinflammatory cytokine, capable of influencing a variety of cell types, both innate and adaptive (35). While many cells can produce IFN-γ, the cell types chiefly associated with its secretion are $CD4^+$ T helper cells (Th), $CD8^+$ cytotoxic T cells, and NK cells. IFN-γ can activate macrophages, stimulate the development of T helper cells, upregulate MHC class I and II on dendritic cells, and cause B cell class switching to IgG2a. Importantly, IFN-γ, which is strongly associated with type 1 Th cells (Th1), can inhibit the development, activation, and proliferation of type 2 Th cells (Th2) (24, 25, 35, 36).

Interleukin-4 and Interleukin-13 (IL-4 and IL-13) are strongly associated with the development and maintenance of Th2 responses (37). Th2 cells and mast cells can produce both cytokines, and these cytokines are capable of inducing B cell class switching to IgE, the isotype most often associated with allergies. In addition, both IL-4 and IL-13 can suppress Th1 responses (24, 25, 37, 38).

Interleukin-10 and Transforming Growth Factor-β (IL-10 and TGF-β), like IL-4 and IL-13, are Th2-associated cytokines. IL-10 is capable of inhibiting Th1 responses by directly reducing T cell secretion of Th1-associated cytokines TNF-α, IL-1β, and IL-2 (39-42). It is also capable of suppressing antigen presentation, cytokine secretion, and phagocytic activities of macrophages. TGF-β can also block cytokine production, as well as cell maturation and proliferation, and cytotoxic activities. However, IL-10 and TGF-β are especially significant as they are the key cytokines produced by Tregs (14). Most studies of Tregs trace suppressive activity to the secretion of these two cytokines. Therefore, while other mechanisms of regulation, such as Fas-FasL interaction, cytokine sequestration, and direct cytotoxicity have been suggested

and/or observed, evidence indicates that secretion of IL-10 and TGF-β is the chief regulatory mechanism used by Tregs (24, 25, 39, 41, 43-46).

Interleukin-17 (IL-17) is traditionally associated with chronic inflammatory conditions, and is not generally considered to be part of the Th1 or Th2 lineage. Rather, IL-17 responses are part of the eponymous Th17 response, though recent work by Bai et al. demonstrated that IL-17 could actually promote a Th1 response in *Chlamydia muridarum* infection (47). The importance of IL-17 in the response to infections is largely due to neutrophil recruitment (48, 49).

Traditionally, IL-17 is not associated with Tregs. However, recent studies indicate that there is a previously unrecognized link between Tregs and IL-17. Studies by Xu et al. demonstrated that in the presence of TGF-β and IL-6, Tregs can not only induce $CD4^+$ T cells to differentiate into Th17 cells, but can also differentiate into Th17 cells themselves (50). This has been linked to Treg uptake of IL-2 (51). Similar results were recently seen in studies involving *Candida albicans*, where Tregs promoted the development of Th17 cells *in vivo* (52). Studies in a murine model of experimental autoimmune encephalomyelitis revealed the presence of Tregs that expressed both IFN-γ and IL-17 in peripheral lymphoid organs, though their role in disease was unclear (53). Furthermore, several studies in humans have shown that Tregs can produce IL-17 under certain conditions (54-57). This connection between Tregs and IL-17 makes sense considering that inducible or adaptive Tregs and Th17 cells develop under similar conditions, namely in environments that contain TGF-β (51, 58).

T Cell Populations

T cells are critical in the induction and regulation of adaptive immunity. They also regulate inflammatory responses and perform effector functions (20). There are numerous T cell populations that mediate different activities. The major T cell populations are T helper cells, cytotoxic T cells, and regulatory T cells. The interaction of these cell populations among themselves and other immune cells often determine the outcome of infection.

$CD4^+$ T Helper (Th) cells can be divided into specific lineages: Th1, Th2, and the previously discussed Th17 (20). Th1 cells are associated with the classic proinflammatory immune response, which is used by the host to protect against a variety of threats including viruses, bacteria, and cancer. The role of Th1 cells is largely based on the secretion of proinflammatory cytokines, including IFN-γ and IL-2 (20, 24, 25). Th2 cells are generally associated with resistance to parasitic infections, though certain types of bacterial and viral infections can also elicit a Th2 response, though this is less common. Th2 cells are known to secrete cytokines such as IL-4 and IL-13 (20, 24, 25). Both of these types of immune responses are generally effective and specific, but both can also cause nonspecific damage if not properly controlled. Many autoimmune diseases have been linked to aggressive Th1 responses, and Th2 responses are strongly associated with allergies and asthma (37, 59). In both of these cases, host damage is due largely to the actions of the immune response rather than any direct action by invading pathogens (a condition known as immunopathology). It is not yet clear why certain immune responses cause nonspecific damage, but the host immune system normally has regulatory controls in place to prevent such occurrences (24, 25, 60, 61).

$CD8^+$ T Cells or Cytotoxic T cells (CTL), are capable of directly killing infected host cells by releasing perforin and apoptosis-inducing granzymes, as well through Fas-FasL

engagement (62, 63). As such, $CD8^+$ T cells are important in intracellular infections, both viral and bacterial, as well as in killing of mutated host cells. In addition, $CD8^+$ T cells are important sources of IFN-γ during infections, serving to bolster Th1 responses (24, 25, 64).

Interestingly, $CD8^+$ T cells were originally thought to be a class of suppressive T cell, due to the observation that they could dampen certain Th cell responses (65). $CD8^+$ T cells have been shown to suppress delayed-type hypersensitivity reactions, and transfer of $CD8^+$ T cells was shown to transfer transplant and graft tolerance from one animal to another. It is believed that $CD8^+$ T suppressor cells can develop in the presence of both antigen and TGF-β. The mechanisms behind suppression by $CD8^+$ T cells are not well understood at this point (65, 66).

Regulatory T Cells are a subset of $CD4^+$ T cells. Tregs comprise a small portion of the cell population, usually encompassing 1-10% of all $CD4^+$ cells in the thymus, blood, and lymph (1). Unlike traditional Th cells, Tregs act to suppress or dampen the immune response. They are believed to exist as controls on the body's immune system, preventing immunopathology. Very early on, Tregs were implicated in autoimmune disorders. A lack of Tregs was thought to be at least related to the onset of autoimmunity, if not the principal cause of it (10). To date, Treg numbers have been found to be either abnormally low or poorly functioning in patients suffering from diseases such as lupus, rheumatoid arthritis, and myasthenia gravis (67-69). Laboratory experiments have shown that adoptive transfer of Tregs into lab animals prone to type I diabetes results in improved prognosis and delay of disease onset (70). Also, the presence of Tregs has been suggested to contribute to the progression of cancer. Again, laboratory experiments have shown that depletion of Tregs can result in enhanced killing of tumor cells by the immune response (71).

First studied in the 1970's, regulatory T cells, known then as suppressor T cells, were identified based on their ability to suppress antigen-specific immune responses. It was some time before it was discovered that the specific type of T cell responsible was a subset of $CD4^+$ T cells. Even after that it was very difficult to study Tregs due to the lack of any known markers specific to the cell type. As a result, the existence of regulatory T cells remained a very controversial idea for a long time. In 1995, it was shown by Sakaguchi et al. that Tregs constitutively express the IL-2 receptor alpha chain, CD25, at a high level (72). It has been theorized that this may be due to the continued T cell receptor (TCR) engagement of self-antigens by these Tregs, resulting in a perpetually active state. Thus, the characterization of Tregs as $CD4^+CD25^{hi}$ cells became widely accepted. Still, it remained somewhat difficult to distinguish Tregs from activated T helper cells, which also express CD25, albeit at lower levels. Recently, the association of the intracellular transcription factor forkhead box P3 (FoxP3) with Tregs has led to the most accurate characterization of Tregs to date: $CD4^+CD25^{hi}FoxP3^+$ (73). While evidence indicates that other cell populations may also have suppressive capabilities, such as NKT cells, γδ T cells, and the aforementioned $CD8^+$ suppressor cells, classical Tregs are by far the most highly studied, the best understood, and, as far as the current evidence indicates, the most important in controlling the body's immune responses (6, 66, 74, 75). $CD4^+CD25^{hi}Foxp3^+$ Tregs also have other distinguishing surface markers such as GITR, CTLA-4, CD103, CCR4, CD62L, and $CD127^{lo}$, though the evidence of these markers as universal has yet to be established (76-82).

The current predominant model dictates there are two main types of Tregs: Natural (central or endogenous) and adaptive (peripheral or inducible) (2, 14, 83-85). Natural Tregs develop in the thymus, in the presence of thymic epithelial cells that express self-antigens.

Evidence indicates that self-reactive T cells are killed in the thymus during negative selection, thus eliminating any cells that could react to self-antigens and cause autoimmunity. However, Tregs are thought to somehow escape this negative selection, since their proposed role in the immune response would require them to be at least minimally reactive in response to self-antigens (2, 14). Once these natural Tregs have been transported to peripheral tissues, they are believed to prevent the development of inflammatory responses by any self-reactive T cells that may have escaped negative selection (14, 86).

Adaptive Tregs differ from natural Tregs in that they develop in the periphery from regular $CD4^+$ cells. Unlike natural Tregs, which are thought to recognize self-antigens, adaptive Tregs may recognize foreign antigens (2). The signals that induce these adaptive Tregs to express Foxp3 are not well understood, though it is known that their development can be induced *in vitro* from $CD4^+$ cells with antigen and IL-10 (2, 14). Adaptive Tregs may be difficult to identify, since they do not always express the same high levels of CD25 as their natural counterparts. This may be related to the fact that adaptive Tregs, unlike their natural counterparts, are thought to recognize foreign antigens rather than self-antigens, and thus do not get activated unless foreign antigens are present. However, it is difficult to prove the accuracy of such a model, since only a few cases of antigen-specific adaptive Tregs have been reported (2, 14, 87, 88).

While the mechanisms of suppression of Tregs are still being defined, research seems to indicate that adaptive Tregs operate mainly through cytokine secretion, with IL-10 and TGF-beta thought to be the critical cytokines (2, 89, 90). Both of these cytokines are associated with Th2 type responses, and, as such, are at odds with the more common Th1 response. Therefore, secretion of these cytokines by Tregs reduces or dampens Th1 inflammatory responses. Natural

Tregs are also known to function via secretion of IL-10, and/or TGF-beta. However, in *in vitro* models, natural Tregs have been shown to have other suppressive mechanisms. This is demonstrated by the fact that blocking of IL-10 and/or TGF-beta does not always abolish the suppressive capabilities of these cells (3). Some studies have suggested that natural Tregs may function through cytokine sequestration, preventing activation of other T cells by effectively consuming local IL-2, and recent studies have confirmed that this does occur under certain conditions (4, 45, 51). Others suggest they may employ cytotoxicity, as they have been shown to express granzyme B and perforin. In fact, in some mouse models, the absence of granzyme B or perforin results in reduced suppressive capability by Tregs(5). These mechanisms have yet to be accurately described (2).

Extensive work has been performed examining the function of Tregs in autoimmunity and cancer. In contrast, relatively little work has been done in the area of Tregs and infectious diseases. An intriguing theory in this field states that chronic infections may develop as a result of Treg actions to dampen the immune response to pathogens. While the immune response may be adequate to prevent further spread of the infection, it may be too weak to clear the pathogen. Thus the possibility exists that one might achieve clearance of a chronic infection by blocking Treg activity, or depleting Tregs altogether. In experiments utilizing *Leishmania major* in a dermal infection model, Tregs were shown to be present in high proportion in chronic infection sites. Transfer of Th cells and Treg cells into Rag knockout mice ($Rag^{-/-}$) resulted in CFU numbers comparable to wild type infection. However, transfer of Th cells alone resulted in significantly lower CFU numbers, suggesting that the presence of the Tregs hinders the ability of the immune response to control the infection (91). In fact, one set of experiments examining the role of Tregs in malaria infection showed that the presence of Tregs allowed for spread of the

parasite and the death of the host, while depletion of the Tregs led to complete clearance of the pathogen and host survival (92). However, there are certain dangers associated with removal of Tregs. Notably, removal of this cell population may result in damaging immunopathology. In models using herpes simplex virus (HSV) ocular infection and *Schistosoma mansoni* chronic infection, removal of Tregs results in damage to the eyes and the liver respectively (93, 94). Experiments examining the role of Tregs in respiratory syncytial virus infection in mice revealed that depletion of Tregs prior to infection led to increased disease, but no reduction in the viral load (95). Thus, the efficacy of Treg-mediated therapies can be unpredictable.

Further complicating the matter, recent studies suggest that Tregs may be capable of producing non-Th2 cytokines. A population of human peripheral blood Tregs was found to secrete IL-17 when stimulated in vitro with IL-1β and IL-6. This was blocked by the addition of TGF-β to the cultures. These IL-17^+ Tregs were found to retain suppressive capability (57). Another recent study in mice showed that Tregs could be stimulated to secrete IL-17 in the presence of TGF-β and IL-6. Whether these cells retained FoxP3 expression or fully differentiated into Th17 cells is unclear (50). In addition, as stated previously, a population of IL-17^+IFN-$\gamma^+$$FoxP3^+$ Tregs has recently been identified in a mouse model of experimental autoimmune encephalomyelitis (53). These studies suggest that the traditional model, which holds that $CD4^+CD25^+FoxP3^+$ cells are Tregs that dampen responses through secretion of Th2-associated cytokines IL-10 and/or TGF-β, may be oversimplified.

Mycoplasma Respiratory Disease

Mycoplasma is a common respiratory bacterium, causing up to 30% of all cases of community-acquired pneumonia. One study detected *M. pneumoniae*, the most common

mycoplasma in humans, in over half of children above the age of 5. This would make mycoplasma the single most common pathogen in humans. While most cases of mycoplasma infection are not life threatening, some do require hospitalization (greater than 100,000 people per year). Mycoplasmas have also been suggested to have a role in the exacerbation of chronic asthma and certain autoimmune conditions (16, 17, 20).

While the human pathogen *M. pneumoniae* is capable of weakly infecting mice and rats, the difficulty of establishing the infection and mild nature of the resultant disease generally drives investigators to use a common mouse model of *Mycoplasma pulmonis* infection instead (96). This well established model produces very clear clinical signs of disease within a few days of infection, and has been shown to very closely mimic the *M. pneumoniae* disease in humans (22, 97).

Research using this model has produced some very interesting results, such as the observation that the particular strain of mouse used in the model can make a drastic difference in the outcome of infection. Common inbred mouse strains such as Balb/c and C3H/HeN are susceptible to intranasal infection with *M. pulmonis*, resulting in a chronic respiratory infection, usually resulting in death after a few weeks (98-100). Clinical disease signs are obvious, such as ruffled fur, weight loss, labored breathing, and joint inflammation (20). Sacrifice and dissection reveals the presence of edema and lesions in the lungs, as well as CFU numbers in the lungs and nasal passages. CFU can also be found in the spleen and liver, indicating spread of the infection (20). In striking contrast, C57/Bl6 mice are highly resistant to *M. pulmonis*, usually clearing the infection within a few days (98-100).

There exists an interesting interplay between *M. pulmonis* and the adaptive immune response in mycoplasma-infected mice. Specifically, immune responses can be

immunopathologic, but can also localize the infection to the lungs, preventing dissemination to other sites. Studies with severe combined immunodeficient (SCID) mice (T and B cell deficient) and athymic mice (T cell deficient) demonstrated that *M. pulmonis* infection resulted in a lower incidence of pulmonary lesions relative to infected wild type mice (23, 101). Adoptive transfer of lymphocytes into SCID mice prior to *M. pulmonis* infection restored the level of mycoplasma disease (21). Importantly, no difference in lung CFU was observed in these studies, providing further evidence that the development of disease is due to immunopathologic immune responses. Depletion of T cells from hamsters showed a similar reduction in lesions after infection with *M. pneumoniae* (102). However, while these immune responses appear to be detrimental in the short term, they have been shown to be important for preventing the spread of mycoplasma from the lungs. These same studies demonstrated that immunodeficient mice did not control dissemination of the infection, leading to the appearance of bacterial CFU in the spleen and liver (21, 23, 101). This is in agreement with observations in human *M. pneumoniae* infections, where immunocompromised patients developed nonrespiratory conditions such as arthritis and meningitis (103, 104).

Studies utilizing *M. pulmonis* have shown that disease severity is directly related to Th cells. Depletion of these cells prior to infection led to significant decreases in disease severity, as measured by weight loss and lesion incidence (22). Since Th cell responses can be separated into Th1 or Th2 lineages, further studies were performed using IFN-$\gamma^{-/-}$ mice and IL-$4^{-/-}$ mice. Mice lacking IFN-γ (which polarizes immune responses towards Th2) developed more severe disease upon infection with *M. pulmonis*, though these differences were observed at an earlier time point (17). In contrast, mice lacking IL-4 (polarizing their

immune responses towards Th1) did not demonstrate any exacerbation of disease (17). These studies suggest that Th2 responses may be related to mycoplasma-associated immunopathology.

$CD8^+$ T cells also play a role in mycoplasma disease, though their role may be regulatory in nature. When $CD8^+$ T cells were depleted from mice prior to infection with *M. pulmonis* disease severity increased (22). Similar results were observed in studies involving rats. F344 rats developed less severe disease after infection with *M. pulmonis* as compared to LEW rats (105-107). This was connected to higher levels of $CD8^+$ T cells in the lungs and draining lymph nodes of F344 rats compared to LEW rats. This effect may be due to the secretion of IFN-γ by $CD8^+$ T cells, which could interfere with immunopathologic Th2 responses.

Recently, studies by Sieve et al. also demonstrated a role for IL-17 in mycoplasma infection. IL-17 production increased during infection with *M. pulmonis*, a finding that agreed with previous work by Sun et al (48, 108). The source of IL-17 was found to be the $CD4^+$ compartment. Mycoplasma CFU was increased in IL-$17R^{-/-}$ mice compared to wild type mice (C57/Bl6). In addition, blockade of IL-23 (a cytokine capable of stimulating IL-17 production by T cells) in a murine *Mycoplasma pneumoniae* infection model led to higher lung CFU at 24 hours post-infection (109).

Given the presence of immunopathology in the lungs of mycoplasma infected animals, and the implication that regulatory cells may be very important in the prevention of this damage, it was important to examine the role of traditional Tregs in mycoplasma respiratory disease. An understanding of the role of Tregs under such conditions could be useful in developing possible immune-mediated therapies for mycoplasma disease, and could contribute to a greater understanding of the behavior of Tregs, particularly regarding their role in infectious diseases. The studies presented here demonstrate an important role for Tregs in controlling damaging

immune responses in mycoplasma respiratory infection. Depletion of these Tregs prior to infection leads to markedly increased disease severity, increased immune cell infiltration into the lungs, and a higher overall level of activation of the immune system. However, depletion of these cells actually shifts the immune response towards a Th2 lineage, rather than a Th1. Surprisingly, the Treg populations that respond during infection actually express IFN-γ or IL-17 rather than IL-10. Data from *in vitro* and *in vivo* experiments suggest that Tregs in this model may also promote the secretion of IFN-γ and/or IL-17 by other cell types, which may act against the normal Th2 immune response against mycoplasma lung infection. These findings may represent a novel mechanism through which Tregs can dampen immune responses.

CHAPTER II

MATERIALS AND METHODS

Mice. Female Balb/cAnNHsd wild-type mice, tested to be virus- and mycoplasma-free, were obtained from Harlan Laboratories (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 8 weeks of age. Female mice were used in all studies. Prior to experimental infection, mice were anesthetized i.p. with diluted ketamine-xylazine. All 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 in mycoplasma pleuropneumonia-like organism medium (Acumedia) and frozen in 1-ml aliquots at -80°C (110). For inoculation, thawed aliquots were dilute to 2×10^5 or 5×10^5 CFU/20 μl. Intranasal inoculations of 20 μl of diluted mycoplasma were given under light anesthesia for experimental infections.

Preparation of *M. pulmonis* antigen. Crude preparations of *M. pulmonis* membrane antigen were used for *in vitro* stimulation of cells. Antigen was prepared as previously described (111). Briefly, *M. pulmonis* was cultured at 37°C in mycoplasma broth medium and harvested at pH 7. Cells were centrifuged at 10,000 rpm for 20 minutes, and resuspended in 5 ml sterile 0.25

M NaCl. Cells were centrifuged again, and resuspended in 4 ml of 2M glycerol at 37°C. Cells were then sonicated at the highest possible setting for 15 seconds using a Vibra cell sonicator (Sonics & Materials/Vibrio Cell, Newtown, CT), and incubated at 37°C for 10 minutes. Cells were then forced through a 27-gauge needle in 0.5 ml amounts into 25 ml aliquots of distilled water. Unlysed organisms were removed by a 20-minute centrifugation at 10,000 rpm. Supernatants containing the actual membrane antigen were centrifuged at 20,000 rpm for 1 hour. Membrane antigen was then resuspended in sterile PBS and stored at -80°C. Protein concentrations were determined using a Bradford protein assay (Bio-Rad, Hercules, CA).

Anti-CD25 antibody-mediated regulatory T cell (Treg) depletion. Mice were given intraperitoneal injections of anti-CD25 (PC61) antibody (BioXCell, West Lebanon, NH), 0.5 mg per mouse (100 μl), at day -1 prior to infection, and again at day 6 post-infection. Depletion was confirmed by staining lymphocytes from spleen, lower respiratory lymph nodes, and lungs with PerCP-labeled anti-CD4 monoclonal antibody (BD Pharmingen, San Diego, CA), PE/Cy7-labeled anti-CD25 mAb (Abcam, Cambridge, MA), and APC-labeled anti-FoxP3 mAb (eBioscience, San Diego, CA) followed by flow cytometry. Control mice were injected with 100 μl sterile PBS.

Determination of mycoplasma numbers. The number of colony forming units (CFU) in the lungs were determined as previously described (110). Briefly, lungs were placed in 1 ml of mycoplasma broth medium and minced. Samples were sonicated (Vibra cell sonicator; Sonics & Materials/Vibro Cell) for 1 minute at 50 amplitudes without pulsing. Serial dilutions were prepared, and 20 μl of each dilution was plated onto mycoplasma agar medium. Plates were incubated at 37°C for 7 days. Colonies were then counted and CFU were calculated.

Assessment of gross lesions. Lungs were removed, and each lobe was examined individually for the presence of gross lesions. The percentage of gross lesions on each lobe was estimated and recorded. Overall gross lesion scores were calculated with each score weighted by the percentage that each lobe contributes to the total lung weight, as previously described (112).

Histopathology. Lungs were fixed in alcohol formalin (4% glacial acetic acid (Fisher Scientific), 6% formaldehyde solution (Fisher Scientific), 40% deionized water, and 50% of 95% ethanol). Tissues were embedded in paraffin, sectioned at a thickness of 5 µm, and stained with H&E for light microscopy by HSRL, Inc. (Mount Jackson, VA). Each lung lobe was sectioned separately. Histology slides were scored for lesion severity (scale of 0–4) on the basis of the characteristic lesions of murine respiratory mycoplasmosis, as described previously (113). Scores refer to: 1) peribronchial and perivascular lymphoid hyperplasia or infiltration (peribronchial infiltrate), or submucosal infiltrate in nasal passages; 2) neutrophilic exudate in airway lumina (airway exudate); 3) hyperplasia of airway mucosal epithelium (epithelial); 4) mixed neutrophilic and histiocytic exudate in alveoli (alveolar exudate). A score for each lesion was weighted according to the percentage each lobe contributes to the total lung weight in arriving at a total lesion score for each set of lungs. For each of the four lesions, a lesion index was calculated by dividing the observed lesion score by the maximum lesion score possible. Thus, the maximum lesion index possible for any lesion was 1.0.

Lymphocyte Isolation. Mononuclear cells from lungs were isolated as previously described, with minor modifications (114). Briefly, lung lobes were dissected and placed into GentleMACS C tubes (Miltenyi Biotec, Auburn, CA), one set of lungs per tube, 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 (Cellgro, Manassas, VA). Lung samples were homogenized using a GentleMACS (Miltenyi Biotec) on the provided setting for mouse lungs, protocol 2. Homogenates were then incubated at 37°C while mixing on a Nutator (Fisher Scientific) for 20 minutes. Subsequently, the homogenates were filtered through a 250-μm nylon mesh. Cells were then purified through density-gradient centrifugation using Lympholyte M (Cedarlane Laboratories, Burlington, NC).

Spleen and lower respiratory lymph nodes were pushed through a 250-μm nylon mesh, and cells isolated through centrifugation. This was followed by red cell lysis using ammonium chloride-potassium carbonate lysis buffer, or ammonium chloride-Tris lysis buffer. Total cells were counted using a Cellometer Auto T4 cell counter (Nexcelom Bioscience, Lawrence, MA).

Flow cytometric analysis. Isolated cells were blocked with Fc block (anti-CD16/32; BD Pharmingen) and/or unconjugated Streptavidin (Invitrogen, Carlsbad, CA) and cell surface staining was performed on live cells resuspended in PBS containing 2% FBS. For intracellular FoxP3 staining, cells were fixed and permeabilized according to manufacturer's instructions using fixation/permeabilization solution (eBioscience). For intracellular cytokine staining, cells resuspended in culture medium [RPMI 1640 media (HyClone Laboratories) containing 10% FBS (HyClone Laboratories), 10 mM HEPES (Fisher Scientific), antibiotic/antimycotic (Cellgro), and 0.005% 2-mercaptoethanol (Sigma-Aldrich)]. Cells were treated with mycoplasma membrane antigen (5 μg/ml) overnight at 37°C with 5% CO_2. The next day cells were treated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich), and 500 ng/ml ionomycin (EMD, Gibbstown, NJ). Cells were incubated for 1 hour, then treated with GolgiPlug, containing brefeldin A (1 μl/ml) (BD Pharmingen). Cells were incubated for 4 more hours.

The following antibodies were used for staining: anti-IFN-γ-Alexa Fluor (AF) 488, anti-IL-13-PE, anti-F4/80-PE, anti-FoxP3-allophycocyanin (APC), anti-IFN-γ-APC, anti-CD127-APC/AF750 (eBioscience), anti-CD11c-AF488, streptavidin-PE/AF610 (Invitrogen), anti-CTLA-4-PE, anti-CD4-peridinin chlorophyll-α protein (PerCP), anti-CD8-PerCP (BD Pharmingen), anti-CD62L-biotin, anti-CD25-PE/Cy7, anti-DX5-APC/Cy7 (Abcam), anti-CD3-PE/Cy7, anti-B220-APC, anti-GITR-AF700, anti-IL-17-AF700, and anti-CD44-APC/Cy7 (Biolegend, San Diego, CA). Cells were analyzed using a BD LSRII Flow Cytometer and BD FACSDiva Software (BD, Fullerton, CA). Further data analysis was performed using FlowJo flow cytometry analysis software (Tree Star, Ashland, OR). To determine the total number of cells in a specific population, the percentage of stained cells was multiplied by the total number of cells recovered.

Cell separations and adoptive transfers. Cells were isolated using paramagnetic bead-conjugated antibodies and AutoMACS (Miltenyi Biotec) following manufacturer's instructions. Tregs and $CD4^+CD25^-$ Th cells were isolated in a two-step process. First, $CD4^+$ cells were isolated by negative selection. Cells were treated with a cocktail of biotin-anti-CD8, biotin-anti-CD11b, biotin-anti-CD45R, biotin-anti-CD49b, and biotin-anti-Ter-119, followed by anti-biotin magnetic microbeads. These labeled cells were depleted from the samples, and the remaining cells were treated with anti-CD25-PE mAb. Cells were then treated with anti-PE magnetic microbeads and separated into two fractions: $CD4^+CD25^+$ Tregs, and $CD4^+CD25^-$ Th cells. For *in vitro* cultures, whole splenocytes were treated with biotin-anti-CD3, followed by anti-biotin magnetic microbeads. $CD3^+$ cells were depleted from the samples, and remaining cells were used as antigen-presenting cells (APC).

Previously isolated Tregs were washed to remove any remaining medium, and resuspended in PBS. Tregs were stained for flow cytometric analysis and confirmed to be >85% FoxP3$^+$. 10^6 Tregs (100 μl) were then injected intravenously into the tail veins of mice using a 1-ml syringe with a 32-gauge needle 1 day prior to infection with *M. pulmonis*.

***In vitro* cultures.** Some 96-well plates were incubated overnight at 37°C with 0.5 μg/ml anti-CD3 antibody (BD Pharmingen) in culture media. Tregs, CD4$^+$ Th cells, or APCs were isolated as described previously. 10^5 of each kind of cell suspended in culture medium were seeded into the wells of a 96-well plate. Samples in anti-CD3 coated wells were treated with anti-CD28 antibody (2.5 μg/ml) (BD Pharmingen), and incubated at 37°C, 5% CO_2 for 4 days. Other samples were treated with APCs and 5 μg/ml of mycoplasma membrane antigen. These plates were incubated for 6 days, and supernatants were collected.

Cytokine measurements. Levels of IFN-γ, IL-4, IL-10, IL-13, and IL-17 were measured in the supernatants from the *in vitro* cultures. Cytokine levels were measured using a custom Bio-Plex kit (Bio-Rad), and 96-well filter-bottom plates. Samples were analyzed according to manufacturer's instructions. Final readings were obtained using a Bio-Plex 100 system (Bio-Rad), and actual cytokine concentrations were determined by comparison with standard curves generated from murine recombinant cytokines. Data were analyzed using Bio-Plex Manager software (Bio-Rad).

Measurement of TGF-β by ELISA. The levels of TGF-β in supernatants were measured by capture ELISA, using an eBioscience Ready-SET-Go ELISA kit (eBioscience). Briefly, Probind 96-well flat-bottom microtiter plates (BD Biosciences) were coated overnight at 4°C with capture antibody. Plates were washed with PBS/0.01% Tween 20 and blocked with assay diluent. Supernatant samples were treated with 1M HCl to activate latent TGF-β, followed by

neutralization with 1N NaOH. Following another wash, samples were added to the plate and incubated. Following another wash, biotinylated detection antibody was added to the plate. After another wash, plate was treated with Avidin-HRP, and incubated. After a final wash, plate was treated with 3,3',5,5'-tetramethylbenzidine substrate (TMB, Moss, Pasadena, MD). After 15 minutes stop solution was added and the plate was read using a Synergy HT Multi-Mode Microplate Reader (Biotek, Winooski, VT) at an absorbance of 450 nm. Cytokine levels were determined by comparison with standard curves generated from recombinant TGF-β after log/log quadratic linear regression analysis using Gen5^{TM} Data Analysis Software (Biotek).

Determination of *M. pulmonis* specific antibody levels. To prepare Ag for ELISA, *M. pulmonis* was cultured at 37°C in mycoplasma broth medium for 3 days and harvested. *M. pulmonis* broth was adjusted to 5 mg/ml protein concentration. Lysis buffer was added (4.2 g $NaHCO_3$/L and 5.3 g Na_2CO_3/L, pH 10.0), warmed to 37°C, and added to the *M. pulmonis* stock. This was incubated at 37°C for 15 minutes. Then 2.2 g of boric acid was added for every 100 mls lysis buffer, and solution was frozen at -80°C. Protein concentration was determined by Bradford assay.

Falcon 96-well flexible assay plates (BD Biosciences) were coated with optimal concentrations of *M. pulmonis* antigen (100 μl at 10 μg/ml) in PBS. After overnight incubation at 4°C, plates were washed three times with PBS-0.05% Tween 20 and blocked overnight at 4°C with 1% milk in PBS. Plates were again washed three times with PBS-0.05% Tween 20. Serum samples were initially diluted 1:400 for IgG and IgM, and 1:200 for IgA. These were then serially diluted 1:2 with 1% milk. 100 μl was placed in each well, and plates were incubated overnight at 4°C. Plates were washed five times with PBS-0.05% Tween 20. Secondary antibody (biotinylated anti-mouse antibody stock reagents of 0.5 mg/ml; Southern Biotechnology

Associates, Birmingham, AL) were diluted 1:5000 for IgG and IgM, and 1:2000 for IgA in 1% milk and added to the appropriate wells (100 μl/well). Plates were incubated at room temperature for five hours. Plates were washed three times with PBS-0.05% Tween 20. A 1:2000 dilution of HRP-conjugated streptavidin (neutralite avidin; Southern Biotechnology Associates) in 1% milk was added to each plate (100 μl/well), and plates were incubated for 1 hour at room temperature. Plates were then washed three times with PBS-0.05% Tween 20, and once with PBS. Reaction mixtures were developed at room temperature by addition of 100 μl of 3,3'5,5'-tetramethylbenzidine peroxidase substrate (Moss, Pasadena, CA) in each well. Plates were read using a BioTek Synergy HT plate reader and Gen5 software (BioTek, Winooski, VT) at an absorbance of 630 nm. Endpoint antibody titers were expressed as the reciprocal dilution of the last dilution that gave an OD of 0.1 U above the OD of negative controls after a 10-15 minute incubation.

Statistical analysis. Data were evaluated by ANOVA, followed by Tukey's post-test comparisons. Analyses were performed using StatView (SAS Institute, Cary, NC) or Prism software (Graphpad Software, La Jolla, CA). A p value ≤ 0.05 was considered statistically significant. Results are expressed as means ± standard errors of the means (SEM). When appropriate, data were logarithmically transformed before statistical analysis.

CHAPTER III

RESULTS

Regulatory T cells (Treg) increase in the draining lymph nodes during the course of *M. pulmonis* infection, and display a classical Treg phenotype.

In order to determine the changes in Treg numbers along the lower respiratory tract during mycoplasma disease pathogenesis, Balb/c mice were infected intranasally with 2×10^5 CFU of *M. pulmonis* and sacrificed at various time points. Cells were isolated from the lungs, lower respiratory lymph nodes (LRN), and spleens. Numbers of $CD4^+CD25^+FoxP3^+$ Tregs were determined using flow cytometry. Cells were also stained for the expression of CTLA-4, glucocorticoid-induced TNF-like receptor (GITR), and CD127.

The absolute number of Treg cells in the LRNs began to increase after day 3 post-infection, reaching almost a three-fold increase by day 7 compared to day 0. This increase continued through day 14 post-infection resulting in more than an eight-fold increase in total $CD4^+CD25^+FoxP3^+$ cells (Figure 1a). This corresponded with an overall increase in total cells in the LRNs through day 14. Total cell numbers in the lungs also increased significantly between days 5 and 14 post-infection (Fig. 1b). Tregs were found to be present in the lungs during infection, but their numbers did not increase significantly.

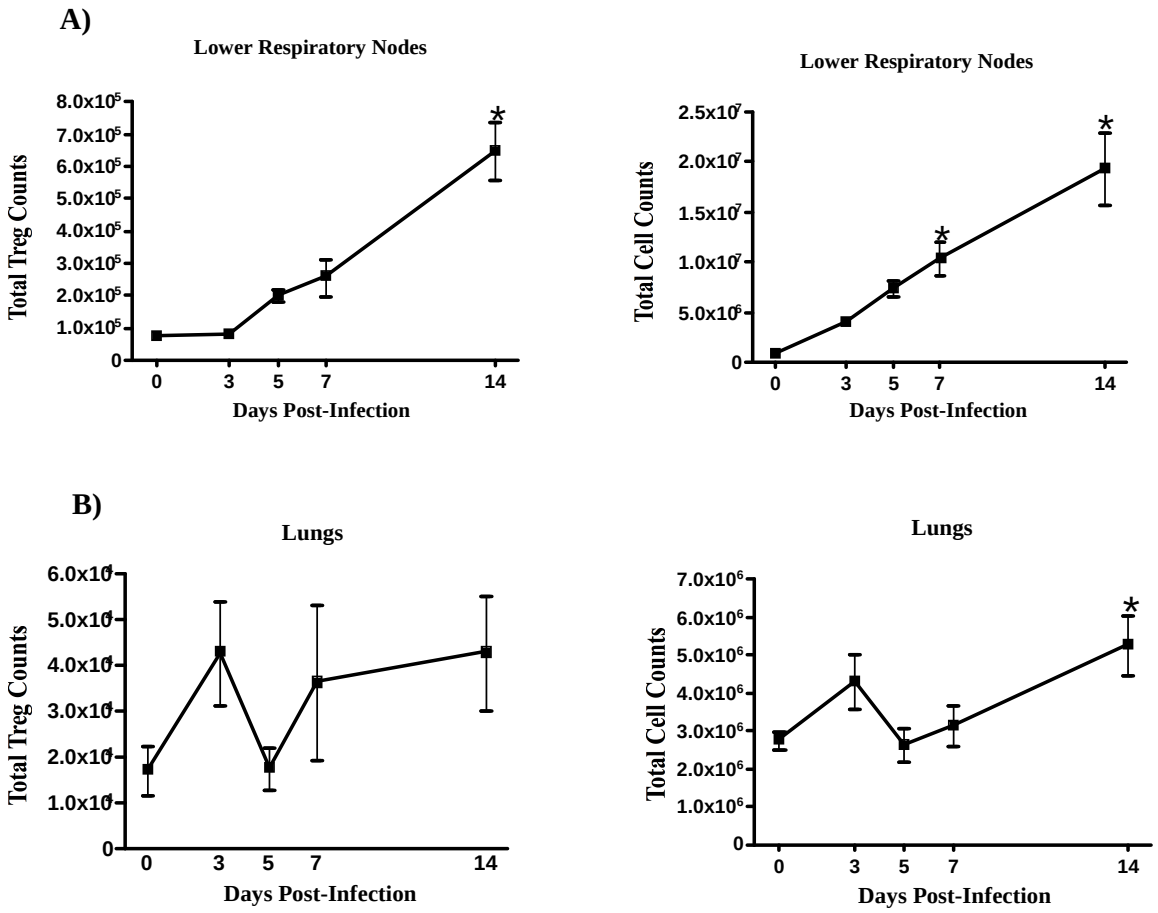


Figure 1. Kinetics of Tregs and total cells in mycoplasma-infected mice. Mice were infected with *M. pulmonis* and sacrificed on days 0, 3, 5, 7, and 14 post-infection. Lymphocytes were isoalted from lower respiratory nodes (LRN) (a) and lungs (b). Tregs are defined as $CD4^{+}CD25^{+}FoxP3^{+}$. An asterisk (*) indicates a significant difference ($p \leq 0.05$) day 14 cell numbers versus day 0 cell numbers. Error bars represent the mean +/- SE (n=9).

No significant increases in either total cells or Treg cells were observed in the spleens at any time point (data not shown).

The majority of Tregs in the LRNs were found to express high levels of CTLA-4 and GITR as compared to $CD4^+$ effector T cells (Fig. 2). In addition, Tregs displayed little to no CD127 as compared to $CD4^+$ cells. Similar results were observed in the lungs (data not shown). This indicates that the dominant phenotype of Tregs in both the lungs and the LRNs during *M. pulmonis* infection is $CD4^+CD25^+FoxP3^+CTLA\text{-}4^+GITR^+CD127^{lo}$. The expression levels of these markers on the Tregs did not significantly change at any time during the infection (data not shown). These data show that *M. pulmonis* infection results in an increase in total lymphocytes in both the LRNs and lungs during the course of infection, as well as an increase in Tregs in the LRNs. The preferential expansion of the Treg population in the draining lymph nodes suggests that these cells do play a role in the immune response to mycoplasma infection. It also suggests that the primary site of action of Tregs may be the LRNs, rather than the lungs. In addition, flow cytometry staining shows that the Tregs found in the lungs and LRNs during infection express all the markers of classical Tregs (70, 73, 76-82).

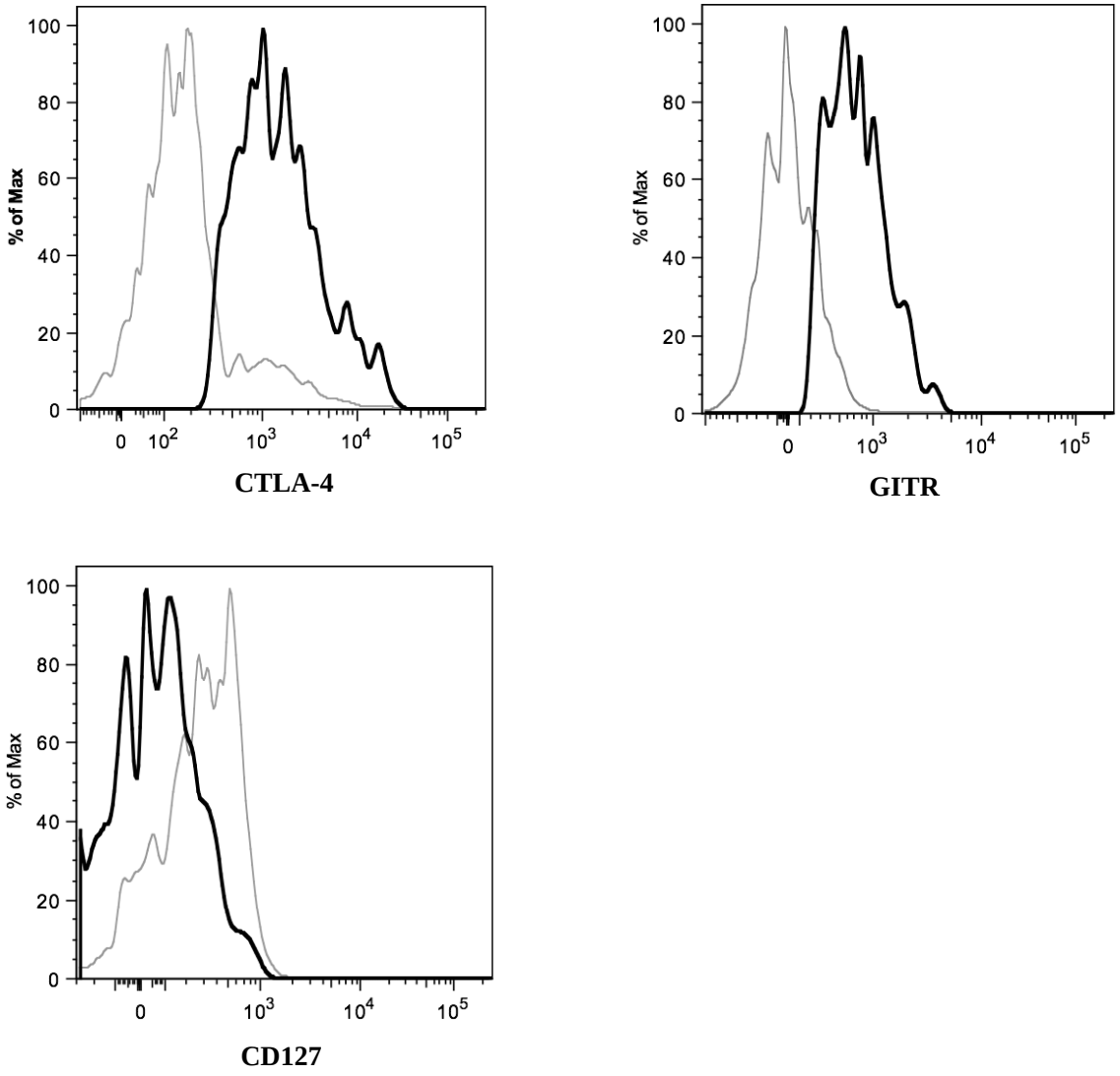


Figure 2. Expression of CTLA-4, GITR, and CD127 on Tregs. Mice were infected with *M. pulmonis* and sacrificed at days 0, 3, 5, 7, and 14 post-infection. Lymphocytes were isolated from LRNs. Black lines represent Tregs ($CD4^+CD25^+FoxP3^+$) while gray lines represent $CD4^+CD25^-FoxP3^-$ Th cells. Data are from a representative LRN sample. Experiment was performed three times (n=9).

Administration of anti-CD25 antibody effectively depletes Tregs without impacting other cell populations.

Since the previous experiment demonstrated that Tregs expand in response to mycoplasma infection, we next wanted to determine whether Tregs play a role in mycoplasma respiratory disease. A common approach for studying Tregs *in vivo* is to use an anti-CD25 antibody to deplete Tregs. CD25, while highly expressed on Tregs, is also expressed on other activated lymphocytes (115). Therefore, it was important to confirm that administration of this antibody did not deplete a significant number of non-Treg cells. Also, it was necessary to confirm the efficacy of the antibody at depleting Tregs themselves.

Balb/c mice were intraperitoneally administered 0.5 mg of anti-CD25 depleting antibody (PC61) or PBS, followed by a second dose 7 days later. Cells were isolated from lungs and LRNs 7 and 14 days after the initial antibody dose, and stained for CD4, CD8, CD25, FoxP3, CD11c, F4/80, B220 (CD45R), and DX5 (CD49b). These cells were analyzed using flow cytometry.

CD25-mediated depletion led to significant decreases in $CD4^{+}CD25^{+}FoxP3^{+}$ cells in both the LRNs and lungs on day 14 (Fig. 3). No significant differences were seen in any of the other cell populations (Table I). This confirms the relative specificity of this antibody for CD25-expressing Tregs.

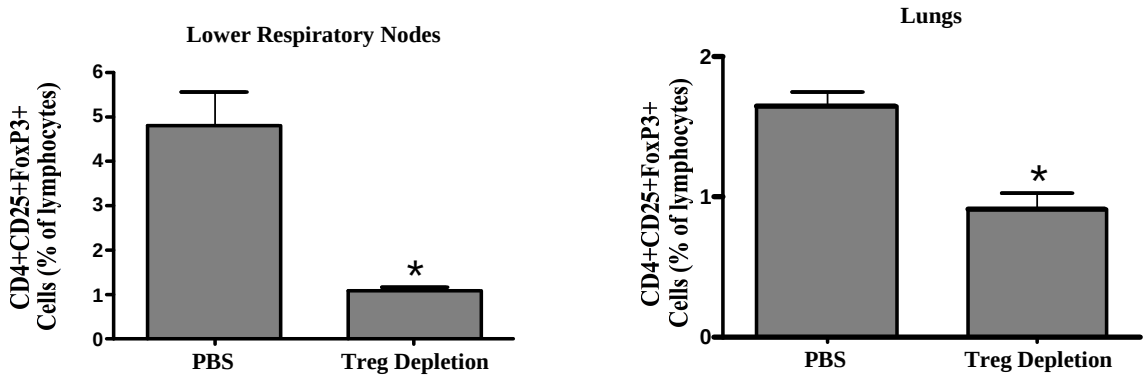


Figure 3. Percentages of Tregs in LRNs and lungs with or without Treg depletion. Mice were given 100 μls (0.5 mg) of anti-CD25 depleting antibody or PBS control on day -1, followed by a second dose at day 6 post-infection. Mice were sacrificed at day 14, and cells were harvested from LRNs and lungs. An asterisk (*) represents a significant ($p \leq 0.05$) difference Treg depletion versus PBS. Vertical bars and error bars represent means +/- SE (n=3).

Table I. Surface Markers in Control versus Treg-Depleted Mice (Uninfected)

Cell Type Percentages[a] (SD)

ORGAN[b]	SURFACE MARKER[c]	Control		Treg Depleted	
		DAY 7[d]	DAY 14	DAY 7	DAY 14
LRN	B220 (B cells)	5.43 (2.4)	9.39 (3.4)	7.84 (2.1)	11.82 (3.3)
	CD11c (DC)	7.50 (4.0)	9.21 (2.7)	7.49 (3.9)	10.19 (2.3)
	F4/80 (Mac)	4.92 (1.0)	2.81 (1.4)	3.67 (0.5)	2.62 (1.5)
	DX5 (NK)	4.88 (2.5)	4.73 (0.8)	3.59 (1.5)	3.96 (0.4)
	CD8 (CTL)	23.97 (22.1)	24.51 (17.5)	15.39 (14.2)	17.37 (11.0)
	CD4 (Th)	46.17 (5.2)	55.83 (9.1)	44.82 (6.3)	49.28 (18.3)
LUNG	B220 (B cells)	6.76 (2.6)	5.69 (1.2)	6.40 (2.6)	4.36 (1.0)
	CD11c (DC)	----[e]	8.66 (0.1)	----	7.43 (0.6)
	F4/80 (Mac)	----	----	----	----
	DX5 (NK)	----	----	----	----
	CD8 (CTL)	1.19 (0.4)	1.52 (0.2)	1.56 (0.6)	1.49 (0.4)
	CD4 (Th)	30.83 (7.3)	42.53 (4.7)	30.78 (8.9)	38.34 (4.8)

[a]Data is expressed as the percentage of live cells (n=3)

[b]Source of cells

[c]Abbreviations: DC- Dendritic Cells; Mac - Macrophages; NK - Natural Killer cells; CTL - Cytotoxic T Lymphocytes; Th - T Helper Cells

[d]Days post-infection

[e]Data was not collected

Treg depletion prior to infection leads to an increase in disease severity.

To determine the effect of Treg depletion on mycoplasma respiratory disease, Balb/c mice were given 0.5 mg anti-CD25 depleting antibody or PBS 1 day prior to infection with 2 x 10^5 CFU *M. pulmonis*, and then given a second dose of antibody at day 6 post-infection. Body weights of individual mice were monitored every other day, and mice were sacrificed at 14 days post-infection. Lungs were visually assessed for the presence of gross lesions, and processed for CFU and histopathology.

$CD25^+$ cell (Treg) depletion caused significantly more severe disease in infected mice. Mice that were Treg-depleted lost significantly more weight over the course of the infection as compared to mice that received no depleting antibody (Fig. 4). Mice that were Treg-depleted and subsequently infected lost >30% of their initial body weight, while infected mice that received no Treg-depleting antibody lost <7% of their body weight. Mice that did not receive depleting antibody prior to infection lost weight steadily over the course of the experiment, while mice that were Treg-depleted prior to infection lost weight steadily through day 5, then began to lose weight very quickly between days 5 and 7. This sharp weight loss continued through day 9, then leveled off. Uninfected control mice that received only the anti-CD25 antibody or PBS did not lose any weight. Thus, Treg depletion resulted in increased disease.

There was also an increase in pulmonary disease in mycoplasma infected mice when treated with anti-CD25 antibody. The lungs of Treg depleted mice had increased severity of gross lesions as compared to mice that were not CD25-depleted (Fig. 5). Furthermore, histopathologic examination showed that Treg-depleted mice had higher peribronchial infiltration, neutrophilic airway exudate, epithelial hyperplasia, and alveolitis lesion indices

relative to infected, non-depleted mice, as determined through histopathology (Figs. 6,7). Thus, there was an increase in all characteristic lesions of mycoplasma disease in the absence of Tregs.

Interestingly, there were no significant differences in the numbers of mycoplasmas recovered from the lungs of Treg depleted and control mice after mycoplasma infection (Fig. 8). Thus, the increase in disease severity seen in Treg depleted mice did not correspond with a change in the bacterial burden in the lungs. This suggests that the increased disease was not due to a change in the levels of bacteria, but rather to increased inflammatory reactions.

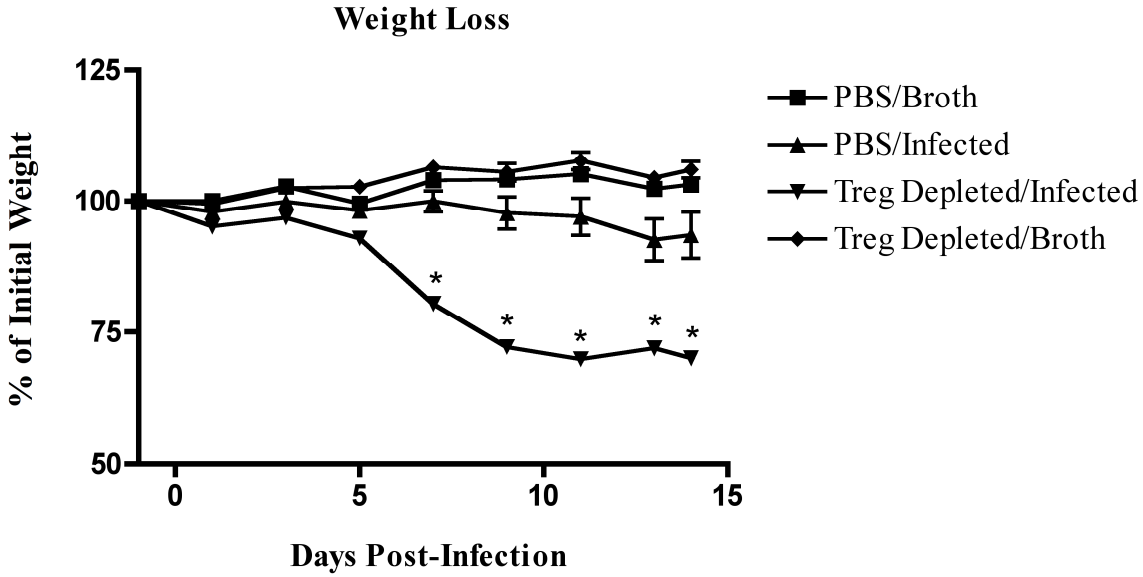


Figure 4. Weight loss of control and Treg-depleted mice during mycoplasma infection. Mice were given 100 μls (0.5 mg) of anti-CD25 depleting antibody or PBS control one day prior to infection with 2 x 10^5 CFU *M. pulmonis*, followed by antibody or PBS at day 6 post-infection. Weights were monitored every other day. Data is expressed as the percentage of initial weight measured at day 0. An asterisk (*) indicates a significant difference ($p \leq 0.05$) from other groups. Error bars represent SE (n=8).

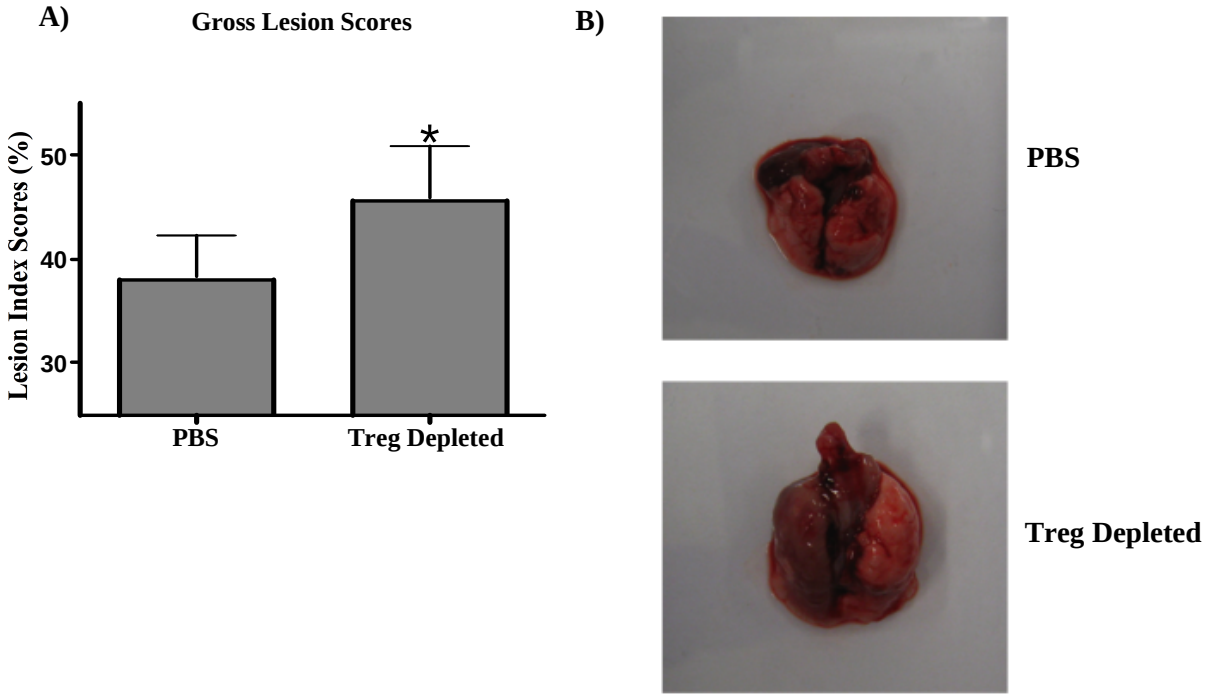
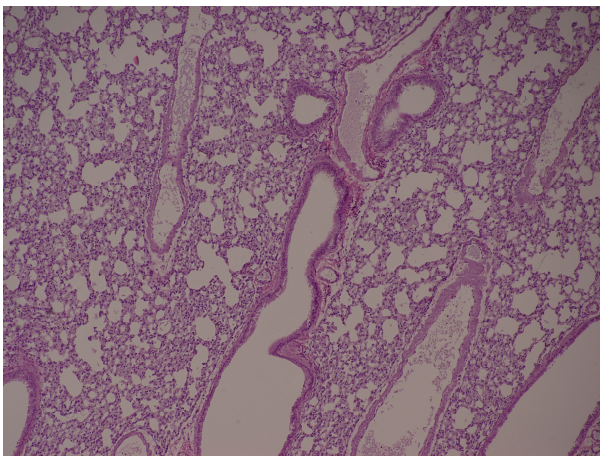
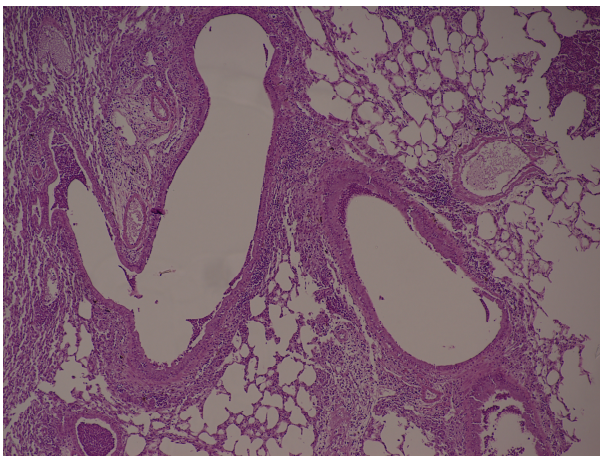


Figure 5. Lung lesion incidence in control and Treg-depleted mice at 14 days post-infection. Mice were administered 100 μls (0.5 mg) of anti-CD25 depleting antibody or PBS control one day prior to infection with 2 x 10^5 CFU *M. pulmonis*, and then given a second dose of antibody or PBS at day 6 post-infection. Lungs were visually assessed for the presence of gross lesions (a). Representative lungs are shown (b). The lungs of the PBS-treated mouse are small and display few gross lesions. The lungs of the Treg-depleted mouse are larger, and the left lobe appears fully necrotic. An asterisk (*) represents a significant ($p \leq 0.05$) difference. Vertical bars and error bars represent means +/- SE (n = 24).

PBS/Broth



PBS/Infected



Treg Depleted/Infected

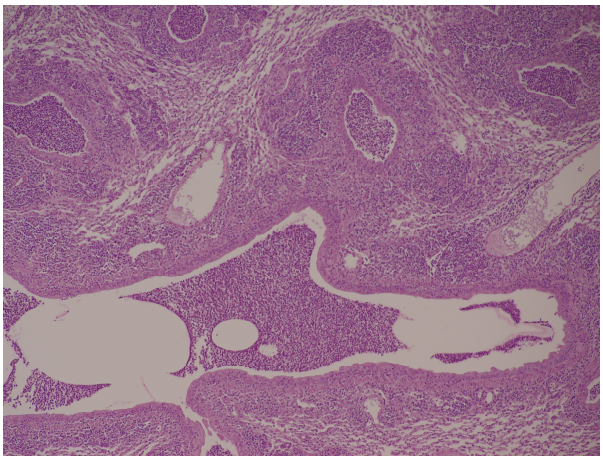


Figure 6. Lung lesions in control, infected, and Treg-depleted/infected mice at 14 days post-infection. Mice were administered 100 μls (0.5 mg) of anti-CD25 depleting antibody or PBS control one day prior to infection with 2 x 10^5 CFU *M. pulmonis*, and then given a second dose of antibody or PBS at day 6 post-infection. At 14 days post-infection lungs were fixed for histology. Representative sections from one experiment are shown.

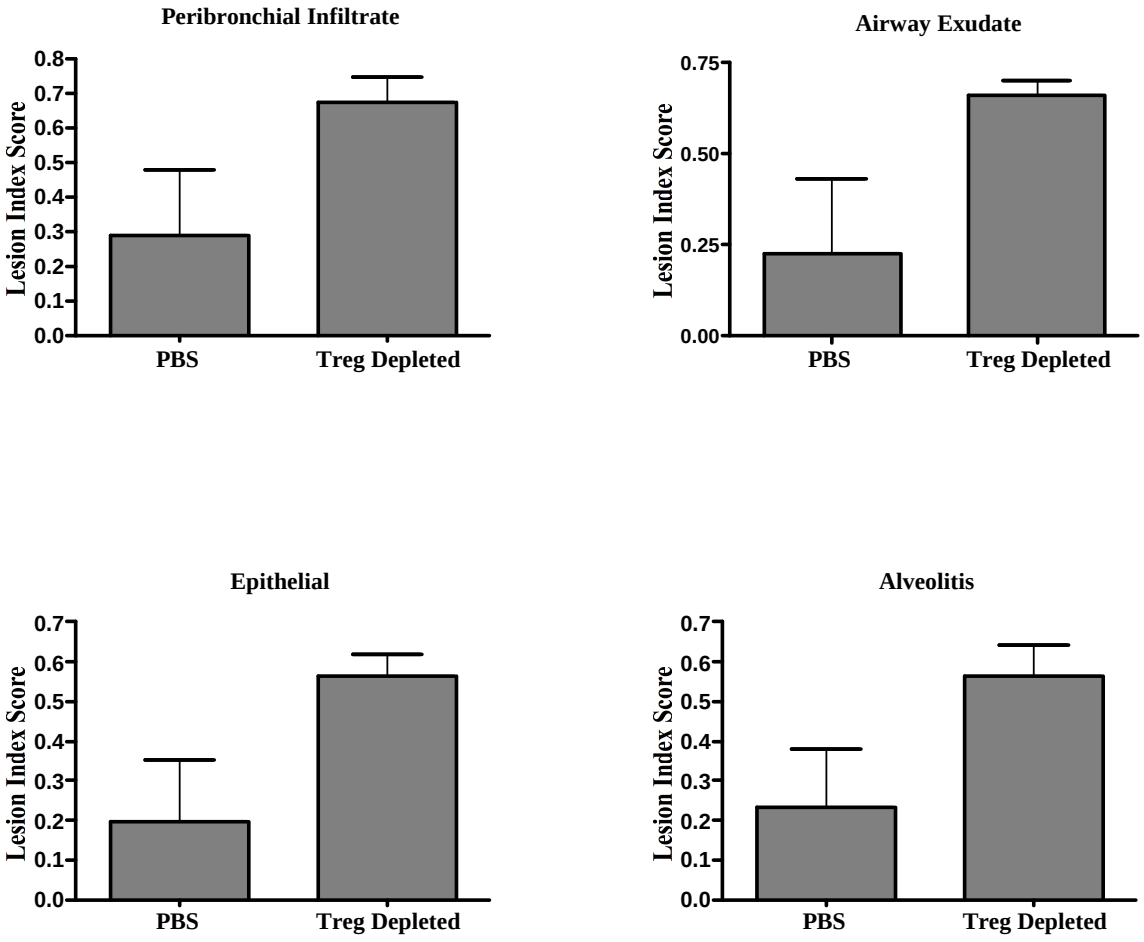


Figure 7. Lesion severity of lungs infected with *M. pulmonis* with or without Treg depletion at day 14 post-infection. Mice were given 100 µls (0.5 mg) anti-CD25 depleting antibody or PBS control 1 day prior to infection with 2 x 10^5 CFU *M. pulmonis*, followed by a second dose of antibody or PBS at day 6 post-infection. Lungs were fixed for histology. Lesion index scores refer to: 1) peribronchial and perivascular lymphoid hyperplasia or infiltration (peribronchial infiltrate) in lung or submucosal infiltrate in nasal passages; 2) neutrophilic exudate in airway lumina (airway exudate); 3) hyperplasia of airway mucosal epithelium (epithelial); 4) mixed neutrophilic and histiocytic exudate in alveoli (alveolitis). Vertical bars and error bars represent means +/- SE (n = 3).

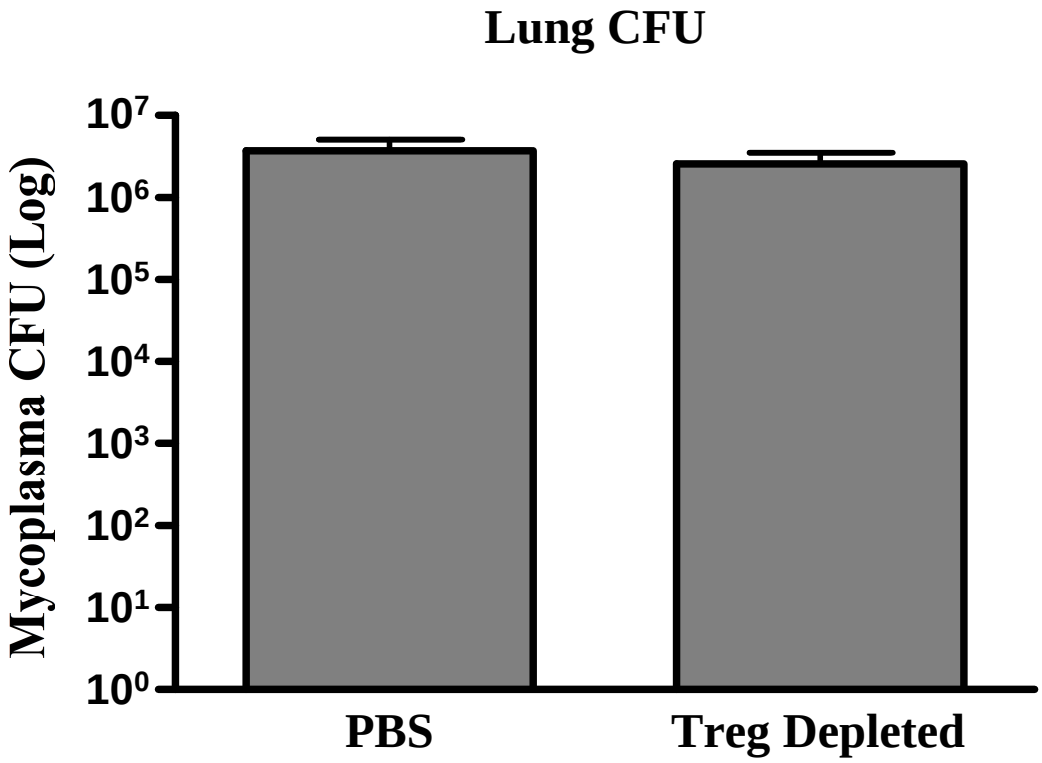


Figure 8. Mycoplasma CFU from the lungs of control or Treg-depleted mice at 14 days post-infection. Mice were given 100 μls (0.5 mg) of anti-CD25 depleting antibody or PBS control one day prior to infection with 2 x 10^5 CFU *M. pulmonis*, followed by a second dose at day 6 post-infection. Vertical bars and error bars represent the mean +/- SE (n=26).

Treg depletion coupled with *M. pulmonis* infection causes increased lung cell infiltration, and increased serum antibody responses.

The previous studies demonstrate that mycoplasma disease is more severe in the absence of $CD25^+$ Treg cells, including an increased infiltration of lymphocytes along the airways. In addition, increased severity of clinical signs corresponded with the time point when adaptive immunity was previously shown to become apparent. To examine whether there was a preferential change in mononuclear cell populations or antibody responses against mycoplasma in the absence of $CD25^+$ cells, Balb/c mice were administered 0.5 mg of anti-CD25 antibody one day prior to infection (day -1) with 2×10^5 or 5×10^5 CFU *M. pulmonis*. Mice were again given 0.5 mg of anti-CD25 antibody at day 6 post-infection. Mice were sacrificed at days 7 and 14 post-infection, and cells were isolated from spleens, LRNs, and lungs. In addition, blood was collected, and serum was used in ELISAs to measure levels of mycoplasma-specific IgG, IgM, and IgA.

Changes in cell numbers in lungs corresponded with increased disease severity due to anti-CD25 antibody treatment. Treg depletion led to an increase in the total cells in the lungs by day 14 post-infection as compared to mice that received no depleting antibody (Fig. 9). Total cells in the LRNs of infected Treg-depleted mice were not significantly higher compared to infected non-depleted mice. However, there were no significant differences in cell numbers in the spleens (data not shown). The increases in cell counts did not appear to be due to the expansion of any specific cell population, as no significant changes in the percentages of CD4, CD8, CD11c, B220, DX5, or F4/80-expressing cells were observed at any time point in any organ (Table II). Thus, the depletion of Treg cells resulted in the increased infiltration of all

major lymphocyte populations (T, B, and NK cells), as well as macrophages into the lungs of mycoplasma infected mice.

Serum levels of mycoplasma-specific IgG, IgM, and IgA were all significantly higher in infected Treg-depleted mice as compared to infected non-depleted mice (Fig. 10). These data show that Treg-depletion prior to infection leads to an increase in the overall magnitude of the immune response, as indicated by higher lung cell counts and higher levels of mycoplasma-specific serum antibodies.

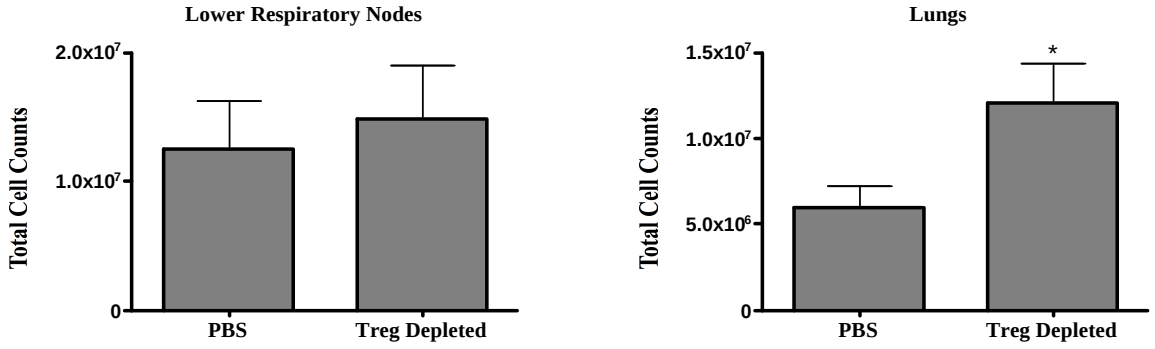


Figure 9. Total cell counts in the LRNs and lungs of Treg-depleted mice at 14 days post-infection. Mice were given 100 μls (0.5 mg) of anti-CD25 depleting antibody or PBS control 1 day prior to infection with 2 x 10^5 CFU *M. pulmonis*, followed by another dose at day 6 post-infection. Mice were sacrificed at day 14 post-infection and cells were isolated from LRNs and lungs. An asterisk (*) represents a significant ($p \leq 0.05$) difference Treg-depleted lungs versus PBS lungs. Vertical bars and error bars represent means +/- SE (n=8).

Table II. Surface Markers in Control versus Treg-Depleted Mice (Infected[a])

Cell Type Percentages[b] (SD)

		Control		Treg Depleted	
ORGAN[c]	**SURFACE MARKER[d]**	**DAY 7[e]**	**DAY 14**	**DAY 7**	**DAY 14**
LRN	B220 (B cells)	11.09 (5.4)	9.52 (2.0)	12.51 (4.7)	9.39 (4.2)
	CD11c (DC)	5.56 (2.2)	12.93 (0.3)	6.92 (2.5)	10.92 (4.8)
	F4/80 (Mac)	3.78 (0.6)	2.99 (0.2)	4.21 (0.8)	2.96 (0.6)
	DX5 (NK)	3.75 (1.2)	4.93 (0.4)	4.34 (1.3)	4.30 (1.0)
	CD8 (CTL)	24.63 (21.2)	19.26 (16.8)	22.84 (12.2)	24.43 (20.7)
	CD4 (Th)	44.88 (5.0)	51.57 (15.0)	47.63 (6.9)	45.57 (13.2)
LUNG	B220 (B cells)	6.58 (1.0)	4.83 (1.3)	5.5 (2.8)	4.42 (1.2)
	CD11c (DC)	----[f]	8.03 (0.9)	----	7.00 (0.4)
	F4/80 (Mac)	----	----	----	----
	DX5 (NK)	----	----	----	----
	CD8 (CTL)	1.43 (0.7)	1.25 (0.3)	1.30 (0.3)	1.41 (0.5)
	CD4 (Th)	30.83 (7.3)	42.22 (4.5)	35.00 (9.0)	41.52 (5.6)

[a]Balb/c mice were intranasally infected with 5 x 10^5 CFU *M. pulmonis*

[b]Data is expressed as the percentage of live cells (n = 3)

[c]Source of cells

[d]Abbreviations: DC- Dendritic Cells; Mac - Macrophages; NK - Natural Killer cells; CTL - Cytotoxic T Lymphocytes; Th - T Helper Cells

[e]Days post-infection

[f]Data was not collected

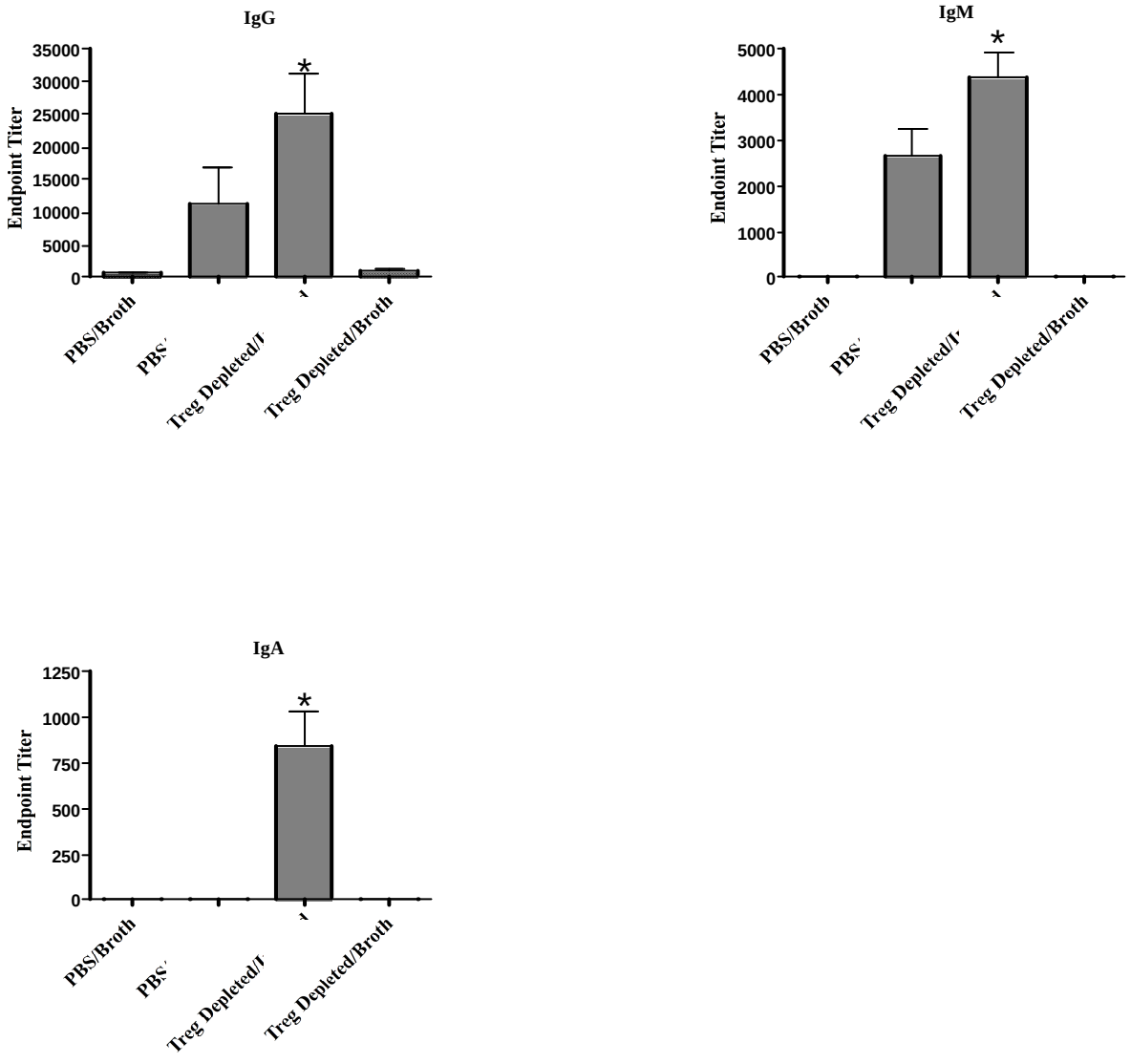


Figure 10. Serum Ig Titers of Treg-depleted or PBS-treated mycoplasma infected mice at 14 days post-infection. Mice were given 100 μls (0.5 mg) anti-CD25 depleting antibody or PBS control 1 day prior to infection with 2 x 10^5 CFU *M. pulmonis*, followed by a second antibody or PBS dose at day 6 post-infection. Antibody titers were determined for mycoplasma-specific IgG, IgM, and IgA. Data was analyzed after $\log_2$ transformation. An asterisk (*) represents a significant ($p \leq 0.05$) difference Treg Depleted/Infected versus PBS/Infected. Vertical bars and error bars represent means +/- SE (n=16).

Treg-depletion does not significantly alter the activation level of lymphocytes.

To examine if Treg depletion affected the activation of cells, mice were administered anti-CD25 antibody as previously, and infected with 5 x 10^5 CFU *M. pulmonis.* Mice were sacrificed at days 7 and 14 post-infection, and cells were isolated from lungs and LRNs. Cells were then stained for expression the activation markers CD44 and CD62L.

No significant increases in the percentage of activated lymphocytes were found in either the LRNs or the lungs, as measured by CD62L expression, at any time point (Fig. 11). Similarly, there were no differences in the percentage of $CD44^+$ cells at any time point (data not shown). This suggests that the increase in immunopathology observed in these mice is likely not due to a higher percentage of activated lymphocytes.

Treg depletion skews the immune response towards a Th2 phenotype.

Since the increase in disease could not be strongly connected with any specific cell type or to the overall levels of cell activation, the possibility existed that the depletion of Tregs was disrupting the Th1/Th2 balance, since this is a mechanism through which Tregs can act (2, 89, 90). Mice were administered anti-CD25 antibody as before, and infected with 5 x 10^5 CFU *M. pulmonis*. Mice were sacrificed at days 7 and 14 post-infection, and $CD4^+$ cells from LRNs and lungs were stained for intracellular IFN-γ and IL-13.

Surprisingly, Treg depletion prior to infection caused a shift in the immune response towards Th2, as evidenced by higher levels of IL-13 relative to IFN-γ in the LRNs (Fig. 12). This was observed at both days 7 and 14 post-infection. No significant differences were observed in

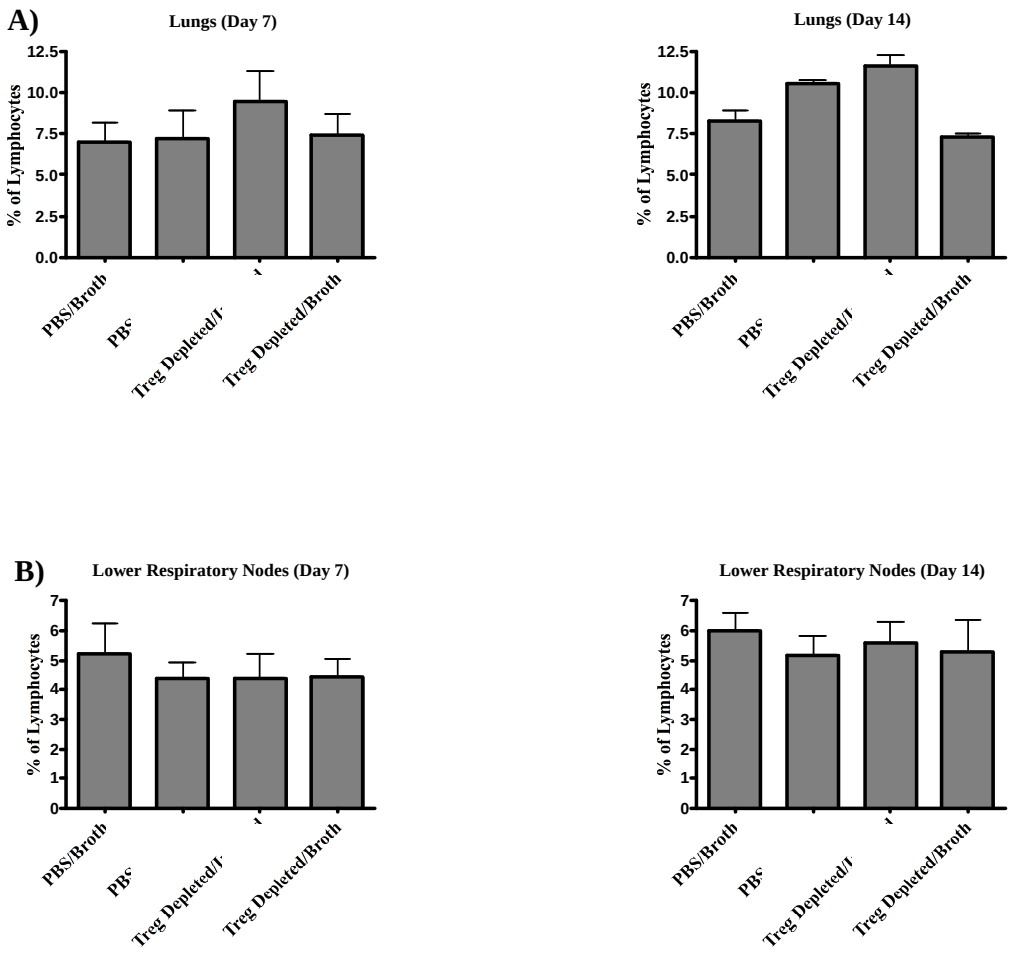


Figure 11. CD62L expression in lungs and LRNs of mice infected with *M. pulmonis* or sham, with or without Treg depletion. Mice were given 100 μls (0.5 mg) anti-CD25 depleting antibody or PBS 1 day prior to infection with 5 x 10^5 CFU *M. pulmonis*, followed by a second antibody dose at day 6 post-infection. Mice were sacrificed at days 7 and 14 post-infection, and lungs (a) and LRNs (b) were harvested. Vertical bars and error bars represent means +/- SE (n=6).

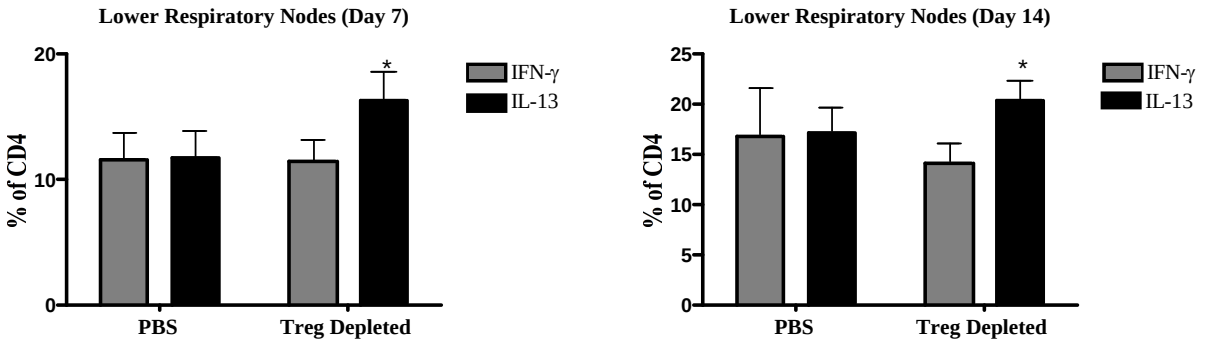


Figure 12. Levels of IFN-γ and IL-13 in the LRNs of *M. pulmonis* infected mice. Mice were given 100 μls (0.5 mg) of anti-CD25 depleting antibody or PBS control 1 day prior to infection with 5 x 10^5 CFU *M. pulmonis*, followed by a second antibody dose or PBS at day 6 post-infection. An asterisk represents a significant ($p \leq 0.05$) difference IL-13 versus IFN-γ. Vertical bars and error bars represent means +/- SE (n=6).

the lungs at any time point (data not shown). These data suggest that the increased immunopathology caused by Treg depletion is due to an enhancement of the Th2 response, which subsequently impacted disease progression.

Tregs from infected mice produce IFN-γ, IL-17, and IL-10 in response to antigen-specific stimulation.

Classical Tregs are known to suppress immune responses through the secretion of IL-10 and/or TGF-β(14). Both of these cytokines are associated with Th2 responses. Therefore, it was surprising to find that the absence of Tregs actually led to an increase in the Th2 immune response rather than the Th1. We decided to examine the cytokines produced by Tregs. Since Tregs can be specific for foreign antigens or self-antigens (2), we examined both antigen specific and polyclonal activation of $CD4^+CD25^+$ Treg cells from LRN. Mice were infected with *M. pulmonis* and sacrificed at day 8 post-infection. Tregs were isolated from LRNs and cultured for 4 days in the presence of anti-CD3 and anti-CD28 antibodies (polyclonal activation), or for 6 days in the presence MMA and antigen-presenting cells (CD3-depleted naïve splenocytes) to assess mycoplasma-specific activation. Supernatants were then assayed for the levels of IL-4, IL-13, IL-10, IL-17, IFN-γ, TGF-β.

Tregs produced a high amount of IL-17 and detectable levels of IFN-γ in response to MMA (Fig. 13). Polyclonal stimulation (anti-CD3/anti-CD28) also caused Tregs to produce IL-17 and IFN-γ. Low levels of IL-10 were also produced in response to both MMA and anti-CD3/anti-CD28 stimulation. Treg cells did not produce either IL-13 or TGF-β in response to polyclonal or mycoplasma antigen stimulation. These data suggest that Tregs are capable of producing IFN-γ and IL-17 in response to mycoplasma.

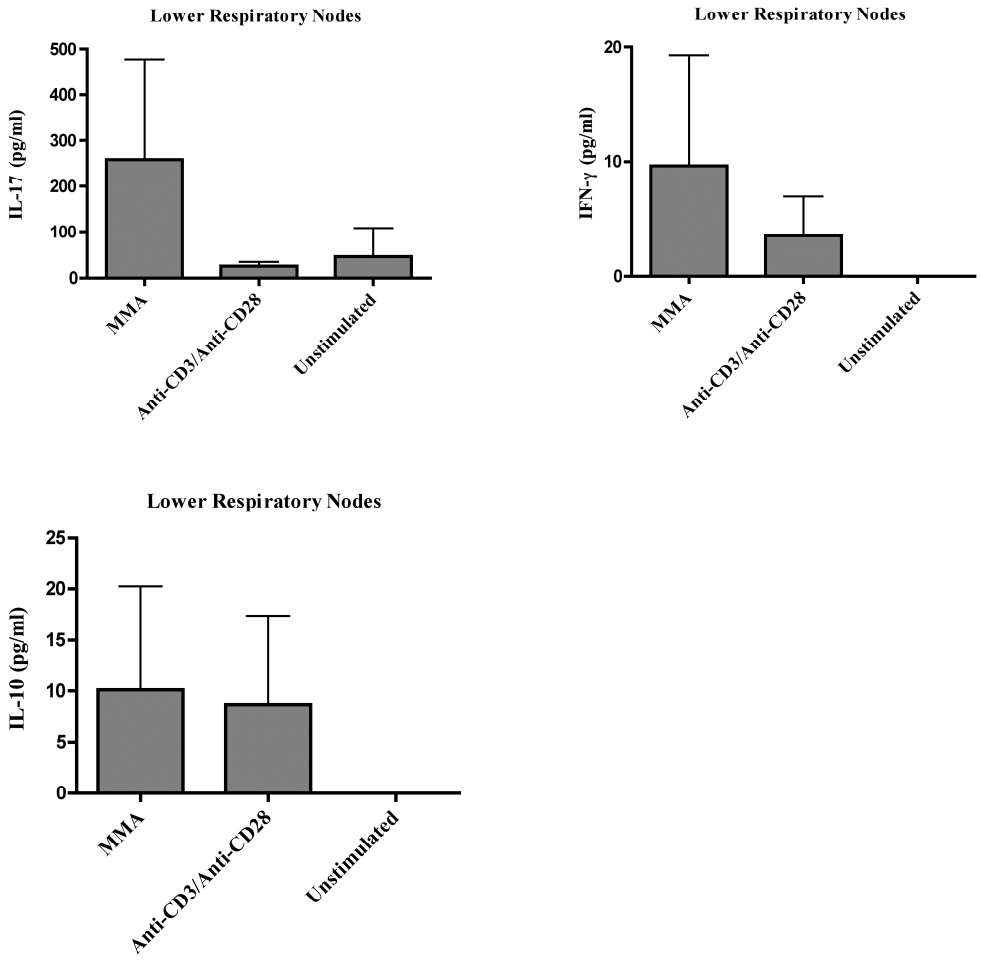


Figure 13. Treg production of IFN-γ, IL-17, and IL-10 in response to MMA and anti-CD3/anti-CD28 stimulation *in vitro*. Mice were infected with 5 x 10^5 CFU *M. pulmonis* and sacrificed at 8 days post-infection. Tregs were isolated from LRN and stimulated with anti-CD3 and anti-CD28 antibodies, or MMA and antigen-presenting cells (APC). Cultures were incubated for either 4 days (anti-CD3/CD28) or 6 days (MMA). IFN-γ, IL-17, and IL-10 concentrations in supernatants were measured. Bars represent means +/- range (n=2).

IL-17$^+$ and IFN-γ$^+$ populations of Tregs expand in response to mycoplasma infection.

To confirm that IL-17$^+$ and/or IFN-γ producing Tregs developed in response to mycoplasma infection, mice were infected with 5 x 10^5 CFU *M. pulmonis*, and sacrificed at 7 and 14 days post-infection. Cells were isolated from lungs and LRNs, and they were immunofluorescently stained for expression CD4, CD25 and intracellular FoxP3, IL-17 and IFN-γ.

IFN-γ$^+$ Tregs and IL-17$^+$ Tregs were found in the LRNs of infected mice (Fig. 14). IFN-γ$^+$ This was also true for Tregs in the lungs (data not shown). There were no double positive (IFN-γ$^+$IL-17$^+$) Tregs observed in either organ. Although a majority of Tregs did not express either of these cytokines, the Treg compartment in mycoplasma-infected mice included a subpopulation of IFN-γ$^+$ Tregs, and a subpopulation of IL-17$^+$ Tregs.

To determine if these subpopulations responded to infection mice were infected with 5 x 10^5 CFU *M. pulmonis*, and sacrificed at days 7 or 14 post-infection. Cells were isolated from LRNs and lungs and stained for IL-17 and IFN-γ.

The percentage of both IFN-γ-expressing Tregs and IL-17-expressing Tregs significantly increased in the LRNs of infected mice relative to uninfected mice at day 7 post-infection (Fig. 15a). No significant differences were seen in the LRNs at day 14 post-infection (Fig. 15b). No significant differences were observed in the lungs at either time point (data not shown). These data demonstrate that IFN-γ-expressing and IL-17-expressing Treg subpopulations preferentially expand in response to infection.

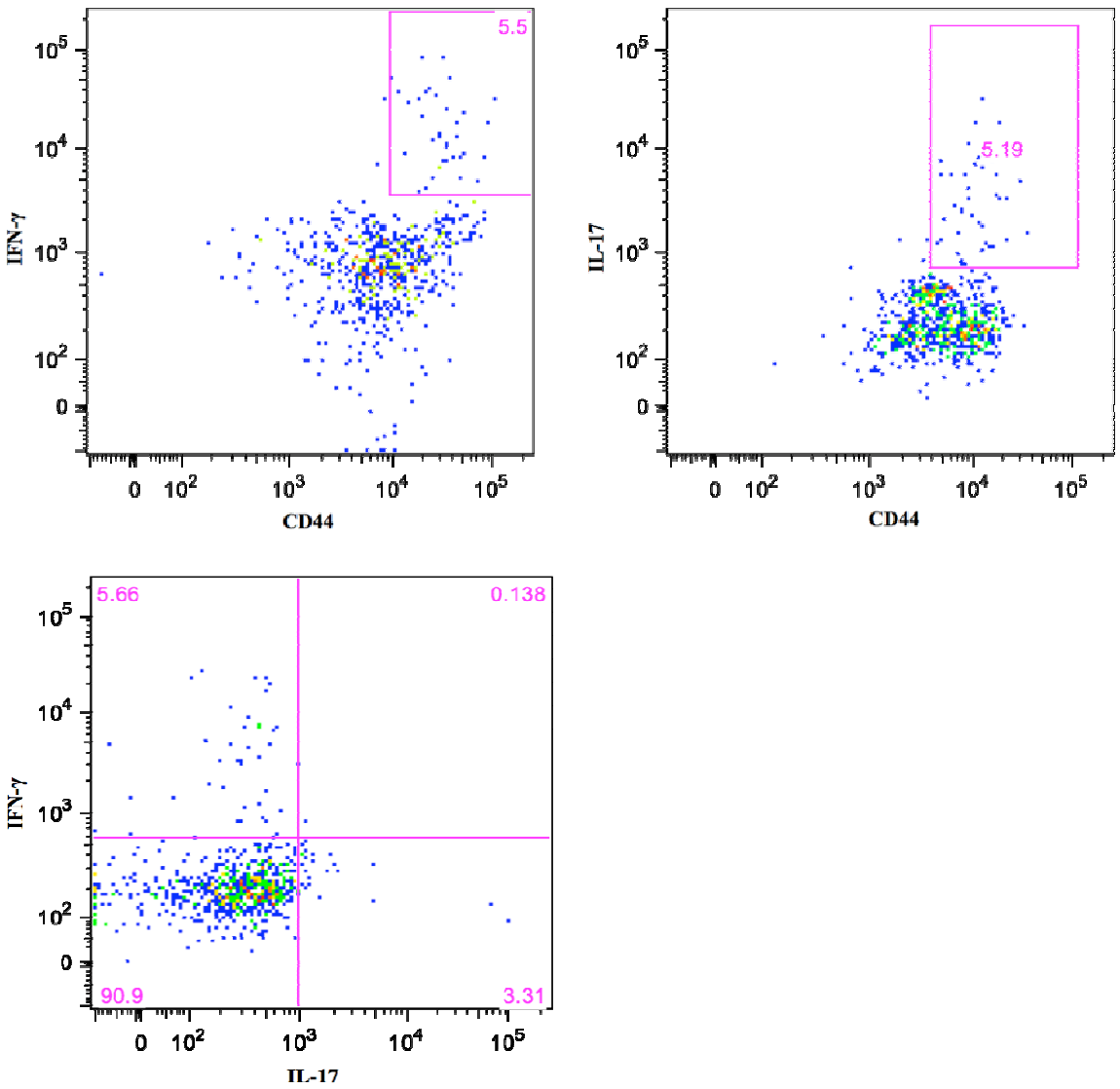


Figure 14. Expression of IL-17 and IFN-γ in LRNs of *M. pulmonis* infected mice. Mice were infected with 5 x 10^5 CFU *M. pulmonis*, and sacrificed at day 8 post-infection. Cells were isolated from LRNs and stained for IL-17 and IFN-γ. Populations are gated on $CD4^+CD25^+FoxP3^+$ cells. One representative LRN sample of two separate experiments is shown.

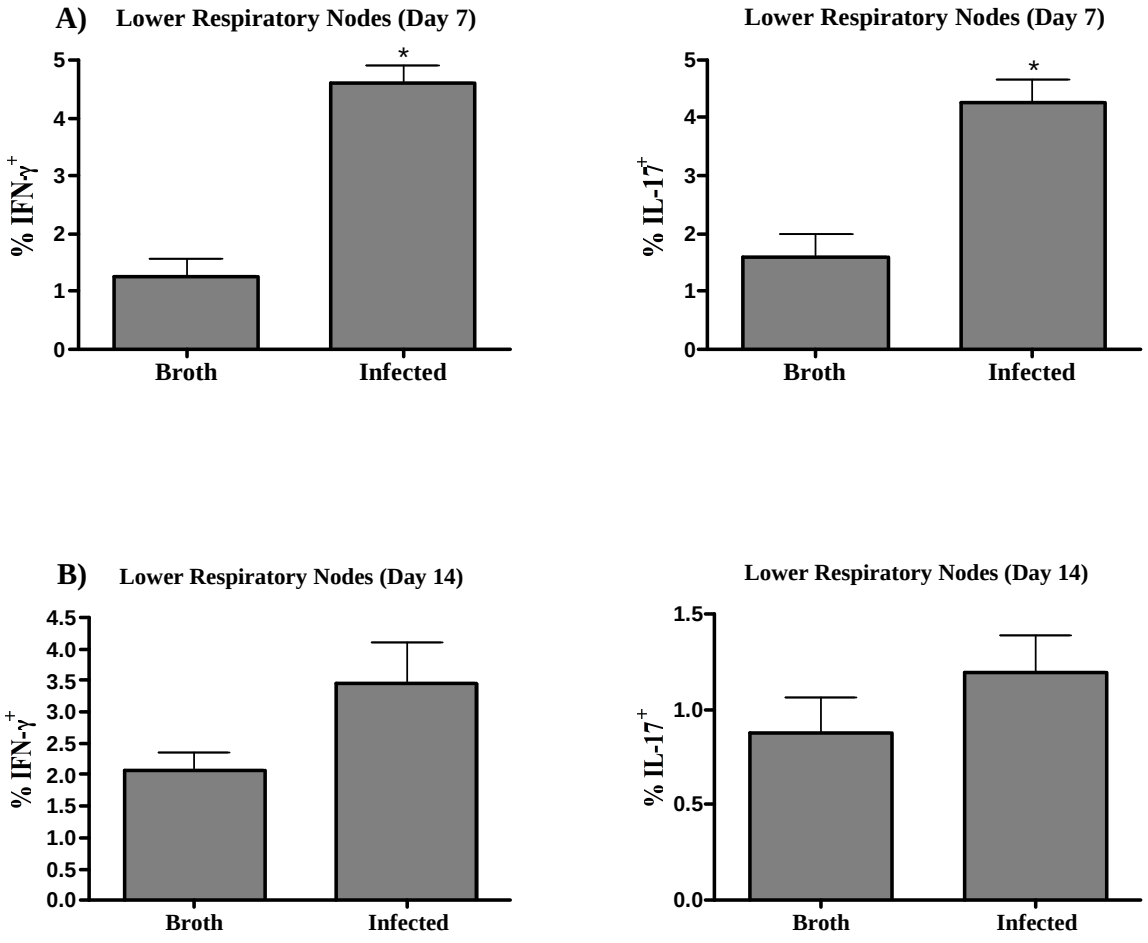


Figure 15. Expression of IL-17 and IFN-γ in Tregs in the LRNs of *M. pulmonis*-infected or control mice. Mice were intranasally infected with 5 x 10^5 CFU *M. pulmonis*. Mice were sacrificed at day 7 (a) or day 14 (b) post-infection, and cells harvested from LRNs. Data are expressed as a percentage of $CD4^+CD25^+FoxP3^+$ cells. An asterisk (*) represents a significant ($p \leq 0.05$) difference Infected versus Broth. Vertical bars and error bars represent means +/- SE (n=6).

$CD25^-$ Tregs express IFN-γ and IL-17 at lower levels, but do not respond to infection.

The previous data demonstrates that CD25-expressing Tregs expand in response to mycoplasma infection and express IFN-γ or IL-17. However, Tregs that do not express CD25 would not be affected by the injection of a CD25-targeted antibody. It was thus necessary to examine the $CD25^-$ Tregs during mycoplasma infection.

Mice were infected with 5×10^5 CFU *M. pulmonis,* and sacrificed at day 7 or 14 post-infection. Cells were isolated from the LRNs and lungs. Cells were then stained for IFN-γ and IL-17.

Overall, $CD25^-$ Tregs expressed IFN-γ and IL-17 in the LRNs at a lower percentage compared to $CD25^+$ Tregs. There was a significant increase in the percentage of IFN-γ-expressing $CD25^-$ Tregs in the LRNs at day 7 (Fig. 16a). No significant differences were observed in the percentages of IL-17-expressing $CD25^-$ Tregs at day 7, and no significant differences in either IFN-γ-expressing or IL-17-expressing $CD25^-$ Tregs were observed at day 14 (Fig. 16b). As before, no significant differences were observed in the lungs at either time point (data not shown). These data suggest that IFN-γ-expressing $CD25^-$ Tregs may also play a role in mycoplasma respiratory disease.

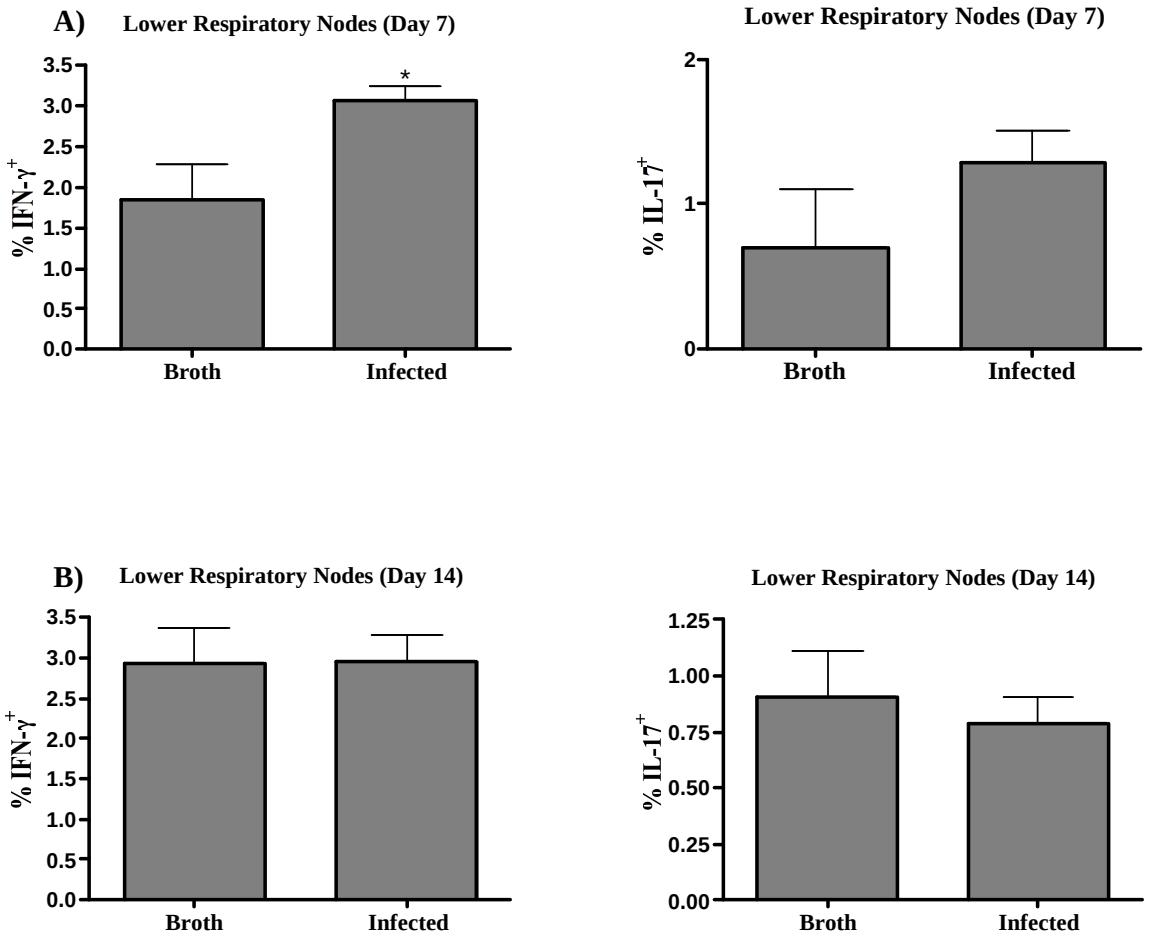


Figure 16. Expression of IL-17 and IFN-γ in $CD25^-$ Tregs in the LRNs of *M. pulmonis*-infected or control mice. Mice were intranasally infected with 5 x 10^5 CFU *M. pulmonis*. Mice were sacrificed at day 7 (a) or day 14 (b) post-infection, and cells harvested from LRNs. An asterisk (*) represents a significant ($p \leq 0.05$) difference Infected versus Broth. Data are expressed as a percentage of $CD4^+CD25^-FoxP3^+$ cells. Vertical bars and error bars represent means +/- SE (n=6).

Depletion of Tregs may decrease the overall levels of IFN-γ and IL-17 in the LRNs.

To explore the possibility that Tregs might promote the secretion of IFN-γ and IL-17 by Th cells. Mice were given anti-CD25 depleting antibody or PBS control 1 day prior to infection with 5 x 10^5 CFU *M. pulmonis*, followed by a second dose or PBS at day 6 post-infection. Mice were sacrificed at days 7 and 14 post-infection, and cells were harvested from LRNs and lungs. Cells were stained for IFN-γ and IL-17.

Treg depletion caused a decrease in the expression of IL-17 in $CD4^+$ LRN cells at day 7 post-infection (Fig. 17a). Treg depletion also caused a decrease in the expression of IFN-γ in $CD4^+$ LRN cells at day 7 post-infection, though the difference was not significant. There were no cytokine differences observed at day 14 in the LRNs (Fig. 17b). No significant differences were seen in the lungs at either time point (data not shown). These data suggest that Tregs may promote the secretion of IFN-γ and IL-17 by Th cells *in vivo*, since their depletion prior to infection resulted in a decrease in the expression of these cytokines.

Antigen-specific Tregs stimulate the production of IFN-γ and IL-17 *in vitro*.

To examine the possibility that Tregs could actually promote Th cells to secrete IFN-γ and/or IL-17, mice were infected with 5 x 10^5 CFU of *M. pulmonis*. Cells from spleens and LRNs were harvested at 8 days post-infection. Cells were sorted into $CD4^+CD25^+$ Treg cells, and $CD4^+CD25^-$ Th cells. Th cells were cultured *in vitro* with or without Tregs, in the presence of plate-bound anti-CD3 antibody and soluble anti-CD28 antibody, or mycoplasma membrane antigen (MMA) with antigen-presenting cells (CD3-depleted naïve splenocytes). Supernatants were then assayed for IL-4, IL-10, IL-13, IL-17, TGF-β, and IFN-γ.

The addition of Tregs led to significant increases in antigen-specific IL-17 secretion and polyclonal IL-17 secretion in both LRN and spleen cultures (Fig. 18). In the case of LRN cultures, the addition of Tregs led to a significant increase in IL-17 even in the absence of any stimulation.

Similar results were with IFN-γ secretion. The addition of Tregs led to significant differences in antigen-specific IFN-γ secretion in both LRN and spleen cultures (Fig. 19). A significant increase in IFN-γ was also observed in polyclonally-stimulated LRN cultures. No significant differences were observed in polyclonally-stimulated spleen cultures.

No antigen-specific IL-10 secretion was observed in any LRN or spleen cultures, though a significant increase in IL-10 was observed in polyclonally-stimulated spleen cultures containing Tregs (Fig. 20). Supernatants were also tested for the presence of TGF-β, but no TGF-β was detected in any cultures (data not shown).

No antigen-specific secretion of IL-4 or IL-13 was observed in either the LRN or spleen cultures (Fig. 21, Fig. 22). A significant increase in IL-4 was observed in polyclonally-stimulated LRN cultures containing Tregs. No significant increases in IL-13 were observed under any conditions.

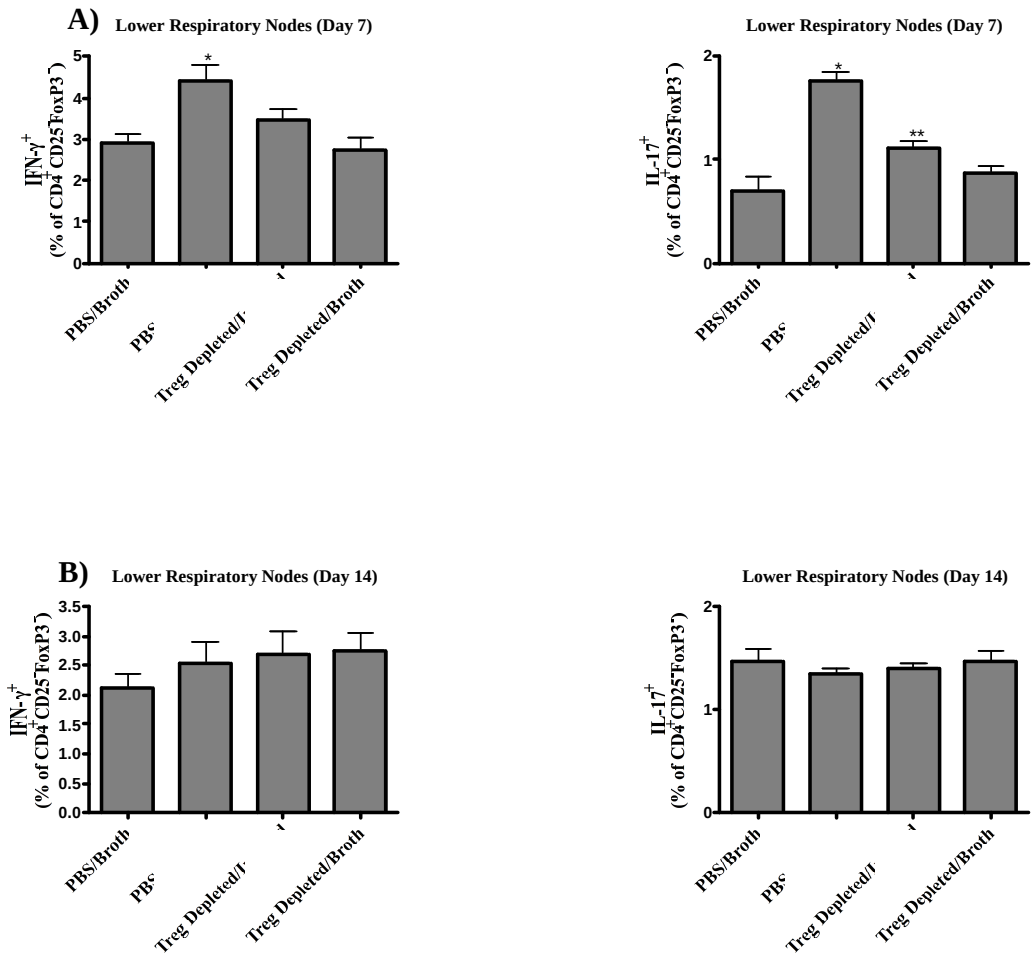


Figure 17. Expression of IL-17 and IFN-γ in $CD4^+CD25^-FoxP3^-$ cells in $CD4^+$ T cells from LRNs of *M. pulmonis*-infected mice, with or without Treg depletion. Mice were given 100 µls (0.5 mg) anti-CD25 depleting antibody or PBS control 1 day prior to infection with 5 x 10^5 CFU *M. pulmonis*, followed by a second dose of antibody or PBS at day 6 post-infection. Mice were sacrificed at day 7 (a) or day 14 (b) post-infection, and cells harvested from LRNs. Data are expressed as a percentage of total lymphocytes. An asterisk (*) represents a significant ($p \leq 0.05$) difference PBS/Infected versus PBS/Broth. A double asterisk (**) represents a significant ($p \leq 0.05$) difference Treg Depleted/Infected versus PBS/Infected. Vertical bars and error bars represent means +/- SE (n=6).

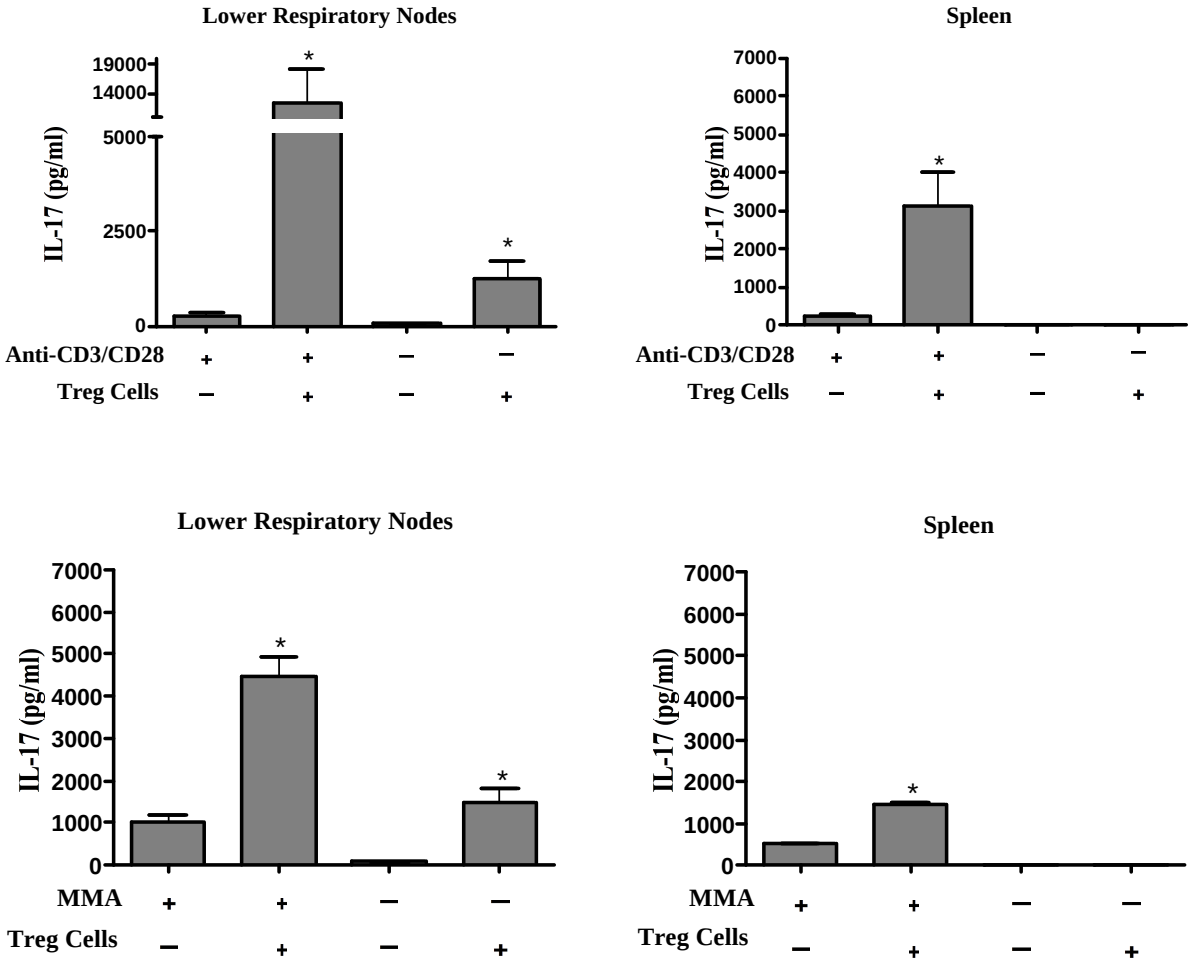


Figure 18. Levels of interleukin-17 in LRN and spleen cultures with and without Tregs. Mice were infected with 5 x 10^5 CFU *M. pulmonis* and sacrificed at 8 days post-infection. Tregs and Th cells were isolated from spleens and LRNs and stimulated with anti-CD3 and anti-CD28 antibodies, or mycoplasma membrane antigen (MMA) and antigen-presenting cells (APC). Cultures were incubated for either 4 days (anti-CD3/CD28) or 6 days (MMA). IL-17 concentration in supernatants was measured. An asterisk (*) represents a significant ($p \leq 0.05$) difference (anti-CD3/28 + Treg versus anti-CD3/28 only, MMA + Treg versus MMA only, Treg only versus no treatment). Vertical bars and error bars represent means +/- SE (n=4).

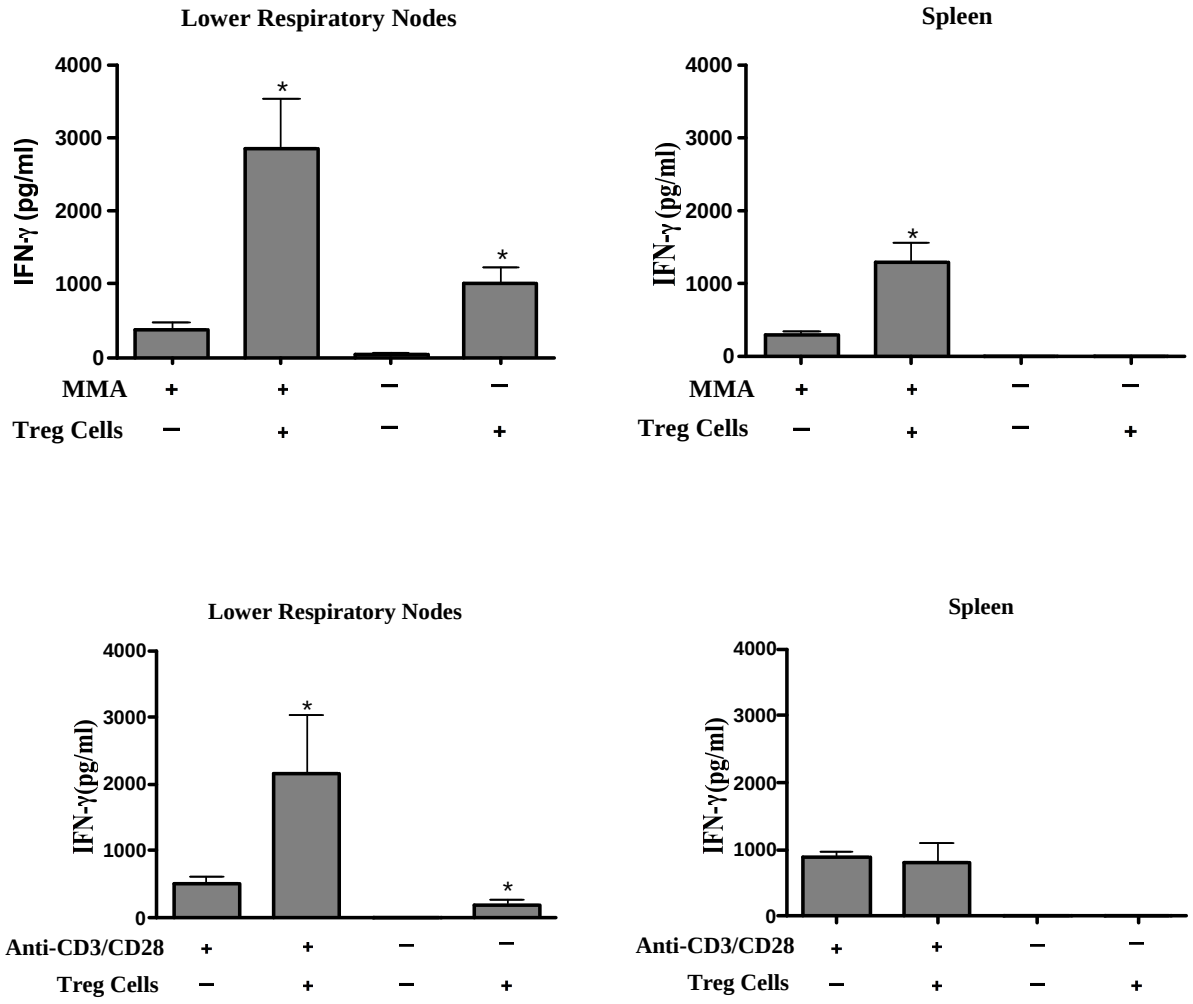


Figure 19. Levels of interferon-γ in LRN and spleen cultures with and without Tregs. Mice were infected with 5 x 10^5 CFU *M. pulmonis* and sacrificed at 8 days post-infection. Tregs and Th cells were isolated from spleens and LRNs and stimulated with anti-CD3 and anti-CD28 antibodies, or mycoplasma membrane antigen (MMA) and antigen-presenting cells (APC). Cultures were incubated for either 4 days (anti-CD3/CD28) or 6 days (MMA). IFN-γ concentration in supernatants was measured. An asterisk (*) represents a significant ($p \leq 0.05$) difference (anti-CD3/28 + Treg versus anti-CD3/28 only, MMA + Treg versus MMA only, Treg only versus no treatment). Vertical bars and error bars represent means +/- SE (n=4).

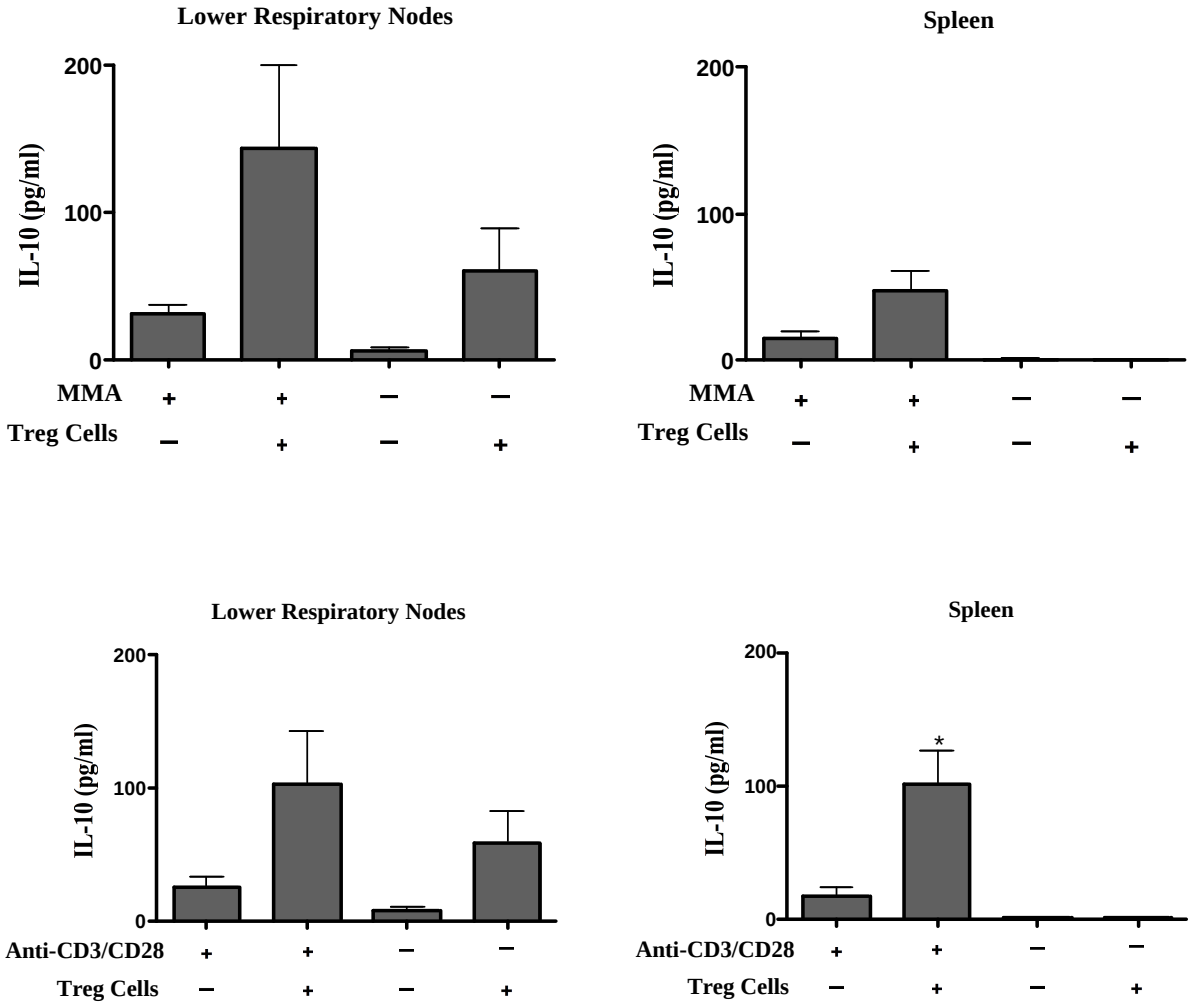


Figure 20. Levels of interleukin-10 in LRN and spleen cultures with and without Tregs. Mice were infected with 5 x 10^5 CFU *M. pulmonis* and sacrificed at 8 days post-infection. Tregs and Th cells were isolated from spleens and LRNs and stimulated with anti-CD3 and anti-CD28 antibodies, or mycoplasma membrane antigen (MMA) and antigen-presenting cells (APC). Cultures were incubated for either 4 days (anti-CD3/CD28) or 6 days (MMA). IL-10 concentration in supernatants was measured. An asterisk (*) represents a significant ($p \leq 0.05$) difference (anti-CD3/28 + Treg versus anti-CD3/28 only). Vertical bars and error bars represent means +/- SE (n=4).

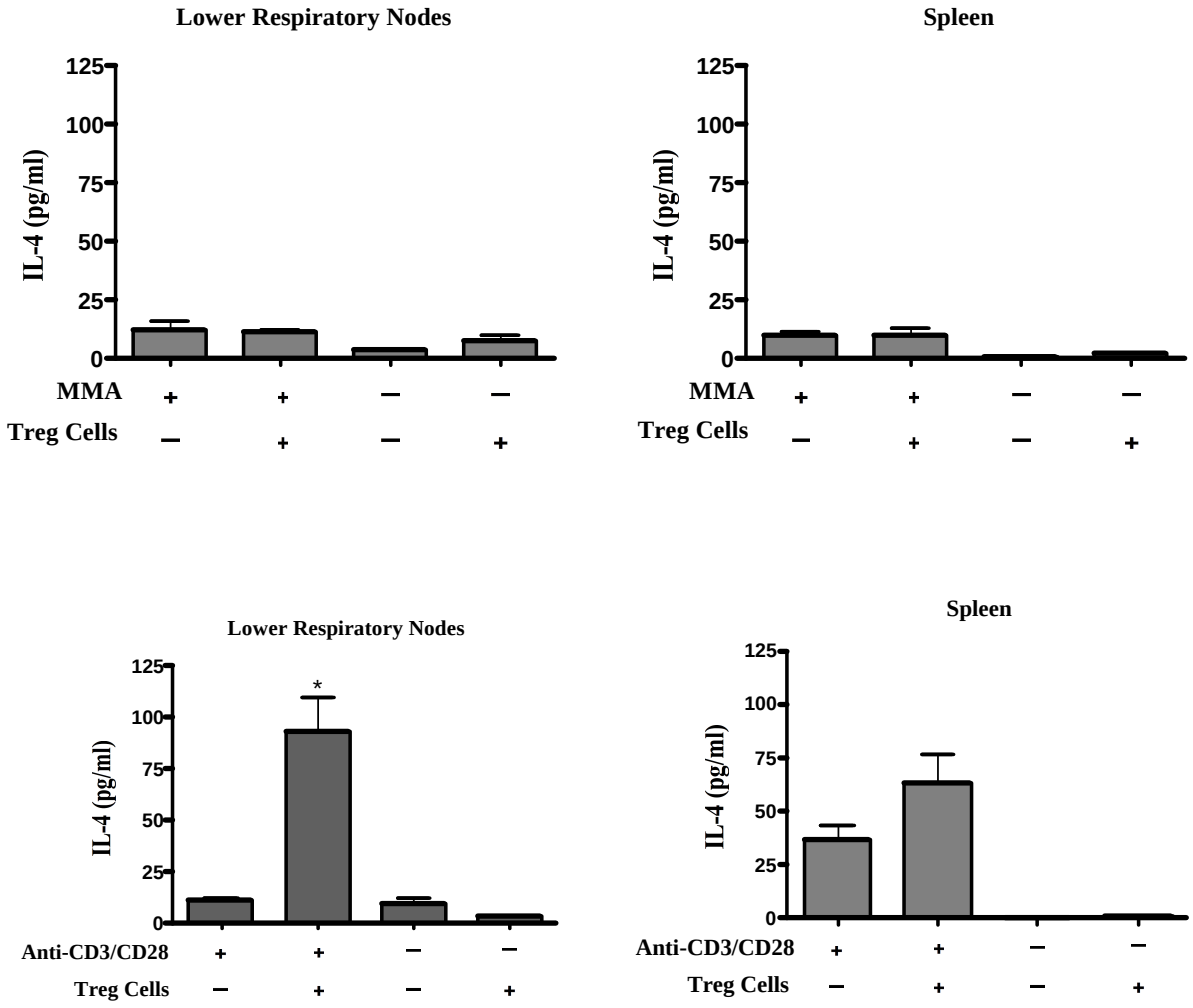


Figure 21. Levels of interleukin-4 in LRN and spleen cultures with and without Tregs. Mice were infected with 5 x 10^5 CFU *M. pulmonis* and sacrificed at 8 days post-infection. Tregs and Th cells were isolated from spleens and LRNs and stimulated with anti-CD3 and anti-CD28 antibodies, or mycoplasma membrane antigen (MMA) and antigen-presenting cells (APC). Cultures were incubated for either 4 days (anti-CD3/CD28) or 6 days (MMA). IL-4 concentration in supernatants was measured. An asterisk (*) represents a significant ($p \leq 0.05$) difference (anti-CD3/28 + Treg versus anti-CD3/28 only). Vertical bars and error bars represent means +/- SE (n=4).

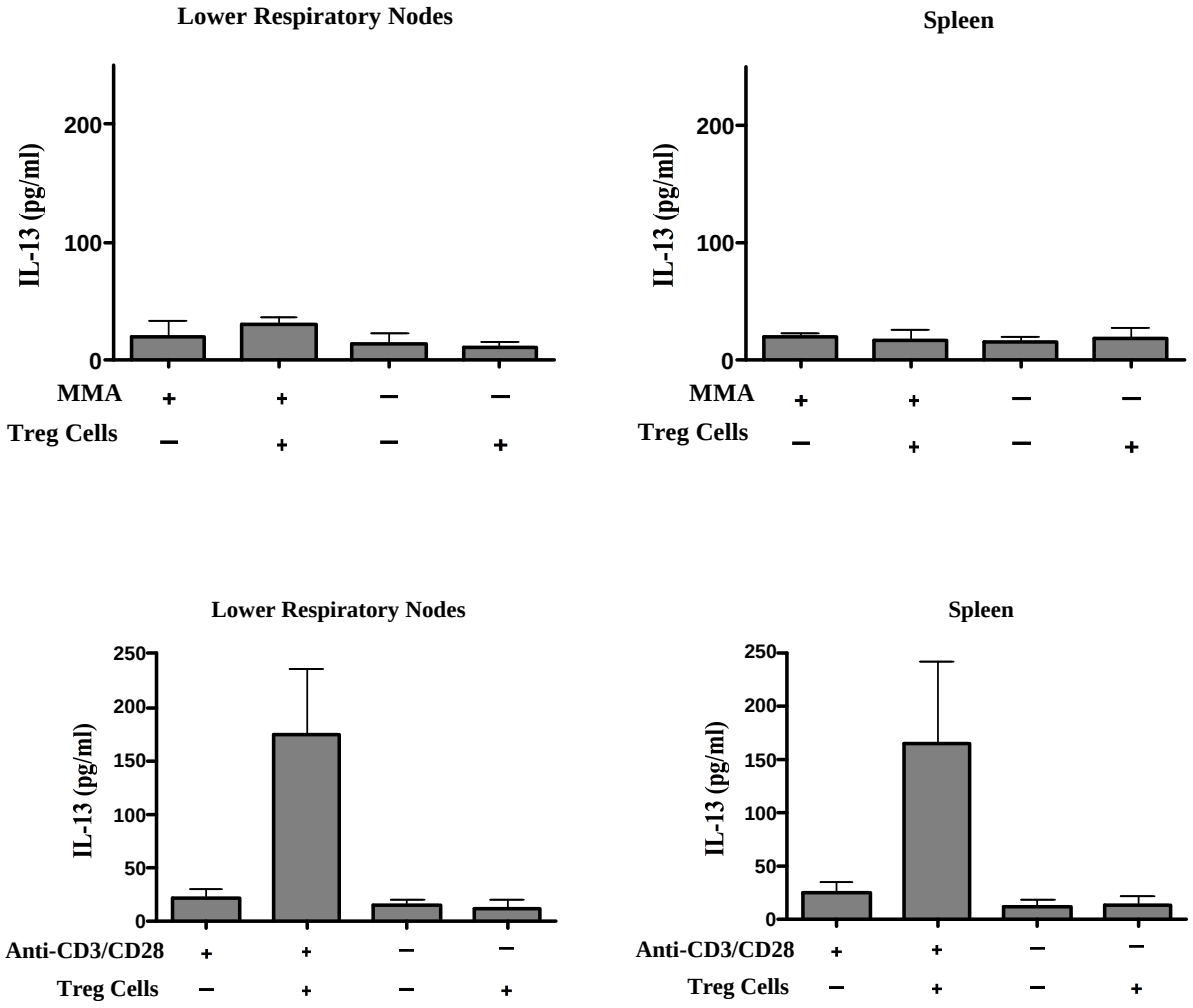


Figure 22. Levels of interleukin-13 in LRN and spleen cultures with and without Tregs.

Mice were infected with 5 x 10^5 CFU *M. pulmonis* and sacrificed at 8 days post-infection. Tregs and Th cells were isolated from spleens and LRNs and stimulated with anti-CD3 and anti-CD28 antibodies, or mycoplasma membrane antigen (MMA) and antigen-presenting cells (APC). Cultures were incubated for either 4 days (anti-CD3/CD28) or 6 days (MMA). IL-13 concentration in supernatants was measured. Vertical bars and error bars represent means +/- SE (n=4).

Therefore, the addition of Tregs to cultures stimulated with MMA did not result in the significant increase of any Th2-associated cytokines. Significant increases were seen in only two instances of polyclonal activation: Spleen cultures secreted more IL-10 after the addition of Tregs, and LRN cultures secreted more IL-4 after the addition of Tregs.

These data suggest that Tregs, in contrast to their previously observed roles, may be secreting the proinflammatory cytokines IFN-γ and IL-17, and/or stimulating their secretion by other cells. Furthermore, the increases in these cytokines in response to MMA stimulation show that a component of this Treg response is foreign antigen-specific.

Adoptive transfer of naïve Tregs from the spleens did not affect mycoplasma disease.

To further examine the potential role of Treg populations in mycoplasma disease, naïve mice were sacrificed, and spleen cells were harvested. This approach has been used in several studies to examine the role of Tregs in disease (116-119). Naïve spleen cells were sorted for the $CD4^+CD25^+$ cells. Recipient mice were intravenously injected with 10^6 Tregs or PBS control 1 day prior to infection with 2 x 10^5 CFU *M. pulmonis*. Recipient mice were then sacrificed at day 14 post-infection.

Adoptive transfer of naïve splenocyte Tregs had no effect on weight loss over the course of the experiment, and there were no differences in the incidence of gross lesions (Fig. 23). Also, there were no differences in cell counts in either the LRNs or the lungs at day 14 post-infection. This indicates that Tregs from naïve spleens are not capable of suppressing the immune response to *M. pulmonis.*

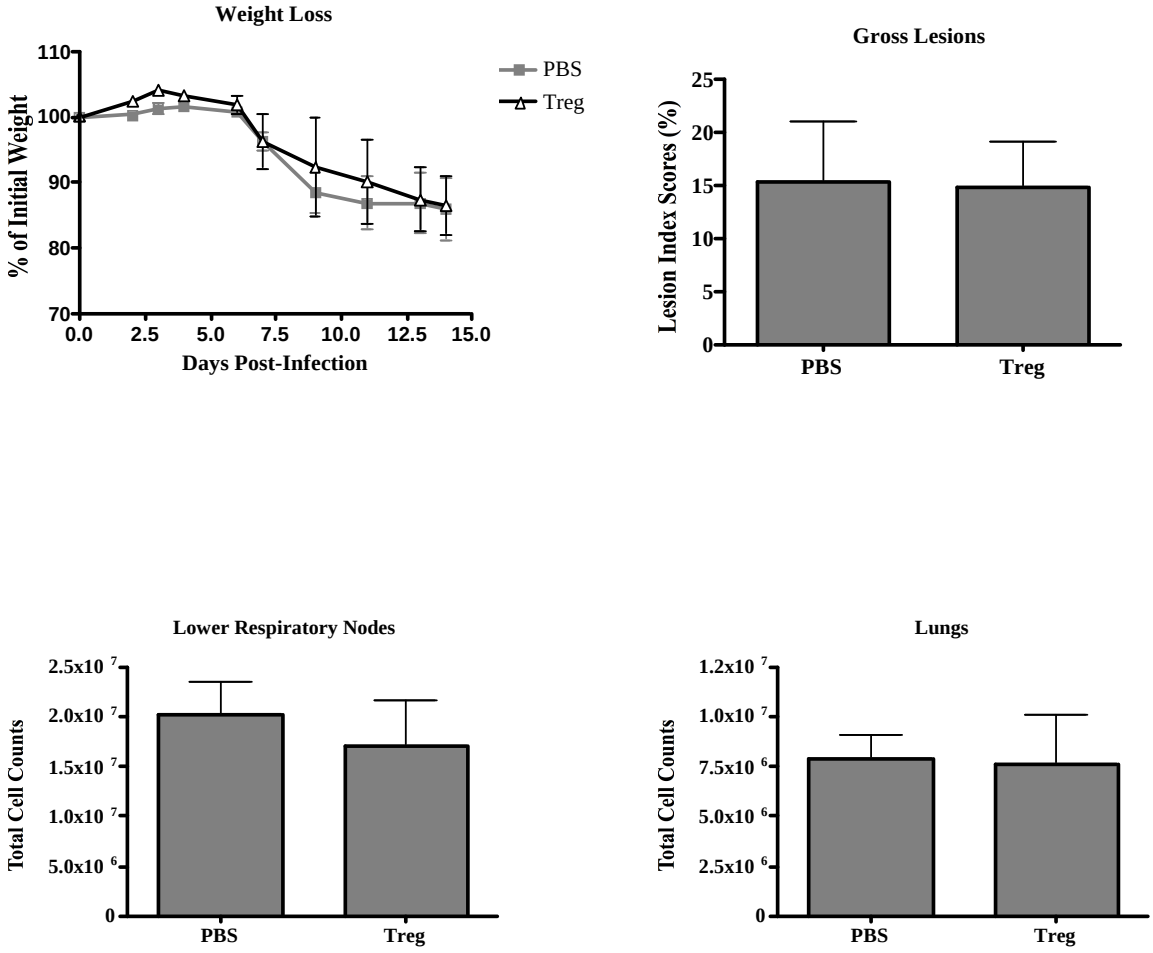


Figure 23. Weight loss, incidence of gross lesions, and cell counts in *M. pulmonis*-infected mice adoptively given Tregs from naïve spleens, or PBS control. Mice were adoptively transferred 10^6 naïve Tregs sourced from spleens 1 day prior to infection with 2 x 10^5 *M. pulmonis*. Mice were sacrificed at 14 days post-infection. Vertical bars and error bars represent means +/- SE (n=9).

Tregs from LRNs of *M. pulmonis*-infected mice cause early disease in recipients.

As shown in the previous studies, Tregs from the spleens of naïve mice were not effective at preventing immunopathology, it was possible that Tregs might need to be exposed to *M. pulmonis* before gaining the ability to suppress the mycoplasma-specific immune responses. Mice were infected with 5 x 10^5 CFU *M. pulmonis* and sacrificed at 14 days post-infection. Cells were harvested from LRNs and sorted. The $CD4^+CD25^+$ cell fraction was collected, and 10^6 Tregs were then injected intravenously into recipient mice 1 day prior to infection with 5 x 10^5 CFU *M. pulmonis.* Mice were sacrificed at 14 days post-infection.

In contrast to the results using Tregs from naïve spleens, Tregs from the LRNs of infected mice actually increased disease. This increase in disease occurred much earlier, with Treg recipients losing weight by day 3 post-infection (Fig. 24). No difference was observed in gross lesion scores at day 14 post-infection, but cell counts were significantly increased in the lungs of Treg recipients compared to control mice, something that was linked to increased disease in previous experiments. Cell counts also increased in the LRNs of Treg recipients, though not significantly. These data demonstrate that the Tregs from LRN in infected mice do respond to mycoplasma infection; however, the results suggest that the development of Tregs and their beneficial activity during *M. pulmonis* infection may be associated with concurrent development of adaptive T cell responses. If added too early in the development of disease, these cells may actually increase disease, most likely through the production of inflammatory cytokines.

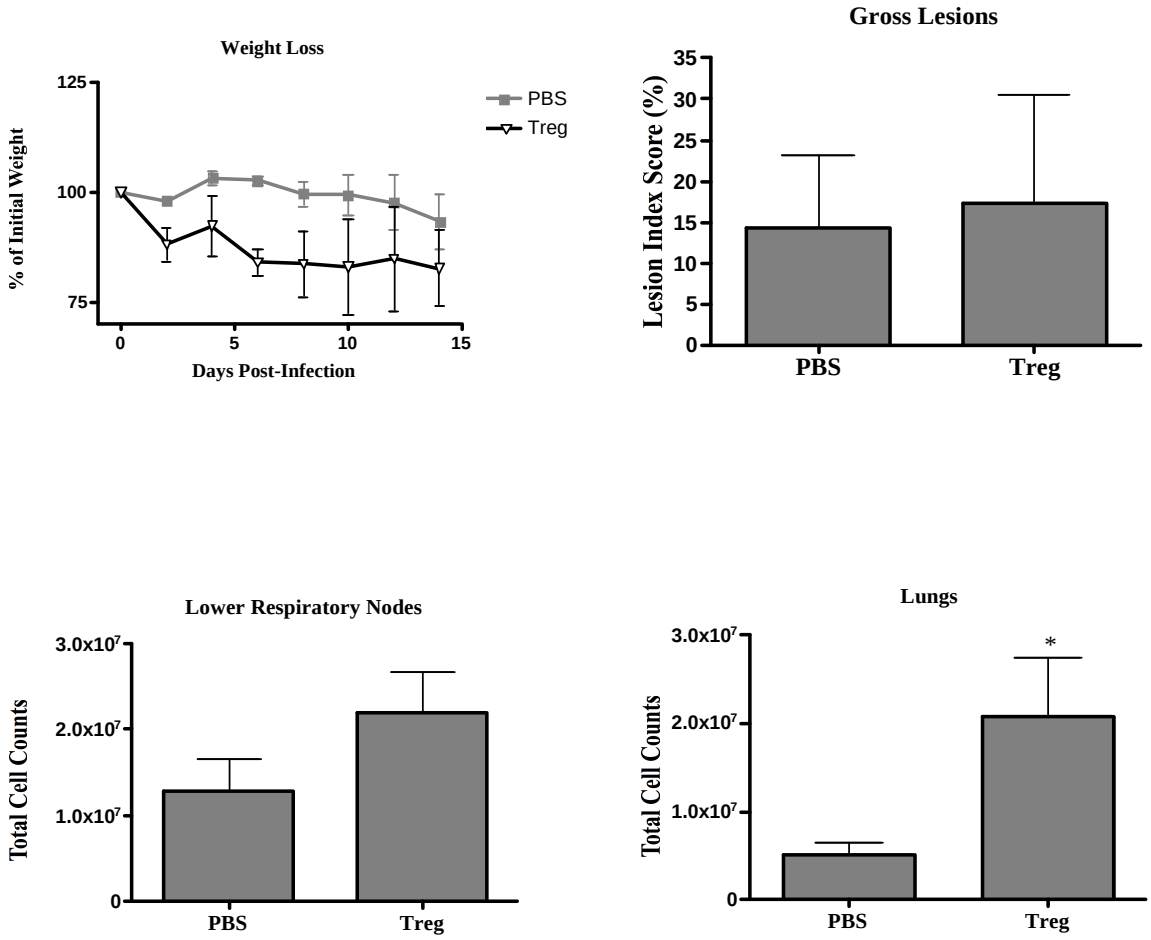


Figure 24. Weight loss, incidence of gross lesions, and cell counts in *M. pulmonis*-infected mice adoptively given Tregs from the LRNs of infected mice, or PBS control. Mice were adoptively transferred 10^6 Tregs sourced from infected LRNs 1 day prior to infection with 2 x 10^5 CFU *M. pulmonis*. Mice were sacrificed at 14 days post-infection. An asterisk (*) represents a significant ($p \leq 0.05$) difference, Treg versus PBS. Vertical bars and error bars represent means +/- SE (n=9).

CHAPTER IV

DISCUSSION

Regulatory T cells, defined as $CD4^+CD25^+FoxP3^+$ cells, are a specialized subset of $CD4^+$ T cells that act against traditional immune responses, normally through the secretion of the cytokines IL-10 and/or TGF-β (2, 89, 90). By suppressing or dampening immune responses, Tregs can often prevent the incidence of damaging immunopathology resulting from a heightened, nonspecific immune response (2). However, Tregs have also been implicated in the development of chronic infections, since weakened immune responses may be insufficient to clear infectious organisms (13, 14, 91). Mycoplasma is a common respiratory bacterium that is responsible for many cases of chronic pneumonia (16, 20). Previous research into mycoplasma has revealed that a large component of mycoplasma respiratory disease is immunopathologic (20, 21). Knowing that Tregs have been shown to be involved in both the control of immunopathology and the persistence of chronic infections, we proposed to study the role of Tregs in mycoplasma respiratory disease. We initially hypothesized that Tregs would play a beneficial role in the control of immunopathologic immune responses, and that depletion of Tregs would lead to a decrease in bacterial CFU.

The results presented here demonstrate an important role for Tregs in the immune response to mycoplasma respiratory infection, and also suggest a novel mechanism of Treg-

mediated immune suppression. In exploring the role of Tregs in mycoplasma respiratory disease, there were several unique findings: 1) The presence of Treg cells dampens immunopathologic immune responses during *M. pulmonis* infection; 2) Depletion of Tregs prior to mycoplasma infection enhances the development of a Th2 response, suggesting that Tregs can suppress the development of Th2 responses in this model; 3) Tregs in mycoplasma-infected animals secrete the cytokines IFN-γ and IL-17, and may promote secretion of these cytokines by other cells. These data clearly show that Tregs are important in controlling immune responses to *M. pulmonis*, and that Tregs may be capable of dampening immune responses through previously unknown mechanisms, a finding which is in agreement with our hypothesis. However, in contrast to our hypothesis, Tregs played no role in the control of infection, as bacterial CFU were unchanged.

Tregs clearly control the level of disease in mycoplasma respiratory infection. Depletion of Tregs using an anti-CD25 depleting antibody prior to infection with *M. pulmonis* resulted in significant increases in clinical disease. Treg-depleted mice lost significantly more weight over the course of the experiment, and displayed a significantly higher incidence of gross lung lesions. In addition, Treg-depleted mice had more severe histological lung lesions in all four of the categories that were examined (peribronchial infiltration, airway exudate, epithelial hyperplasia, alveolitis). This effect was clearly related to a decrease in the level of Tregs, as the anti-CD25 antibody was shown to specifically deplete $CD4^+CD25^+FoxP3^+$ cells without affecting other cell populations.

Treg depletion prior to infection led to an increase in the percentage of IL-13^+ cells in the lower respiratory lymph nodes (LRN), suggesting that immune responses in these mice had been skewed towards the Th2 lineage. Increased disease in Treg-depleted mice was also concurrent

with increases in lung cell infiltration and increases in the levels of mycoplasma-specific serum antibodies. However, Treg-depleted mice displayed no differences in lung CFU. These data show that Tregs in mycoplasma respiratory disease control inflammation and immunopathology, but have no effect on the control of the actual level of infection.

Tregs preferentially expanded in the host in response to mycoplasma infection, suggesting that they do play a role in the disease. Characterization of Tregs in mycoplasma-infected mice showed that they express high levels of both CTLA-4 and glucocorticoid-induced TNF-like receptor (GITR), and low levels of CD127, demonstrating that their expression pattern matches that of traditional Tregs (70, 73, 76-82). Interestingly, these Tregs included two subpopulations that expressed either IFN-γ or IL-17.

In vitro cultures containing Tregs and Th cells from LRNs of infected mice secreted significantly higher levels of IFN-γ and IL-17 when stimulated with MMA as compared to Th cells alone. No increases in IL-4, IL-13, or IL-10 were observed in response to MMA. These data provided evidence that Tregs might stimulate the secretion of IFN-γ and IL-17 cytokines by Th cells. Depletion of Tregs from infected mice led to a decrease in the *in vivo* secretion of IFN-γ and IL-17 by $CD4^+CD25^-FoxP3^-$ T helper (Th) cells. These data suggest that two unique populations of Tregs, IFN-γ^+ or IL-17^+, develop in mycoplasma-infected mice, and that they participate in control of the disease. Furthermore, these data suggest that IFN-γ^+ and IL-17^+ Tregs may promote the secretion of these cytokines by Th cells.

Based on these results we propose a model for the role of Tregs in mycoplasma respiratory disease. The development of IFN-γ^+ or IL-17^+ Tregs is likely due to stimulation by mycoplasma antigens and/or changes in the cytokine environment as a result of mycoplasma infection. This development may take several days, during which time natural Tregs may receive

signals that alter their phenotype to that of IFN-γ^+ or IL-17^+ Tregs. Alternatively, $CD4^+$ Th cells may be induced to differentiate into IFN-γ^+ or IL-17^+ Tregs. Tregs may then begin to stimulate Th1 responses in $CD4^+$ Th cells in the LRNs, or suppress Th2 responses, through secretion of IFN-γ or IL-17 (47, 120, 121), and/or through other mechanisms that are currently unknown. By preventing the development of a strong Th2 response, Tregs can prevent some of the immunopathology associated with mycoplasma respiratory disease.

Conversely, when Tregs are depleted from the host, the Th2 response is no longer suppressed. The mycoplasma infection elicits a strong Th2 response, which begins to act between days 5 and 7 post-infection. It is at this time that cell infiltration into the lungs begins to increase, coinciding with the first signs of more severe disease. The level of disease increases through day 14 as immunopathologic Th2 responses cause the development of severe lung lesions.

There have been a number of previous studies demonstrating the importance of the Th1/Th2 ratio in the pathogenesis of mycoplasma respiratory disease. *M. pulmonis*-infected IFN-$\gamma^{-/-}$ mice demonstrated increased disease severity compared to *M. pulmonis*-infected IL-$4^{-/-}$ mice (17). Since IFN-$\gamma^{-/-}$ mice will preferentially mount a Th2-type response, these data suggested that immunopathology in mycoplasma infections may be related to Th2 immune responses. Further experiments by Jones et al. showed that depletion of $CD8^+$ T cells prior to infection also led to increased disease(22). This may have been related to the fact that $CD8^+$ T cells are a source of IFN-γ, and the fact that their removal could allow for increased Th2 responses. Bodhankar et al. conducted further studies demonstrating that the depletion of natural killer (NK) cells prior to immunization with mycoplasma membrane antigen (MMA) resulted in increased protection against *M. pulmonis* and a lower level of clinical disease (122). This was correlated with higher

levels of IFN-γ and lower levels of IL-4 in the LRNs. In addition, immunization of IFN-$\gamma^{-/-}$ mice prior to *M. pulmonis* infection resulted in severe exacerbation of disease, while immunization of IL-$4^{-/-}$ were significantly protected, displaying a much lower level of disease (123). Experiments by Dobbs et al. demonstrated that STAT$6^{-/-}$ mice, which have a deficiency in IL-4, were also better protected against mycoplasma infection after immunization and subsequent *M. pulmonis* infection (124). This was connected to a lower level of IL-13^{+} Th cells in the LRNs. In other experiments, dendritic cells (DC) pulsed with MMA, granulocyte macrophage colony stimulating factor (GM-CSF), TGF-β, and IL-10 were adoptively transferred into mice prior to infection with *M. pulmonis*. These mice developed more severe disease relative to control mice, and this difference was again linked to a higher level of IL-13^{+} Th cells in the LRNs (124). Thus, the connection between Th2 responses and increased mycoplasma respiratory disease severity is well established at this point.

Mycoplasma respiratory disease shares a number of characteristics with asthma. Both are chronic respiratory conditions characterized by an increase in airway hyperreactivity. Both are Th2-mediated inflammatory diseases, with each shown to be related to levels of IL-4. In addition, mycoplasma infections are known to exacerbate asthma. Thus, the research performed in asthma models may be relevant to studies of mycoplasma respiratory infections.

Tregs have long been implicated in the control of asthma, though the mechanisms through which they accomplish this control are still being studied (125-127). In one set of experiments, Lewkowich et al. demonstrated that the depletion of Tregs in a murine model of asthma greatly exacerbated symptoms (128). This was connected to a decrease in IL-12 (a Th1-promoting cytokine) and an increase in IL-13. These data suggest that Tregs in respiratory disease can promote Th1 responses, a finding which is in agreement with our data.

IFN-γ has also been shown to suppress immune responses in asthma. One study in humans found that asthmatic individuals had lower levels of IFN-γ as measured by ELISA (129). Another study found that asthma treatments that decreased airway hyperreactivity and inflammation in mice were correlated with increased levels of IFN-γ (130). In addition, one study showed decreased asthmatic symptoms when mice were treated with IFN-γ and macrophage-activating lipopeptide-2 (131). Finally, in two separate experiments, systemic administration of an IFN-γ-expressing plasmid reduced asthma symptoms in mice (120, 121). Thus, the role of IFN-γ as a Th2-suppressing cytokine in respiratory disease models is well supported.

IL-17 has recently been shown to have beneficial effects in both asthma and respiratory infections. Bai et al. demonstrated that IL-17 could promote Th1 responses in *Chlamydia muridarum* lung infection (47). Neutralization of IL-17 in this model led to an increased Th2 response and an increase in disease. The authors also noted an effect on DC function, where IL-17 neutralization led to the development of IL-10-secreting DCs that were unable to protect mice from infection after adoptive transfer. The role of IL-17 in asthma is complex, but one study showed that IL-17 neutralization in a mouse model of asthma led to exacerbation of disease (132). Another study found that adoptive transfer of IL-17-producing γδ T cells accelerated the resolution of asthma-related airway inflammation and airway hyperreactivity in a mouse model, while blockade of γδ T cells exacerbated disease (133). Studies by Sieve et al. have demonstrated a role for IL-17 in mycoplasma infection (48). Mycoplasma CFU were increased in IL-17R$^{-/-}$ mice compared to wild type mice, though this infection was taken to a later time point. In another study, blockade of IL-23 (a cytokine capable of stimulating IL-17 production by T cells) in a murine model of acute *Mycoplasma pneumoniae* infection led to higher lung CFU at 24

hours post-infection (109). Thus IL-17 has been previously shown to play a beneficial role in models of respiratory disease.

The secretion of IFN-γ and IL-17 by Tregs has been previously observed in a few instances. In a mouse model of experimental autoimmune encephalomyelitis (EAE), a population of Tregs expressing both IFN-γ and IL-17 was observed infiltrating into the central nervous system, though their role in disease was unclear (53). Another study found that human Tregs could secrete IL-17 when stimulated *in vitro* with IL-1β and IL-6, though they were not found to occur in the host in response to any natural disease (57). A number of other studies have shown that human Tregs can secrete IL-17 after stimulation with cytokines (54-56), but none have shown that these Tregs respond to any foreign antigens, or that they play a role in any specific disease. Finally, another mouse model showed that Tregs could secrete IL-17 when stimulated *in vitro* with TGF-β and IL-6, though it is unknown if these cells retained FoxP3 expression, or if they merely differentiated into Th17 cells (50). To our knowledge this is the first time that IFN-γ^+ or IL-17^+ Tregs have been found in an animal with a natural disease, the first time that IFN-γ^+ or IL-17^+ Tregs have been shown to have specificity for a foreign antigen, and the first time that this type of Treg has been shown to play a role in the course of the disease.

Transfer of Tregs from naïve spleens has been successful in dampening immune responses in other models (116-119). However, adoptive transfer of Tregs from the spleens of naïve mice was ineffective in preventing mycoplasma respiratory disease in our model. In this case, the problem may be due to the fact that the Treg phenotype (IFN-γ^+ or IL-17^+) does not appear to be the natural or default state for Tregs. Rather, traditional Tregs express IL-10 and/or TGF-β (2, 89, 90). The development of the IFN-γ^+ or IL-17^+ phenotype may be a consequence of *in vivo* Treg activation in the specific cytokine environments that result from *M. pulmonis*

infection. As such, naïve Tregs may not be primed for this kind of activity, and thus may not have an effect when adoptively transferred. Another possibility is that the spleen may not be the source of the Tregs that participate in this immune response. It is not yet known where these Tregs originate, and it is plausible that they may not come from the spleen at all. It is also uncertain as to whether these Tregs develop from natural Tregs or whether they are induced (though their expression of CD25 suggests that the former is true (2, 14)). Thus, the inability of naïve splenic Tregs to suppress immune responses *in vivo*, as has been seen in other models(116-119) provides further evidence that our model involves a unique Treg population.

Adoptive transfer of Tregs from LRNs of infected mice actually led to increases in disease. In this case, disease actually occurred at a much earlier time point (by day 3 post-infection). This may be due to an issue involving the timing of Treg administration. Treg depletion experiments suggested that these cells may not play a role until around days 5-7 post-infection, around the time at which the adaptive immune response begins. During the innate period, endogenous Tregs may develop into IFN-γ-secreting or IL-17-secreting Tregs, and thus might not promote anti-Th2 responses until midway through the infection. Introduction of exogenous IFN-γ-secreting or IL-17-secreting Tregs prior to infection may actually be harmful to the host, possibly causing Th1 or Th17-mediated inflammation within a few days. In other words, if the host has not yet begun to mount a competing Th2 response, the introduction of Th1-promoting cells may be detrimental. This increased disease could also be exacerbated by the high number of cells transferred into recipients. Mice were given 10^6 Tregs from infected hosts. It is unknown how long these high Treg levels persist in the host animal, or how many of them actually have an effect at the primary infection site or the corresponding draining lymph nodes, but these studies have shown that Tregs normally make up 3-5% of the $CD4^+$ T cell population

in the LRNs of infected mice (less than 2.5 x 10^5 cells). Future studies should be performed to determine if any protection from disease can be achieved by transfer of Tregs at a later point in the infection, and/or by transfer of a smaller number of Tregs. Adoptive transfer of Tregs from infected LRNs may still be a viable strategy for future studies, but the number of Tregs administered and the timing of the administration need to be optimized.

There are a number of studies that can be performed to better understand the role of Tregs and their associated cytokines in this model. Future studies can focus on three specific areas: 1) The role of IFN-γ and IL-17 in the dampening of Th2-associated immunopathology in mycoplasma-infected mice, 2) The effect of Tregs on dendritic cell (DC) function in mycoplasma respiratory disease, and 3) the development of IFN-γ^+ or IL-17^+ Tregs in response to cytokine stimulation and mycoplasma antigen.

The mechanisms through which Tregs in mycoplasma-infected mice exert their suppressive effects need to be studied further to show conclusively that IFN-γ and IL-17 are involved in the dampening of Th2-mediated immunopathology. First, coculture experiments could be repeated with Th cells and Tregs from the LRNs of infected mice in order to determine the sources of IFN-γ and IL-17. While it is likely that the Th cells in the cultures are being promoted to secrete these cytokines, it is also possible that culture conditions are activating Tregs to a very high level, causing them to secrete large amounts of IFN-γ or IL-17. To establish the true source of these cytokines, Tregs could be labeled with a dye such as CFSE before being placed in culture. After MMA stimulation and incubation, cells could be stained for IFN-γ and IL-17 and analyzed using flow cytometry. If Tregs are secreting the bulk of these two cytokines, then IFN-γ and IL-17 would co-stain strongly with CFSE.

Another possible study would make use of Th cells from IFN-$\gamma R^{-/-}$ and IL-$17R^{-/-}$ mice. Tregs from wild type, mycoplasma-infected mice could be cocultured with IFN-$\gamma R^{-/-}$ or IL-$17R^{-/-}$ Th cells from both naïve and mycoplasma-infected mice. If the promotion of Th cytokine secretion involves the secretion of IFN-γ or IL-17 by Tregs, then coculture of these Tregs with IFN-$\gamma R^{-/-}$ or IL-$17R^{-/-}$ Th cells should not lead to the previously seen increases in the secretion of these two cytokines. If the IFN-γ or IL-17 levels still increase, this may suggest that Tregs promote secretion of these cytokines though a different mechanism.

Another approach would involve the treatment of Tregs with siRNA against IFN-γ or IL-17. These Tregs could be isolated from mycoplasma-infected mice, treated with siRNA *in vitro*, and then transferred into recipient mice. Recipients could be infected with *M. pulmonis* either before or after adoptive transfer of these Tregs. This approach has the advantage of allowing the Tregs to develop during the course of a normal infection prior to blockade of the target cytokine. Thus, this approach is preferable to the isolation of Tregs from IFN-$\gamma^{-/-}$ or IL-$17^{-/-}$ mice, since the overall absence of these cytokines in the host could result in an environment that does not produce the needed Treg phenotype.

The effect of Tregs on this immune response may also be related to the function of DCs. In addition to the study by Bai et al.(47), a number of other studies have shown that Tregs exert an effect on DCs. Tadokoro et al. demonstrated that Tregs in draining lymph nodes could prevent the formation of stable contacts between DCs and Th cells (134, 135). Experiments from our lab showed that MMA-pulsed DCs that were adoptively transferred into recipient mice exacerbated disease during subsequent mycoplasma infection (124). This suggests that interference with antigen presentation could be an effective strategy for prevention of mycoplasma respiratory disease. Thus, it is possible that Tregs in *M. pulmonis* infections serve to suppress Th2 responses

by affecting the interactions of DCs and Th cells. This could be examined using two-photon microscopy techniques, though this is difficult.

Tregs could also suppress immune responses by delaying the development of and expansion of Th cell populations. Recent work by Shafiani et al. demonstrated that Tregs in a model of *Mycobacterium tuberculosis* infection actually delayed the infiltration of antigen-specific Th cells into the lungs by several days (136). In another experiment, interaction of DCs with Tregs caused the DCs to home to lymph nodes (137). In our studies, depletion of Tregs prior to infection resulted in a significant increase in total cells into the lungs by day 14 as compared to non-depleted mice. However, the number of total cells in the LRNs remained the same regardless of the level of Tregs. This could be the result of delayed activation and delayed expansion of Th cells as a result of Treg action. Tregs might normally act in the LRNs to block or slow Th activation, but, in their absence, Th cells might rapidly proliferate and travel to the primary site of infection. Future studies could easily be performed following the infection to a later time point. If Tregs are simply delaying the development of disease in this way, then non-depleted, infected mice should eventually develop a similar level of disease.

It is more likely, though, that Tregs are promoting Th1-type responses by promoting the development of Th1-associated DCs. As stated previously, studies in an asthma model revealed that Treg depletion led to the development of DCs that promoted the secretion of Th2 cytokines (128). The previously mentioned study by Bai et al. demonstrated that Treg depletion in *Chlamydia* lung infection enhanced the development of IL-10-expressing DCs (47). It is already known through previous experiments in our lab that DCs can significantly exacerbate disease when cultured in the presence of Th2 cytokines (124). DCs cultured in Th1-polarizing conditions caused no such increase in disease. Tregs secreting and promoting the secretion of IL-17 and

IFN-γ could create an environment in the LRNs that favors the development of Th1-associated DCs. These Th1 DCs could then stimulate the development of antigen-specific Th1 cells, which could then act against Th2 responses.

One approach to evaluate these ideas is to coculture Tregs from mycoplasma-infected mice with MMA-pulsed DCs from naïve mice, with and without Th cells from infected animals. These DCs could then be adoptively transferred into recipient mice that would then be infected with *M. pulmonis*. These mice would then be monitored for signs of disease, as well as processed for lung CFU. In addition, cells could be stained for IFN-γ and IL-13 to determine if the immune response is trending towards Th1 or Th2. If Tregs do promote the development of Th1-associated DCs, then transfer of these DCs into mice prior to infection should result in a lower level of disease, and possibly even a reduction in lung CFU. This experiment could also show whether Tregs require Th cells to promote this response, or whether Tregs alone are sufficient. In addition, DCs cultured under these conditions could simply be stained for their expression of IL-12 and IL-10, thus providing an indication of whether they are Th1 or Th2-associated.

The conditions under which IFN-γ^+ and IL-17^+ Tregs can develop are not clear. Presumably, this phenotype is the result of specific cytokine environments combined with stimulation by *M. pulmonis* antigens. If Tregs have evolved to dampen immune responses, it is possible that they may be more adaptable than once thought, developing according to their specific environment to allow for different kinds of suppression. Thus, in a Th2 environment, as is found in *M. pulmonis*-infected mice, Tregs may adapt to promote Th1 responses.

To study this aspect, future experiments could once again make use of IFN-$\gamma^{-/-}$ and IL-$4^{-/-}$ mice. Mice could be infected with *M. pulmonis*, and cells could be harvested at 14 days post-infection. Tregs could be stained for their expression of IL-10, IFN-γ, and IL-17, or sorted and

run through real time-PCR to measure mRNA levels of IL-10, IFN-γ, IL-17, and TGF-β. If Tregs develop an IL-17^{+} phenotype under Th2 conditions as hypothesized, then these cells, their cytokines, and their cytokine mRNA should be easily detected in cells from Th2-polarized IFN-γ$^{-/-}$ mice. Conversely, Tregs from IL-4$^{-/-}$ mice might express little to no IFN-γ or IL-17, appearing instead as traditional IL-10/TGF-β-expressing Tregs. The development of IFN-γ^{+} Tregs could obviously not be studied in this model, since Tregs from IFN-γ$^{-/-}$ mice would not express IFN-γ.

Another option, and a study that is currently ongoing in our lab, is to adoptively transfer Th1 or Th2 polarized Th cell lines into recipient mice, followed by infection with *M. pulmonis*. This could skew the cytokine environment towards Th1 or Th2 similar to the effect seen in IFN-γ$^{-/-}$ and IL-4$^{-/-}$ mice. The same studies could then be performed to evaluate the effect on the development of Tregs.

The data presented here, as well as the data from these proposed experiments, can contribute to science on two different fronts. First, these data further illuminate the mechanisms behind the immune response to mycoplasma. This is important because, in addition to causing several different diseases in both humans and animals, mycoplasma has been linked to exacerbation of a number of other diseases, and thus has a profound impact on health care as well as agriculture (20). The complexity of the immune response to mycoplasma, as well as the failure of numerous attempts to create vaccines, necessitates the further study of this pathogen. The results from our experiments will contribute to a greater understanding of mycoplasma diseases, which will hopefully lead to advancements in this field.

In terms of Treg research, the results presented here demonstrate that Tregs are capable of secreting IFN-γ and IL-17, two cytokines that can act against Th2 responses. This shows a plasticity and adaptability of Tregs that was previously unknown, and suggests that Tregs may be

able to undergo changes that allow them to suppress a number of different types of immune responses. Thus, the possibility exists that Tregs may play different roles in a number of diseases in which they were previously not thought to be involved. These experimental results combined with other research involving Tregs, IL-17, and IFN-γ, can lead to a greater understanding of Treg function, and eventually lead to the development of new Treg-mediated therapies for diseases of all kinds.

BIBLIOGRAPHY

1. McHugh, R. S. and E. M. Shevach. 2002. The role of suppressor T cells in regulation of immune responses. *J. Allergy Clin. Immunol.* 110: 693-702.

2. Bluestone, J. A. and A. K. Abbas. 2003. Natural versus adaptive regulatory T cells. *Nat. Rev. Immunol.* 3: 253-257.

3. Shevach, E. M. 2002. CD4+ CD25+ suppressor T cells: more questions than answers. *Nature Rev. Immunol.* 2: 389-400.

4. de la Rosa, M., S. Rutz, H. Dorninger, and A. Scheffold. 2004. Interleukin-2 is essential for CD4+CD25+ regulatory T cell function. *Eur. J. Immunol.* 34: 2480-2488.

5. Cao, X., S. F. Cai, T. A. Fehniger, J. Song, L. I. Collins, D. R. Piwnica-Worms, and T. J. Ley. 2007. Granzyme B and perforin are important for regulatory T cell-mediated suppression of tumor clearance. *Immunity* 27: 635-646.

6. Bach, J. F. 2003. Regulatory T cells under scrutiny. *Nat. Rev. Immunol.* 3: 189-198.

7. Modigliani, Y., A. Coutinho, P. Pereira, N. Le Douarin, V. Thomas-Vaslin, O. Burlen-Defranoux, J. Salaun, and A. Bandeira. 1996. Establishment of tissue-specific tolerance is driven by regulatory T cells selected by thymic epithelium. *Eur. J. Immunol.* 26: 1807-1815.

8. Nagahama, K., E. Nishimura, and S. Sakaguchi. 2007. Induction of tolerance by adoptive transfer of treg cells. *Methods Mol. Biol.* 380: 431-442.

9. Stephens, L. A., D. Gray, and S. M. Anderton. 2005. CD4+CD25+ regulatory T cells limit the risk of autoimmune disease arising from T cell receptor crossreactivity. *Proc. Natl. Acad. Sci. U. S. A.* 102: 17418-17423.

10. Ellner, J. J. 1981. Suppressor cells of man. *Clin. Immunol. Rev.* 1: 119-214.

11. Onizuka, S., I. Tawara, J. Shimizu, S. Sakaguchi, T. Fujita, and E. Nakayama. 1999. Tumor rejection by in vivo administration of anti-CD25 (interleukin-2 receptor alpha) monoclonal antibody. *Cancer Res.* 59: 3128-3133.

12. Strauss, L., C. Bergmann, M. Szczepanski, W. Gooding, J. T. Johnson, and T. L. Whiteside. 2007. A unique subset of CD4+CD25highFoxp3+ T cells secreting interleukin-10 and transforming growth factor-beta1 mediates suppression in the tumor microenvironment. *Clin. Cancer Res.* 13: 4345-4354.

13. Joosten, S. A. and T. H. Ottenhoff. 2008. Human CD4 and CD8 regulatory T cells in infectious diseases and vaccination. *Hum. Immunol.* 69: 760-770.

14. Wohlfert, E. and Y. Belkaid. 2008. Role of endogenous and induced regulatory T cells during infections. *J. Clin. Immunol.* 28: 707-715.

15. Krause, D. C. and M. F. Balish. 2004. Cellular engineering in a minimal microbe: structure and assembly of the terminal organelle of Mycoplasma pneumoniae. *Mol. Microbiol.* 51: 917-924.

16. Atkinson, T. P., M. F. Balish, and K. B. Waites. 2008. Epidemiology, clinical manifestations, pathogenesis and laboratory detection of Mycoplasma pneumoniae infections. *FEMS Microbiol. Rev.* 32: 956-973.

17. Woolard, M. D., L. M. Hodge, H. P. Jones, T. R. Schoeb, and J. W. Simecka. 2004. The upper and lower respiratory tracts differ in their requirement of IFN-gamma and IL-4 in controlling respiratory mycoplasma infection and disease. *J. Immunol.* 172: 6875-6883.

18. Freymuth, F., A. Vabret, J. Brouard, F. Toutain, R. Verdon, J. Petitjean, S. Gouarin, J. F. Duhamel, and B. Guillois. 1999. Detection of viral, Chlamydia pneumoniae and Mycoplasma pneumoniae infections in exacerbations of asthma in children. *J. Clin. Virol.* 13: 131-139.

19. Martin, R. J., M. Kraft, H. W. Chu, E. A. Berns, and G. H. Cassell. 2001. A link between chronic asthma and chronic infection. *J. Allergy Clin. Immunol.* 107: 595-601.

20. Dobbs, N. A., A. N. Odeh, X. Sun, and J. W. Simecka. 2010. The Multifaceted Role of T cell-mediated Immunity in Pathogenesis and Resistance to Mycoplasma Respiratory Disease. In *Current Trends in Immunology,* Vol. 10. N. Joseph ed. Research Trends, Kerala, India.

21. Cartner, S. C., J. R. Lindsey, J. Gibbs-Erwin, G. H. Cassell, and J. W. Simecka. 1998. Roles of innate and adaptive immunity in respiratory mycoplasmosis. *Infect. Immun.* 66: 3485-3491.

22. Jones, H. P., L. Tabor, X. Sun, M. D. Woolard, and J. W. Simecka. 2002. Depletion of CD8+ T cells exacerbates CD4+ Th cell-associated inflammatory lesions during murine mycoplasma respiratory disease. *J. Immunol.* 168: 3493-3501.

23. Keystone, E. C., D. Taylor-Robinson, M. F. Osborn, L. Ling, C. Pope, and V. Fornasier. 1980. Effect of T-cell deficiency on the chronicity of arthritis induced in mice by Mycoplasma pulmonis. *Infect. Immun.* 27: 192-196.

24. Janeway, C. A., P. Travers, M. Walport, and M. J. Shlomchik. 2005. Immunobiology: The Immune System in Health and Disease. 6th ed.ed.Garland Science Publishing, New York, NY.

25. Kindt, T. J., R. A. Goldsby, B. A. Osborne, and J. Kuby. 2007. Immunology. 6th ed.ed.W.H. Freeman and Co., New York, NY.

26. Vivier, E., D. H. Raulet, A. Moretta, M. A. Caligiuri, L. Zitvogel, L. L. Lanier, W. M. Yokoyama, and S. Ugolini. 2011. Innate or adaptive immunity? The example of natural killer cells. *Science* 331: 44-49.

27. O'Neill, L. A. 2006. How Toll-like receptors signal: what we know and what we don't know. *Curr. Opin. Immunol.* 18: 3-9.

28. Barber, G. N. 2011. Innate immune DNA sensing pathways: STING, AIMII and the regulation of interferon production and inflammatory responses. *Curr. Opin. Immunol.* 23: 10-20.

29. Magalhaes, J. G., M. T. Sorbara, S. E. Girardin, and D. J. Philpott. 2011. What is new with Nods? *Curr. Opin. Immunol.* 23: 29-34.

30. Purdy, A. K. and K. S. Campbell. 2009. Natural killer cells and cancer: regulation by the killer cell Ig-like receptors (KIR). *Cancer. Biol. Ther.* 8: 2211-2220.

31. Chevalier, N., D. Jarrossay, E. Ho, D. T. Avery, C. S. Ma, D. Yu, F. Sallusto, S. G. Tangye, and C. R. Mackay. 2011. CXCR5 Expressing Human Central Memory CD4 T Cells and Their Relevance for Humoral Immune Responses. *J. Immunol.*

32. Rosado, M. M., M. Scarsella, E. Pandolfi, S. Cascioli, E. Giorda, P. Chionne, E. Madonne, F. Gesualdo, M. Romano, C. M. Ausiello, M. Rapicetta, A. R. Zanetti, A. Tozzi, and R. Carsetti. 2011. Switched memory B cells maintain specific memory independently of serum antibodies: the Hepatitis B example. *Eur. J. Immunol.*

33. Nishimura, M., A. Murakami, Y. Hara, and T. Azuma. 2011. Characterization of memory B cells responsible for affinity maturation of anti- (4-hydroxy-3-nitrophenyl)acetyl (NP) antibodies. *Int. Immunol.* 23: 271-285.

34. Billingham, M. E. 1987. Cytokines as inflammatory mediators. *Br. Med. Bull.* 43: 350-370.

35. Saha, B., S. Jyothi Prasanna, B. Chandrasekar, and D. Nandi. 2010. Gene modulation and immunoregulatory roles of interferon gamma. *Cytokine* 50: 1-14.

36. Teixeira, L. K., B. P. Fonseca, B. A. Barboza, and J. P. Viola. 2005. The role of interferon-gamma on immune and allergic responses. *Mem. Inst. Oswaldo Cruz* 100 Suppl 1: 137-144.

37. Kuperman, D. A. and R. P. Schleimer. 2008. Interleukin-4, interleukin-13, signal transducer and activator of transcription factor 6, and allergic asthma. *Curr. Mol. Med.* 8: 384-392.

38. Deepak, P., S. Kumar Jr, D. Kishore, and A. Acharya. 2010. IL-13 from Th2-type cells suppresses induction of antigen-specific Th1 immunity in a T-cell lymphoma. *Int. Immunol.* 22: 53-63.

39. Sabat, R. 2010. IL-10 family of cytokines. *Cytokine Growth Factor Rev.* 21: 315-324.

40. Sabat, R., G. Grutz, K. Warszawska, S. Kirsch, E. Witte, K. Wolk, and J. Geginat. 2010. Biology of interleukin-10. *Cytokine Growth Factor Rev.* 21: 331-344.

41. Yoshimura, A., Y. Wakabayashi, and T. Mori. 2010. Cellular and molecular basis for the regulation of inflammation by TGF-beta. *J. Biochem.* 147: 781-792.

42. Chaudhury, A. and P. H. Howe. 2009. The tale of transforming growth factor-beta (TGFbeta) signaling: a soigne enigma. *IUBMB Life* 61: 929-939.

43. Shevach, E. M. 2009. Mechanisms of foxp3+ T regulatory cell-mediated suppression. *Immunity* 30: 636-645.

44. Strauss, L., C. Bergmann, and T. L. Whiteside. 2009. Human circulating CD4+CD25highFoxp3+ regulatory T cells kill autologous CD8+ but not CD4+ responder cells by Fas-mediated apoptosis. *J. Immunol.* 182: 1469-1480.

45. Pandiyan, P., L. Zheng, S. Ishihara, J. Reed, and M. J. Lenardo. 2007. CD4+CD25+Foxp3+ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4+ T cells. *Nat. Immunol.* 8: 1353-1362.

46. Boissonnas, A., A. Scholer-Dahirel, V. Simon-Blancal, L. Pace, F. Valet, A. Kissenpfennig, T. Sparwasser, B. Malissen, L. Fetler, and S. Amigorena. 2010. Foxp3+ T cells induce perforin-dependent dendritic cell death in tumor-draining lymph nodes. *Immunity* 32: 266-278.

47. Bai, H., J. Cheng, X. Gao, A. G. Joyee, Y. Fan, S. Wang, L. Jiao, Z. Yao, and X. Yang. 2009. IL-17/Th17 promotes type 1 T cell immunity against pulmonary intracellular bacterial infection through modulating dendritic cell function. *J. Immunol.* 183: 5886-5895.

48. Sieve, A. N., K. D. Meeks, S. Bodhankar, S. Lee, J. K. Kolls, J. W. Simecka, and R. E. Berg. 2009. A novel IL-17-dependent mechanism of cross protection: respiratory infection with mycoplasma protects against a secondary listeria infection. *Eur. J. Immunol.* 39: 426-438.

49. Hirota, K., B. Martin, and M. Veldhoen. 2010. Development, regulation and functional capacities of Th17 cells. *Semin. Immunopathol.* 32: 3-16.

50. Xu, L., A. Kitani, I. Fuss, and W. Strober. 2007. Cutting edge: regulatory T cells induce CD4+CD25-Foxp3- T cells or are self-induced to become Th17 cells in the absence of exogenous TGF-beta. *J. Immunol.* 178: 6725-6729.

51. Chen, Y., C. J. Haines, I. Gutcher, K. Hochweller, W. M. Blumenschein, T. McClanahan, G. Hammerling, M. O. Li, D. J. Cua, and M. J. McGeachy. 2011. Foxp3(+) Regulatory T Cells Promote T Helper 17 Cell Development In Vivo through Regulation of Interleukin-2. *Immunity* 34: 409-421.

52. Pandiyan, P., H. R. Conti, L. Zheng, A. C. Peterson, D. R. Mathern, N. Hernandez-Santos, M. Edgerton, S. L. Gaffen, and M. J. Lenardo. 2011. CD4(+)CD25(+)Foxp3(+) Regulatory T Cells Promote Th17 Cells In Vitro and Enhance Host Resistance in Mouse Candida albicans Th17 Cell Infection Model. *Immunity* 34: 422-434.

53. Esposito, M., F. Ruffini, A. Bergami, L. Garzetti, G. Borsellino, L. Battistini, G. Martino, and R. Furlan. 2010. IL-17- and IFN-gamma-secreting Foxp3+ T cells infiltrate the target tissue in experimental autoimmunity. *J. Immunol.* 185: 7467-7473.

54. Voo, K. S., Y. H. Wang, F. R. Santori, C. Boggiano, Y. H. Wang, K. Arima, L. Bover, S. Hanabuchi, J. Khalili, E. Marinova, B. Zheng, D. R. Littman, and Y. J. Liu. 2009. Identification of IL-17-producing FOXP3+ regulatory T cells in humans. *Proc. Natl. Acad. Sci. U. S. A.* 106: 4793-4798.

55. Ayyoub, M., F. Deknuydt, I. Raimbaud, C. Dousset, L. Leveque, G. Bioley, and D. Valmori. 2009. Human memory FOXP3+ Tregs secrete IL-17 ex vivo and constitutively express the T(H)17 lineage-specific transcription factor RORgamma t. *Proc. Natl. Acad. Sci. U. S. A.* 106: 8635-8640.

56. Kitani, A. and L. Xu. 2008. Regulatory T cells and the induction of IL-17. *Mucosal Immunol.* 1 Suppl 1: S43-6.

57. Beriou, G., C. M. Costantino, C. W. Ashley, L. Yang, V. K. Kuchroo, C. Baecher-Allan, and D. A. Hafler. 2009. IL-17-producing human peripheral regulatory T cells retain suppressive function. *Blood* 113: 4240-4249.

58. Belkaid, Y. and W. Chen. 2010. Regulatory ripples. *Nat. Immunol.* 11: 1077-1078.

59. Buckner, J. H. 2010. Mechanisms of impaired regulation by CD4(+)CD25(+)FOXP3(+) regulatory T cells in human autoimmune diseases. *Nat. Rev. Immunol.* 10: 849-859.

60. Singh, V. K., S. Mehrotra, and S. S. Agarwal. 1999. The paradigm of Th1 and Th2 cytokines: its relevance to autoimmunity and allergy. *Immunol. Res.* 20: 147-161.

61. Monick, M. M., L. S. Powers, I. Hassan, D. Groskreutz, T. O. Yarovinsky, C. W. Barrett, E. M. Castilow, D. Tifrea, S. M. Varga, and G. W. Hunninghake. 2007. Respiratory syncytial virus synergizes with Th2 cytokines to induce optimal levels of TARC/CCL17. *J. Immunol.* 179: 1648-1658.

62. Morishima, N., T. Owaki, M. Asakawa, S. Kamiya, J. Mizuguchi, and T. Yoshimoto. 2005. Augmentation of effector CD8+ T cell generation with enhanced granzyme B expression by IL-27. *J. Immunol.* 175: 1686-1693.

63. Shrestha, B. and M. S. Diamond. 2007. Fas ligand interactions contribute to CD8+ T-cell-mediated control of West Nile virus infection in the central nervous system. *J. Virol.* 81: 11749-11757.

64. Rottinghaus, E. K., B. Vesosky, and J. Turner. 2009. Interleukin-12 is sufficient to promote antigen-independent interferon-gamma production by CD8 T cells in old mice. *Immunology* 128: e679-90.

65. Kapp, J. A. and R. P. Bucy. 2008. CD8+ suppressor T cells resurrected. *Hum. Immunol.* 69: 715-720.

66. Wang, Y. M. and S. I. Alexander. 2009. CD8 regulatory T cells: what's old is now new. *Immunol. Cell Biol.* 87: 192-193.

67. Mudd, P. A., B. N. Teague, and A. D. Farris. 2006. Regulatory T cells and systemic lupus erythematosus. *Scand. J. Immunol.* 64: 211-218.

68. Flores-Borja, F., C. Mauri, and M. R. Ehrenstein. 2008. Restoring the balance: harnessing regulatory T cells for therapy in rheumatoid arthritis. *Eur. J. Immunol.* 38: 934-937.

69. Wang, H. B., J. B. Zhang, and L. Y. Chui. 2008. Identification of correlations between numbers of CD4+ CD25+ Treg cells, levels of sera anti-AChR antibodies and transfer growth factor-beta in patients with myasthenia gravis. *Zhonghua Yi Xue Za Zhi* 88: 1036-1040.

70. Sakaguchi, S., R. Setoguchi, H. Yagi, and T. Nomura. 2006. Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in self-tolerance and autoimmune disease. *Curr. Top. Microbiol. Immunol.* 305: 51-66.

71. Nomura, T. and S. Sakaguchi. 2005. Naturally arising CD25+CD4+ regulatory T cells in tumor immunity. *Curr. Top. Microbiol. Immunol.* 293: 287-302.

72. Curiel, T. J. 2007. Regulatory T-cell development: is Foxp3 the decider? *Nat. Med.* 13: 250-253.

73. Hori, S., T. Nomura, and S. Sakaguchi. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299: 1057-1061.

74. Oh, S. J., J. H. Kim, C. K. Min, and D. H. Chung. 2008. Role of type II NKT cells in the suppression of graft-versus-host disease. *Crit. Rev. Immunol.* 28: 249-267.

75. Born, W. K., M. Lahn, K. Takeda, A. Kanehiro, R. L. O'Brien, and E. W. Gelfand. 2000. Role of gammadelta T cells in protecting normal airway function. *Respir. Res.* 1: 151-158.

76. Shimizu, J., S. Yamazaki, T. Takahashi, Y. Ishida, and S. Sakaguchi. 2002. Stimulation of CD25(+)CD4(+) regulatory T cells through GITR breaks immunological self-tolerance. *Nat. Immunol.* 3: 135-142.

77. Wing, K., Y. Onishi, P. Prieto-Martin, T. Yamaguchi, M. Miyara, Z. Fehervari, T. Nomura, and S. Sakaguchi. 2008. CTLA-4 control over Foxp3+ regulatory T cell function. *Science* 322: 271-275.

78. Zhao, D., C. Zhang, T. Yi, C. L. Lin, I. Todorov, F. Kandeel, S. Forman, and D. Zeng. 2008. In vivo-activated CD103+CD4+ regulatory T cells ameliorate ongoing chronic graft-versus-host disease. *Blood* 112: 2129-2138.

79. Sather, B. D., P. Treuting, N. Perdue, M. Miazgowicz, J. D. Fontenot, A. Y. Rudensky, and D. J. Campbell. 2007. Altering the distribution of Foxp3(+) regulatory T cells results in tissue-specific inflammatory disease. *J. Exp. Med.* 204: 1335-1347.

80. Fu, S., A. C. Yopp, X. Mao, D. Chen, N. Zhang, D. Chen, M. Mao, Y. Ding, and J. S. Bromberg. 2004. CD4+ CD25+ CD62+ T-regulatory cell subset has optimal suppressive and proliferative potential. *Am. J. Transplant.* 4: 65-78.

81. Liu, W., A. L. Putnam, Z. Xu-Yu, G. L. Szot, M. R. Lee, S. Zhu, P. A. Gottlieb, P. Kapranov, T. R. Gingeras, B. Fazekas de St Groth, C. Clayberger, D. M. Soper, S. F. Ziegler, and J. A. Bluestone. 2006. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *J. Exp. Med.* 203: 1701-1711.

82. Bayer, A. L., J. Y. Lee, A. de la Barrera, C. D. Surh, and T. R. Malek. 2008. A function for IL-7R for CD4+CD25+Foxp3+ T regulatory cells. *J. Immunol.* 181: 225-234.

83. Apostolou, I., P. Verginis, K. Kretschmer, J. Polansky, J. Huhn, and H. von Boehmer. 2008. Peripherally induced Treg: mode, stability, and role in specific tolerance. *J. Clin. Immunol.* 28: 619-624.

84. Curotto de Lafaille, M. A. and J. J. Lafaille. 2009. Natural and adaptive foxp3+ regulatory T cells: more of the same or a division of labor? *Immunity* 30: 626-635.

85. Horwitz, D. A., S. G. Zheng, and J. D. Gray. 2008. Natural and TGF-beta-induced Foxp3(+)CD4(+) CD25(+) regulatory T cells are not mirror images of each other. *Trends Immunol.* 29: 429-435.

86. Belkaid, Y. 2008. Role of Foxp3-positive regulatory T cells during infection. *Eur. J. Immunol.* 38: 918-921.

87. Kretschmer, K., I. Apostolou, D. Hawiger, K. Khazaie, M. C. Nussenzweig, and H. von Boehmer. 2005. Inducing and expanding regulatory T cell populations by foreign antigen. *Nat. Immunol.* 6: 1219-1227.

88. Kretschmer, K., I. Apostolou, E. Jaeckel, K. Khazaie, and H. von Boehmer. 2006. Making regulatory T cells with defined antigen specificity: role in autoimmunity and cancer. *Immunol. Rev.* 212: 163-169.

89. Vignali, D. A., L. W. Collison, and C. J. Workman. 2008. How regulatory T cells work. *Nat. Rev. Immunol.* 8: 523-532.

90. von Boehmer, H. 2005. Mechanisms of suppression by suppressor T cells. *Nat. Immunol.* 6: 338-344.

91. Belkaid, Y., C. A. Piccirillo, S. Mendez, E. M. Shevach, and D. L. Sacks. 2002. CD4+CD25+ regulatory T cells control Leishmania major persistence and immunity. *Nature* 420: 502-507.

92. Walther, M., J. E. Tongren, L. Andrews, D. Korbel, E. King, H. Fletcher, R. F. Andersen, P. Bejon, F. Thompson, S. J. Dunachie, F. Edele, J. B. de Souza, R. E. Sinden, S. C. Gilbert, E. M. Riley, and A. V. Hill. 2005. Upregulation of TGF-beta, FOXP3, and CD4+CD25+ regulatory T cells correlates with more rapid parasite growth in human malaria infection. *Immunity* 23: 287-296.

93. Suvas, S., A. K. Azkur, B. S. Kim, U. Kumaraguru, and B. T. Rouse. 2004. CD4+CD25+ regulatory T cells control the severity of viral immunoinflammatory lesions. *J. Immunol.* 172: 4123-4132.

94. Belkaid, Y. 2007. Regulatory T cells and infection: a dangerous necessity. *Nat. Rev. Immunol.* 7: 875-888.

95. Lee, D. C., J. A. Harker, J. S. Tregoning, S. F. Atabani, C. Johansson, J. Schwarze, and P. J. Openshaw. 2010. CD25+ natural regulatory T cells are critical in limiting innate and adaptive immunity and resolving disease following respiratory syncytial virus infection. *J. Virol.* 84: 8790-8798.

96. Hardy, R. D., H. S. Jafri, K. Olsen, J. Hatfield, J. Iglehart, B. B. Rogers, P. Patel, G. Cassell, G. H. McCracken, and O. Ramilo. 2002. Mycoplasma pneumoniae induces chronic respiratory infection, airway hyperreactivity, and pulmonary inflammation: a murine model of infection-associated chronic reactive airway disease. *Infect. Immun.* 70: 649-654.

97. Lindsey, J. R. and H. Cassell. 1973. Experimental Mycoplasma pulmonis infection in pathogen-free mice. Models for studying mycoplasmosis of the respiratory tract. *Am. J. Pathol.* 72: 63-90.

98. Parker, R. F., J. K. Davis, D. K. Blalock, R. B. Thorp, J. W. Simecka, and G. H. Cassell. 1987. Pulmonary clearance of Mycoplasma pulmonis in C57BL/6N and C3H/HeN mice. *Infect. Immun.* 55: 2631-2635.

99. Cartner, S. C., J. W. Simecka, J. R. Lindsey, G. H. Cassell, and J. K. Davis. 1995. Chronic respiratory mycoplasmosis in C3H/HeN and C57BL/6N mice: lesion severity and antibody response. *Infect. Immun.* 63: 4138-4142.

100. Cartner, S. C., J. W. Simecka, D. E. Briles, G. H. Cassell, and J. R. Lindsey. 1996. Resistance to mycoplasmal lung disease in mice is a complex genetic trait. *Infect. Immun.* 64: 5326-5331.

101. Evengard, B., K. Sandstedt, G. Bolske, R. Feinstein, I. Riesenfelt-Orn, and C. I. Smith. 1994. Intranasal inoculation of Mycoplasma pulmonis in mice with severe combined immunodeficiency (SCID) causes a wasting disease with grave arthritis. *Clin. Exp. Immunol.* 98: 388-394.

102. Taylor, G., D. Taylor-Robinson, and G. W. Fernald. 1974. Reduction in the severity of Mycoplasma pneumoniae-induced pneumonia in hamsters by immunosuppressive treatment with antithymocyte sera. *J. Med. Microbiol.* 7: 343-348.

103. Johnston, C. L., A. D. Webster, D. Taylor-Robinson, G. Rapaport, and G. R. Hughes. 1983. Primary late-onset hypogammaglobulinaemia associated with inflammatory polyarthritis and septic arthritis due to Mycoplasma pneumoniae. *Ann. Rheum. Dis.* 42: 108-110.

104. Roifman, C. M., C. P. Rao, H. M. Lederman, S. Lavi, P. Quinn, and E. W. Gelfand. 1986. Increased susceptibility to Mycoplasma infection in patients with hypogammaglobulinemia. *Am. J. Med.* 80: 590-594.

105. Davis, J. K., J. W. Simecka, J. S. Williamson, S. E. Ross, M. M. Juliana, R. B. Thorp, and G. H. Cassell. 1985. Nonspecific lymphocyte responses in F344 and LEW rats: susceptibility to murine respiratory mycoplasmosis and examination of cellular basis for strain differences. *Infect. Immun.* 49: 152-158.

106. Davis, J. K., R. B. Thorp, P. A. Maddox, M. B. Brown, and G. H. Cassell. 1982. Murine respiratory mycoplasmosis in F344 and LEW rats: evolution of lesions and lung lymphoid cell populations. *Infect. Immun.* 36: 720-729.

107. Davis, J. K. and G. H. Cassell. 1982. Murine respiratory mycoplasmosis in LEW and F344 rats: strain differences in lesion severity. *Vet. Pathol.* 19: 280-293.

108. Sun, X., H. P. Jones, L. M. Hodge, and J. W. Simecka. 2006. Cytokine and chemokine transcription profile during Mycoplasma pulmonis infection in susceptible and resistant strains of mice: macrophage inflammatory protein 1beta (CCL4) and monocyte chemoattractant protein 2 (CCL8) and accumulation of CCR5+ Th cells. *Infect. Immun.* 74: 5943-5954.

109. Wu, Q., R. J. Martin, J. G. Rino, R. Breed, R. M. Torres, and H. W. Chu. 2007. IL-23-dependent IL-17 production is essential in neutrophil recruitment and activity in mouse lung defense against respiratory Mycoplasma pneumoniae infection. *Microbes Infect.* 9: 78-86.

110. Davidson, M. K., J. K. Davis, J. R. Lindsey, and G. H. Cassell. 1988. Clearance of different strains of Mycoplasma pulmonis from the respiratory tract of C3H/HeN mice. *Infect. Immun.* 56: 2163-2168.

111. Simecka, J. W. and G. H. Cassell. 1987. Serum antibody and cellular responses in LEW and F344 rats after immunization with Mycoplasma pulmonis antigens. *Infect. Immun.* 55: 731-735.

112. Overcash, R. G., J. R. Lindsey, G. H. Cassel, and H. J. Baker. 1976. Enhancement of natural and experimental respiratory mycoplasmosis in rats by hexamethylphosphoramide. *Am. J. Pathol.* 82: 171-190.

113. Pinson, D. M., T. R. Schoeb, J. R. Lindsey, and J. K. Davis. 1986. Evaluation by scoring and computerized morphometry of lesions of early Mycoplasma pulmonis infection and ammonia exposure in F344/N rats. *Vet. Pathol.* 23: 550-555.

114. Jones, H. P., L. M. Hodge, K. Fujihashi, H. Kiyono, J. R. McGhee, and J. W. Simecka. 2001. The pulmonary environment promotes Th2 cell responses after nasal-pulmonary immunization with antigen alone, but Th1 responses are induced during instances of intense immune stimulation. *J. Immunol.* 167: 4518-4526.

115. Zelenay, S., T. Lopes-Carvalho, I. Caramalho, M. F. Moraes-Fontes, M. Rebelo, and J. Demengeot. 2005. Foxp3+ CD25- CD4 T cells constitute a reservoir of committed regulatory cells that regain CD25 expression upon homeostatic expansion. *Proc. Natl. Acad. Sci. U. S. A.* 102: 4091-4096.

116. Watanabe, K., V. P. Rao, T. Poutahidis, B. H. Rickman, M. Ohtani, S. Xu, A. B. Rogers, Z. Ge, B. H. Horwitz, T. Fujioka, S. E. Erdman, and J. G. Fox. 2008. Cytotoxic-T-lymphocyte-associated antigen 4 blockade abrogates protection by regulatory T cells in a mouse model of microbially induced innate immune-driven colitis. *Infect. Immun.* 76: 5834-5842.

117. Wang, H., T. Peters, A. Sindrilaru, D. Kess, T. Oreshkova, X. Z. Yu, A. M. Seier, H. Schreiber, M. Wlaschek, R. Blakytny, J. Rohrbein, G. Schulz, J. M. Weiss, and K. Scharffetter-Kochanek. 2008. TGF-beta-dependent suppressive function of Tregs requires wild-type levels of CD18 in a mouse model of psoriasis. *J. Clin. Invest.* 118: 2629-2639.

118. Miao, C. H., B. R. Harmeling, S. F. Ziegler, B. C. Yen, T. Torgerson, L. Chen, R. J. Yau, B. Peng, A. R. Thompson, H. D. Ochs, and D. J. Rawlings. 2009. CD4+FOXP3+ regulatory T cells confer long-term regulation of factor VIII-specific immune responses in plasmid-mediated gene therapy-treated hemophilia mice. *Blood* 114: 4034-4044.

119. Kim, M. G., S. Y. Lee, Y. S. Ko, H. Y. Lee, S. K. Jo, W. Y. Cho, and H. K. Kim. 2011. CD4+ CD25+ regulatory T cells partially mediate the beneficial effects of FTY720, a sphingosine-1-phosphate analogue, during ischaemia/reperfusion-induced acute kidney injury. *Nephrol. Dial. Transplant.* 26: 111-124.

120. Hayashi, T., K. Maeda, K. Hasegawa, S. Nakai, T. Hamachi, and H. Iwata. 2002. Systemic administration of interferon-gamma-expressing plasmid reduces late allergic bronchitis in a mouse model of asthma. *Int. J. Exp. Pathol.* 83: 81-86.

121. Nakagome, K., K. Okunishi, M. Imamura, H. Harada, T. Matsumoto, R. Tanaka, J. Miyazaki, K. Yamamoto, and M. Dohi. 2009. IFN-gamma attenuates antigen-induced overall immune response in the airway as a Th1-type immune regulatory cytokine. *J. Immunol.* 183: 209-220.

122. Bodhankar, S., M. D. Woolard, X. Sun, and J. W. Simecka. 2009. NK cells interfere with the generation of resistance against mycoplasma respiratory infection following nasal-pulmonary immunization *J. Immunol.* 183: 2622-2631.

123. Bodhankar, S., X. Sun, M. D. Woolard, and J. W. Simecka. 2010. Interferon gamma and interleukin 4 have contrasting effects on immunopathology and the development of protective adaptive immunity against mycoplasma respiratory disease. *J. Infect. Dis.* 202: 39-51.

124. Dobbs, N. A. 2010. Setting Us Up to Fail: Pulmonary Dendritic Cells Promote Immunopathology During Mycoplasma Respiratory Disease.

125. McGuirk, P., S. C. Higgins, and K. H. Mills. 2010. The role of regulatory T cells in respiratory infections and allergy and asthma. *Curr. Allergy Asthma Rep.* 10: 21-28.

126. Ray, A., A. Khare, N. Krishnamoorthy, Z. Qi, and P. Ray. 2010. Regulatory T cells in many flavors control asthma. *Mucosal Immunol.* 3: 216-229.

127. Lloyd, C. M. and C. M. Hawrylowicz. 2009. Regulatory T cells in asthma. *Immunity* 31: 438-449.

128. Lewkowich, I. P., N. S. Herman, K. W. Schleifer, M. P. Dance, B. L. Chen, K. M. Dienger, A. A. Sproles, J. S. Shah, J. Kohl, Y. Belkaid, and M. Wills-Karp. 2005. CD4+CD25+ T cells protect against experimentally induced asthma and alter pulmonary dendritic cell phenotype and function. *J. Exp. Med.* 202: 1549-1561.

129. Yong, J., G. Q. Chen, B. Huang, and S. Wu. 2011. Correlation between the ratio of T-bet/GATA-3 and the levels of IL-4 and IFN-gamma in patients with allergic asthma. *Mol. Med. Report*

130. Srivastava, K., T. Zhang, N. Yang, H. Sampson, and X. M. Li. 2010. Anti-Asthma Simplified Herbal Medicine Intervention-induced long-lasting tolerance to allergen exposure in an asthma model is interferon-gamma, but not transforming growth factor-beta dependent. *Clin. Exp. Allergy* 40: 1678-1688.

131. Weigt, H., C. Nassenstein, T. Tschernig, P. F. Muhlradt, N. Krug, and A. Braun. 2005. Efficacy of macrophage-activating lipopeptide-2 combined with interferon-gamma in a murine asthma model. *Am. J. Respir. Crit. Care Med.* 172: 566-572.

132. Schnyder-Candrian, S., D. Togbe, I. Couillin, I. Mercier, F. Brombacher, V. Quesniaux, F. Fossiez, B. Ryffel, and B. Schnyder. 2006. Interleukin-17 is a negative regulator of established allergic asthma. *J. Exp. Med.* 203: 2715-2725.

133. Murdoch, J. R. and C. M. Lloyd. 2010. Resolution of allergic airway inflammation and airway hyperreactivity is mediated by IL-17-producing {gamma}{delta}T cells. *Am. J. Respir. Crit. Care Med.* 182: 464-476.

134. Belkaid, Y. and K. V. Tarbell. 2009. Arming Treg cells at the inflammatory site. *Immunity* 30: 322-323.

135. Tadokoro, C. E., G. Shakhar, S. Shen, Y. Ding, A. C. Lino, A. Maraver, J. J. Lafaille, and M. L. Dustin. 2006. Regulatory T cells inhibit stable contacts between CD4+ T cells and dendritic cells in vivo. *J. Exp. Med.* 203: 505-511.

136. Shafiani, S., G. Tucker-Heard, A. Kariyone, K. Takatsu, and K. B. Urdahl. 2010. Pathogen-specific regulatory T cells delay the arrival of effector T cells in the lung during early tuberculosis. *J. Exp. Med.* 207: 1409-1420.

137. Bayry, J., F. Triebel, S. V. Kaveri, and D. F. Tough. 2007. Human dendritic cells acquire a semimature phenotype and lymph node homing potential through interaction with CD4+CD25+ regulatory T cells. *J. Immunol.* 178: 4184-4193.