
UNIVERSITY OF NORTH TEXAS
HEALTH SCIENCE CENTER AT FORT WORTH
1970

Ivey, James A.,THE CHARACTERIZATION OF T CELL RESPONSES ALONG THE RESPIRATORY TRACT. Masters (Immunology). May, 2004. 63 pages. 2 tables, 19 figures. 55 titles.

ABSTRACT

This research converged on T cell responses to respiratory agents, tobacco smoke and allergens in humans, and T cell responses to an extracellular bacterium, *M. bovis*, in calves. In the first chapter, we will discuss the basic functions of the immune system. In the second chapter, we characterized the impact of tobacco smoke on T cell responses in atopic smokers and atopic nonsmokers. The results were inconclusive and the study was postponed until the proper allergens were found. We also characterized the T cell responses of calves infected with *Mycoplasma bovis* in the third chapter. The results showed an increase in T lymphocytes in the upper respiratory tract of infected calves, which correlated with sites of infection.

THE CHARACTERIZATION OF T CELL RESPONSES ALONG THE RESPIRATORY TRACT

James A. Ivey Jr. BS

APPROVED:

Major Professor

Committee Member

Committee Member

University Member

Chair, Department of Molecular Biology and Immunology

Dean, Graduate School of Biomedical Sciences

THE CHARACTERIZATION OF T CELL RESPONSES ALONG THE RESPIRATORY TRACT

THESIS

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

MASTER OF SCIENCE

By

James A. Ivey Jr.

ACKNOWLEDGMENT

First and foremost, I would like to bestow my humble gratitude to my mentor, Dr. Jerry Simecka, for his guidance and patience throughout my graduate career. In addition, I would like to thank Dr. Fling and Dr. Brown for their advice and assistance. I would like to also thank the members of the laboratory: Matthew Woolard, Xiangle Sun, and Wes Love.

I would also like to thank the members who served on my committee, Dr. Mary B. Brown, Dr. John Fling, and Dr. Ming-Chi Wu.

Lastly, I would like to thank my family for supporting me in my endeavor to grow spiritually and intellectually. I want to say a special thank you to my wife, the woman who owns my love and devotion. Thank you for your unconditional love, patience and support for a very long two years. Most importantly, I want to thank God for His mercy, grace, strength and love.

TABLE OF CONTENTS

CHAPTER I

INTRODUCTION

Ultimately, it is the immune system's response that will determine the direction and the outcome of an infection. Immune function becomes especially vital when in the context of the respiratory tract. Both the upper and lower respiratory tract are bombarded with insults from bacteria, viruses, and allergens every second of life. To acquire an understanding of how the immune response of the respiratory tract interacts with various insults would open doors to new or better vaccines and improve quality of life for individuals with lung disorders. In this study, we:

1) Characterized the impact of tobacco smoke on T cell responses in atopic smokers and atopic nonsmokers.
2) Characterized the T cell response of calves infected with *Mycoplasma bovis.*

The mucosal surface of the lungs is responsible for many tasks that include absorption, secretory functions, and protective barrier. Mucosal surfaces accomplish these tasks by maintaining an exceptional immune system that closely regulates the balance between responsiveness and hypo-responsiveness [1]. The immune system is comprised of both lymphatic and non-lymphatic tissues, effector molecules like antibodies, and messengers like cytokines. Through these measures, immunity can be acquired by two distinct responses of the immune system, the innate or the adaptive immune response.

The innate immune system is the mediator of the initial protection against invading organisms. Most of what is considered an innate response occurs in the epithelium and in the mucosal linings of the respiratory and the intestinal tract. What makes innate immunity so different from the adaptive response is the fact it is not specific. The innate response is the same every time it is stimulated by a bacteria or virus, enabling it to respond quickly and without discrimination. Another characteristic of innate immunity that makes it effective as a defense system is the components of innate immunity recognize structures of microbes that are necessary for the microbe's survival. Therefore, a mutation in these sites would make the innate system ineffective but may also render the microbe unable to infect and cause disease.

As mentioned before, the components of the innate system are the physical barriers like the epithelia and certain cell types in the tissues and circulation. The epithelium forms tight junctions that help prevent organisms from entering the host. The epithelium also has cells that secrete antibody like proteins that help combat microbes. Another cell type at the disposal of the epithelium is a unique group of T cells termed γδ-T cells. γδ-T cells are believed to be involved with the initial protection against invading microbes [2, 3]. γδ-T cells are found in low titers throughout the bodies of most organisms, excluding ruminants [3]. Other cells of the innate immune system include natural killer (NK) cells, macrophages, and neutrophils.

The adaptive immune response is very specific for a particular antigen. The primary cells of an adaptive response are the lymphocytes, B cells and T cells for example. The main properties of the adaptive immune system are its specificity, and its

ability to produce memory of prior exposure to an antigen. The adaptive immune response can be divided into two responses, the cell-mediated and the humoral. The humoral response is characterized by antibody production. The humoral response is responsible for the elimination of extracellular antigens as well as toxins. Antibodies are a secreted product of mature B cells. In short, a naïve B cell engages an antigen and becomes an activated or primed B cell. The B cell proliferates and undergoes clonal expansion, and depending on the cytokines produced by T helper cells (T_h), B cells will differentiate into a particular immunoglobulin (Ig) class expressing B cell. There are five classes of immunoglobulins, IgA, IgE, IgG, IgM, and IgD and each perform specific functions from hypersensitivity reactions to maintaining mucosal immunity. B cells also play an important role in T helper cell activation. The B cells can act as antigen presenting cells (APC), bringing antigen peptides to the T helper cells and aiding in their activation and maturation.

The second type of adaptive immunity is the cell-mediated response. This arm of the immune system is primarily responsible for mounting a response against intracellular pathogens. The main cells of the cell-mediated response are T cells. T cells can be divided into two phenotypes, $CD8^+$, or cytotoxic T lymphocytes (CTL), and $CD4^+$ or T helper cells (T_h). Briefly, the activation of T_h cells occurs inside a lymphatic organ, where APC's present antigen proteins to T_h cells. T_h cells need two signals to mature into effector T_h cells. The first signal is generated by ligation of the T cell receptor (TCR) and unique complexes on antigen presenting cells (APC). The second signal, or costimulatory signal, is delivered by CD28 engagement by B-7 (B7-1 or CD80 and B7-2

or CD86) molecules on APC. The APC must interact with a T cell long enough for the T cell to receive a strong signal. The result is the activation of a T cell capable of eliminating foreign pathogens. The T_h cell then leaves the lymphatic organ and patrols for a particular antigen. If and when it recognizes the pathogen, the T_h cell becomes fully activated. The cytokine interleukin 2 (IL-2) tells the T_h cell to proliferate. Depending on the type of antigen, genetic predisposition, or an environmental factor, certain cytokines are secreted and the T_h cell will become either a T_h1 or a T_h2 cell.

T_h1 cells secrete cytokines like IL-2, IFN-γ and TNF-α which activate phagocytic cells to kill ingested pathogens or enhance the production of antibodies that stimulate the ingestion of pathogens. This type of response is believed to help activate macrophages and cytotoxic T cells. T_h1 cells can also promote chronic inflammatory responses. The secretion of cytokines, IL-4, IL-5, IL-6, IL-10, and IL-13, characterizes a type 2 response. These secreted cytokines are responsible for blocking macrophage activation either by direct inhibition, or by functioning as antagonists of IFN-γ and therefore block the T cell-mediated activation of macrophages. Th2 cells and their cytokine secretions are also alleged to play pivotal roles in the induction of some types of inflammatory responses. This transpires when the Th2 cytokines activate antigen specific IgE antibodies that ultimately lead to the de-granulation of mast cells and eosinophils.

Cytotoxic T lymphocytes (CTL) or $CD8^+$ T cells play another role in the cell-mediated adaptive response. The activation is similar to a $CD4^+$ cell, but CTL need a stronger second signal. Once activated, the CTL will search out infected cells and kill them with two mechanisms. The CTL can release the granule proteins, perforin and

granzymes. Perforin is the protein that will form pores in infected cell membranes and allow the entry of granzymes (proteases). The aperture perforin also allows the inward movement of Ca^{2+} causing the infected cell to swell due to changes in osmotic pressure. Granzymes are responsible for the activation of the caspase cascade that will cleave several substrates, and instigate the target cell to undergo apoptosis. Infected cells may also be eradicated via the FAS-FAS-ligand interaction. This mechanism of attack involves the direct communication by receptors on the CTL and the infected target cell. The interface activates the caspase cascade that sends signals to the target cell to undergo programmed cell death.

While the immune system can be split into two distinct responses, the constant interactions between the innate system and the adaptive cannot be overlooked. The innate response is the first to respond, but adaptive immunity is there to aid and destroy anything that has slipped by the innate system. Another tie between the two responses is that the innate response provides the cytokines, antibodies and other signals that initiate the activation of adaptive immune cells. Therefore, the innate response provides costimulatory signals but also helps to guide the nature of the adaptive response. Inversely, T cells of the adaptive response aid the further stimulation of innate immune cells like macrophages. Thus, these two immune systems form an interactive and mutually supportive affiliation that mounts an effective and formidable defense to infectious agents.

This research focuses on T cell responses to respiratory agents, respiratory allergens in smoking and non-smoking humans, and T cell responses to an extracellular bacterium, *M. bovis*, in calves. While the two seem divergent in nature, the immune response elicited by both is believed to be centered on T cells. T cells are the conductors of the immune response, regulating the duration and the strength of the response [4]. T cells are implicated in most documented diseases caused by infectious pathogens. T cells may also be the foremost culprit of the allergic and inflammatory responses seen in asthma and allergies caused by tobacco smoke [5]. It is these reasons that it is imperative that T cell responses be characterized to determine a possible means to control the immune response and better control pathogenic agents.

CHAPTER II

Immunological Effects of Smoking on T helper Cell Responses

Summary: Tobacco use remains the leading preventable cause of death in the United States, causing more than 440,000 deaths each year [6]. Forty percent of all who are considered heavy smokers will die before they reach retirement age, as compared to only eighteen percent of nonsmokers [6-8]. There is hardly a part of the human body that is not affected by the chemicals in tobacco products. There are 1,000 known chemical agents in tobacco; more than half are classified as carcinogenic agents [9]. When a tobacco product is lit, the tobacco product is transformed into a toxic chemical factory. It is when these harmful agents enter the airways and begin damaging the body that the immune system is activated and attempts protection.

In this project, the area of interest was tobacco smoke, and its impact on the immune system. Tobacco smoke is a significant factor for many diseases in the respiratory tract of infectious, malignant, and inflammatory etiologies. The United States spends approximately $100 billion annually on tobacco related medical conditions that include lung cancer, heart disease and atherosclerosis, as well as chronic obstructive pulmonary disease (COPD), which may be caused by or augmented by the affects of tobacco smoke [10]. However, the direct effect of smoking on the immune response is still unclear. It is clear that tobacco smoke damages the epithelium of the upper and lower respiratory tract, altering the immune response [11]. It is these alterations that we believe to be a major contributor to the pathological symptoms of diseases, more

specifically, T helper cell responses. We utilized flow cytometry to measure cytokine production and cell surface molecules to determine the type of T cell response that occurs when a system is subjected to a particular allergen.

Problem/Hypothesis: It has been observed in the scientific and medical fields that smoking affects the immune system, especially within the lungs and respiratory tract. Smoking increases the risk of various diseases including infection, cardiovascular disease, cancer, asthma and COPD [10]. The contribution of tobacco smoke to the development of these diseases may, in part, be facilitated through suppressive or altered immune responses. For example, smoking was reported to decrease the activity of natural killer (NK) cells and to induce a failure of T cells to respond normally to antigens and allergens [9]. It was also reported that smoking decreases serum levels of most immunoglobulin classes, with the exception of IgE [12, 13]. The antibody IgE is usually found at low levels within blood serum. Despite the low serum levels, IgE is a potent mediator of the immediate hypersensitivity reactions that are responsible for the symptoms of hay fever, asthma, and in severe cases, anaphylactic shock. Thus, smoking may influence immune responses to respiratory allergens and contribute to the symptoms associated with allergic and inflammatory diseases.

T_h cells responses are a critical component in asthma and allergies. In allergic diseases, there is a polarization of the Th2 response and an increase in the production of type 2 cytokines (IL-4, IL-5, IL-10), which are involved in the production of IgE [9, 13]. IgE production ultimately will lead to inflammatory disease. It is observations like these

that led to the theory that the Th2 cytokine arsenal is responsible for the inflammation and pathogenesis associated with allergic diseases. However, recent studies suggest that Th1 cells may also contribute to the pathological responses associated with allergic diseases [14]. Focusing not only on the Th2 response, but also on total T helper cell responses will explicate an important component in the pathologic foundation of smoking-related diseases, in addition to other allergic and autoimmune diseases. We hypothesized that T helper cell responses against respiratory allergens are preferentially Th2 in nonsmokers but smoking either enhances these responses in smokers or generates mixed Th1/ Th2 responses.

Background: It is surprising that immunological studies on the effects of tobacco smoke on the human immune system are rare. It is known that smokers have an increased susceptibility and severity of medical problems both in the lower and upper respiratory tracts. Children of smokers that are exposed to environmental tobacco smoke (ETS) or passive smoke are at a higher risk of developing of childhood asthma and allergies, and at an increased risk of sudden infant death syndrome (SIDS) [15, 16]. This observation correlates to the theory that when the immune system is subjected to repeated insults by an allergen, there is an immune dysregulation that can exacerbate or facilitate the development of inflammatory diseases [17].

Research has shown that chemicals used in the making of tobacco products affect the ability of T lymphocytes to proliferate by inhibiting ribonucleotide reductase, and preventing the enzymatic reduction of ribonucleotides to deoxyribonucleotides [7, 18,

19]. Further, research also suggests that long term exposure to tobacco smoke impairs T cell signaling by depleting the IP-3-Sensitive Ca^{2+} stores [7]. These aberrations in turn hinder a T cells response to an antigen receptor ligation, weakening or altering the immune response. Numerous studies have linked tobacco smoking with the increase frequency of infections within the lungs of smokers compared to that of non-smokers [9, 15]. Asthma, for example, is an immune mediated problem of the airways in the lower respiratory tract, and smoking is believed to be a contributing factor to this disease [5, 15]. The incidence of asthma is not necessarily affected by smoking, but rather the severity and frequency of asthma attacks are increased in smokers and in the children of smokers. These studies indicate that smoking has an adjuvant effect after exposure to environmental allergens, which results in enhanced levels of IgE [20, 21]. The levels of IgE should correlate with a Th2 response, triggered by IL-4.

Since T helper cells are such a pivotal player in immunity, the modification of the intensity or the type of T helper response can have a significant effect on respiratory disease as mentioned earlier. It was believed for many years that the Th2 subset of the immune response was solely responsible for the abnormal allergic responses [5, 19]. Recent studies however, have begun looking at the effects the Th1 cells have on inflammatory diseases [20]. Studies that involved the examination of the severity of asthma in smokers show that smokers produce Th1 and Th2 responses to allergen stimulation [5, 15, 20, 21]. Even though the major percentage of total inflammatory cells recruited were of the Th2 subtype and the levels increased over a function of time and with successive challenges, there was still a significant amount of Th1 cytokines present.

Other studies have looked at the levels of IFN-γ in smokers. The results show that the levels of IFN-γ secreted by $CD4^+$ and $CD8^+$ cells in smokers with asthma are increased as compared to that of nonsmokers [14, 20, 22]. Further studies investigating T cells derived from the airways are required to render the importance of Th1 and Th2 cell cytokine production in the pathogenesis of inflammatory diseases. Understanding the cytokine production within atopic smokers and non-smokers with allergies to respiratory allergens will confer an understanding of how tobacco smoke suppresses, or over expresses pulmonary T cell responses.

The immune response to an allergen is influenced by the nature of the T helper subsets participating in the response. New techniques in this study were used to look at human T helper cell responses and give a better understanding of how tobacco smoke affects respiratory tract immunity of humans. Research of this nature can also be extended to the immunity against other respiratory agents and the pathogenesis of asthma. Thus, because both subsets of T helper cells may be involved in the pathogenesis of inflammatory diseases, understanding the cellular basis of the altered immune response is critical in the treatment of the symptoms and prevention of the diseases.

The purpose of this study was to further establish how smoking affects respiratory T helper cell responses against respiratory allergens in humans. We hypothesize that T helper cell responses against respiratory allergens are preferentially Th2-type in nonsmokers but smoking either enhances these responses or results in the generation of a mixed Th1 and Th2 response. By addressing these hypotheses, we attempted to

determine if T helper cell responses against respiratory allergens are different between smoking and nonsmoking atopic (allergic) subjects. We examined both T helper cell responses to respiratory allergens in smokers and non-smokers, and the level of T helper cytokine responses using flow cytometry.

Methods and Materials:

Subjects: Smokers with atopy (N=10), and non-smokers with atopy (N=10) were selected on basis of age and history of habitual tobacco use. Diagnosis of atopy was determined by a positive skin prick test against a battery of respiratory allergens comprised of trees, grass, pollen, weeds, and common household pets. A positive skin test was determined by a certain wheal diameter 15-min after allergen contact.

Cell Isolation: A blood sample (20 ml) collected from the subjects by peripheral venous puncture and diluted with wash media (RPMI supplemented with 5% fetal bovine serum). The blood was placed in Accuspin tubes (Sigma Aldrich, St. Louis, MO) containing Histopaque (specific gravity 1.077, Sigma), and centrifuged at 800 g for 20 min at 4°C, to separate the cells of the blood sample by their density. The mononuclear layer was aspirated and placed in (1X) HyQ RPMI 1640 supplemented with 2 mM L-Glutamine (HyClone, Logan Utah), antibiotic/antimytotic, HEPES (Sigma Aldrich) and 10% fetal bovine serum.

Lysing of RBC's was performed, if necessary, using BD FACS Lysing Solution (BD Pharmingen, San Diego, CA). The total number of viable cells was counted on hemacytometer.

An adjusted cell count of 2-4 x10 ^{6} cells per milliliter were divided into three parts: one for the intracellular staining, one for the cytometric bead array, and the last for freezing for future assays. The cells were stimulated with the particular allergen that gave the greatest reaction on the skin test. Tetanus toxoid (TT) was used as a control for the procedures. The stimulated cells were incubated in 5% CO_2 at 37° C, 24 hours for intracellular staining and 72 hours for the detection and characterization of cytokine levels in the cell supernatants

Intracellular Staining: To analyze the subject's T_h cell subset response, the cell surface was labeled with PerCp-conjugted anti-human CD3 antibody and FITC-conjugated anti-human CD4 antibody (Both 1:10, BD Pharmingen). Cell suspensions (200 µl) were incubated for 30 min. at 4°C with 20 µl of antibodies for cell surface markers. After the cells were washed with staining buffer (filtered PBS and 3% fetal calf serum); 500 µl of permeabilization solution (BD Pharmingen) was added to each tube for 20 min. in the dark. Cells were washed and 20 µl of anti-cytokine, PE-conjugated IL-4 (TH_2) or IFN-γ (TH_1) were added (Both 1:10, BD Pharmingen) for 30 min. at 4°C in the dark. PE, FITC, and PerCP-conjugated anti-mouse IgG1 antibodies were used as a negative control. The cells were then analyzed on the flow cytometer (Coulter EPICS XL/XL-MCL Module 1) to determine the cytokine environment of the subjects and to determine which T

lymphocyte subset the subjects' immune systems were primed towards. Electronic gates were set on a forward scatter and side scatter plot. 10,000 cells were computed in the gates and analyzed with the appropriate software. The number of cells stained for each cytokine was expressed as a percentage of $CD3^+$ and $CD4^+$ populations.

Cytometric Bead Array (CBA): BD Pharmingen developed a means to investigate many analytes from a small, single sample. By utilizing the capacity of flow cytometry, and combining it with the CBA assay resulted in a useful, multiplexed assay. The basic principle of the assay is similar to that of a sandwich style ELISA. Six bead populations, each with individual fluorescence intensities, were coated with capture antibodies that are specific for IL-2, IL-4, IL-6, IL-10, TNF-α and IFN-γ. These beads are termed the capture antibodies, because they are mixed with a PE-conjugated detection antibody, forming a sandwich complex when and if a cytokine is bound. After the cells were incubated in 96 well plates, they were washed with assay diluent (BD Pharmingen). Th1/Th2 cytokine standards were used to determine the sensitivity of the assay. Dilutions were made from a top standard of 5000 pg/ml to a negative control of 0 pg/ml. 50 µl of the cytokine standards and the PE detection reagent were added to the assay tubes. 50 µl of cytokine standards were added to the control assay tubes and 50 µl of each test sample was added to the appropriate test assay tubes. The tubes incubated at room temperature in the dark for 3 hours. The cells were washed with wash buffer (BD Pharmingen).

300 µl of wash buffer was added to the cell pellet and the samples were run on a flow cytometer. The results are analyzed on software provided by BD Pharmingen.

Statistical analysis: The data was evaluated by ANOVA, followed by Fisher protected least significant difference multigroup comparison or Tukey's multigroup comparison. These analyses were performed using the StatView (SAS Institute, Cary, NC) computer program. A p value of less than or equal to 0.05 ($p \leq 0.05$) was considered statistically significant. If the data was analyzed after logarithmic transformation, the standard error of the transformed data was used to present the data.

Results: The above experiment was designed to investigate the type of T cell responses within the lungs of smokers and non-smokers. Twenty subjects were evaluated and chosen based on their smoking history and on the results of the allergen skin test. Blood was drawn from the patients into 10 ml heparinized tubes. The blood was then processed and through density gradient separation, the lymphocytes were isolated. The cells were then washed and prepared for intracellular cytokine staining and stained for analysis of the cell supernatant cytokine levels.

The effects of allergens on the intracellular production of cytokines skewed towards a type 2 response. To investigate the type of immune response elicited by lung lymphocytes to allergen stimulation, cells were stimulated with either PHA for a positive control, TT, or an allergen that was specific for each subject. The cells were stained with PerCp-conjugated anti-human CD3 antibody and FITC-conjugated anti-human CD4

antibody for cell differentiation, and anti-cytokine, PE-conjugated IL-4 (TH_2) or IFN-γ (TH_1). The data showed an immune response that is associated with a Th2 immune response marked by a greater percentage of $CD4^+$ T cells stained positive for IL-4 *(Figure 1)*. The percentage of $CD4^+$ cells secreting IFN-γ was unusually high as compared to the percentage of cells secreting IL-4 (*Figure 1*). It was also observed that there was no significant difference between the results of smokers versus non-smokers.

To determine the cytokine levels in cell culture supernatants, cells were cultured in 96-well round-bottom plates. After 4 days in culture, lung lymphocytes supernatants were removed and analyzed on the flow cytometer. The results are interpreted by the populations of capture beads shifting along the FL2 (PE) axis. Levels of several Th1 cytokines IL-2, IL-6, IL-10, TNF-α, and IFN-γ. Levels of IL-2 were elevated indicating T cells were activated, and levels of TNF-α and IL-6, which are innate cytokines believed to play key roles in innate inflammation, were slightly elevated. Surprisingly, we were unable to detect much IL-4 in the culture supernatants. This could be due to the extended time for culture, or the different intensity of florescent beads for IL-4. In addition, a response by cell types other than T helper cells, natural killer cells (NK) for example, could account for this uncanny shift. Figure 2 shows the outcome of the assay showing a marginal level of IFN-γ over that of IL-4. There are numerous reasons for these findings; however, because of the allergen quandary no significant deductions can be made concerning this project and the results.

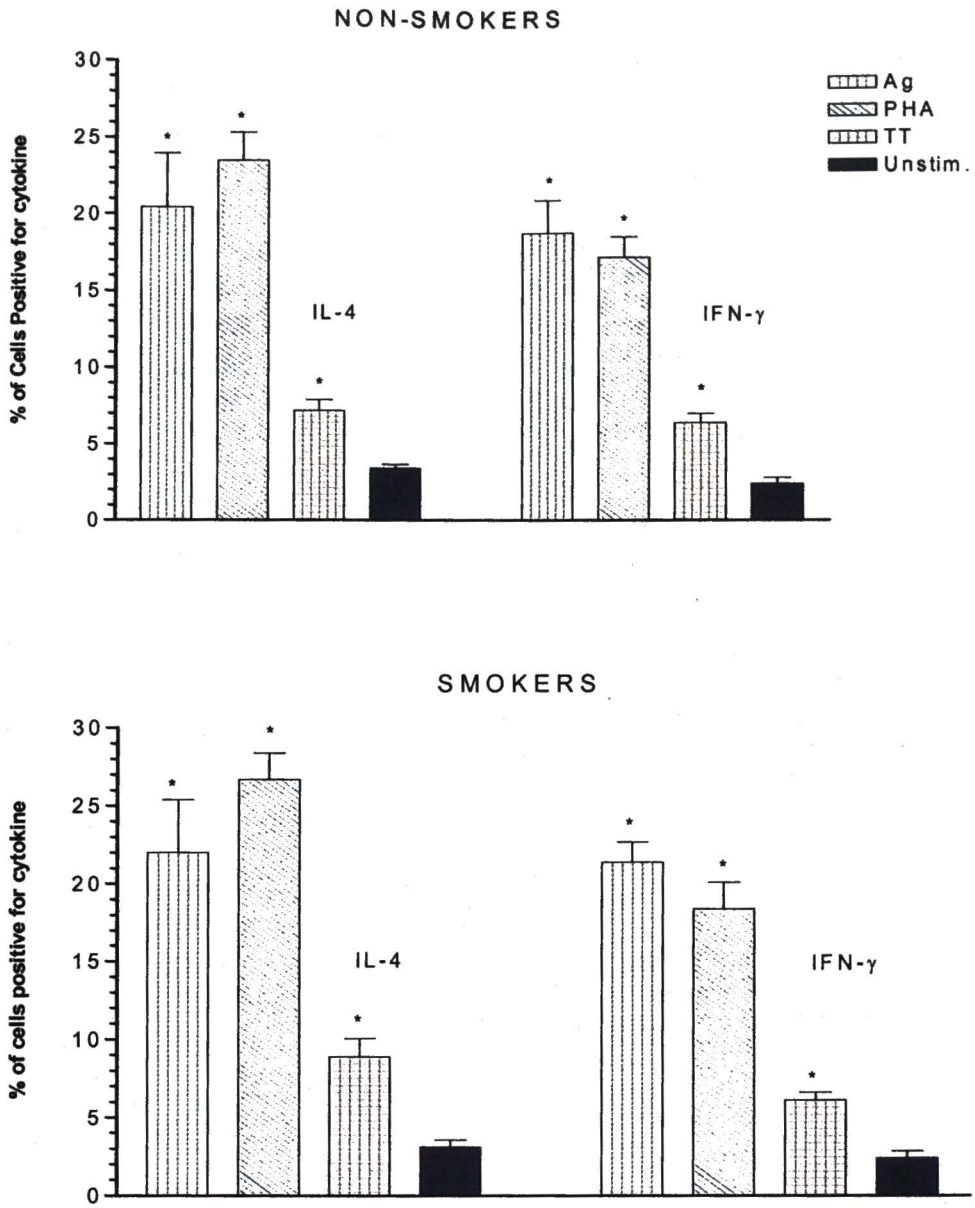

Figure 1. The results from the smoking and nonsmoking group's intracellular staining after 24 hour stimulation with an allergen (Ag), Tetanus toxoid (TT), PHA, or non-stimulated (Unstim.), showed that the allergen stimulated cells were skewing towards a Th2 response (increased levels of IL-4). There was no difference between smokers and nonsmokers. Results are represented as the percentage of $CD3^+/CD4^+$ cell detected positive for either IL-4 or IFN-γ.

(* denotes statistical significance.)

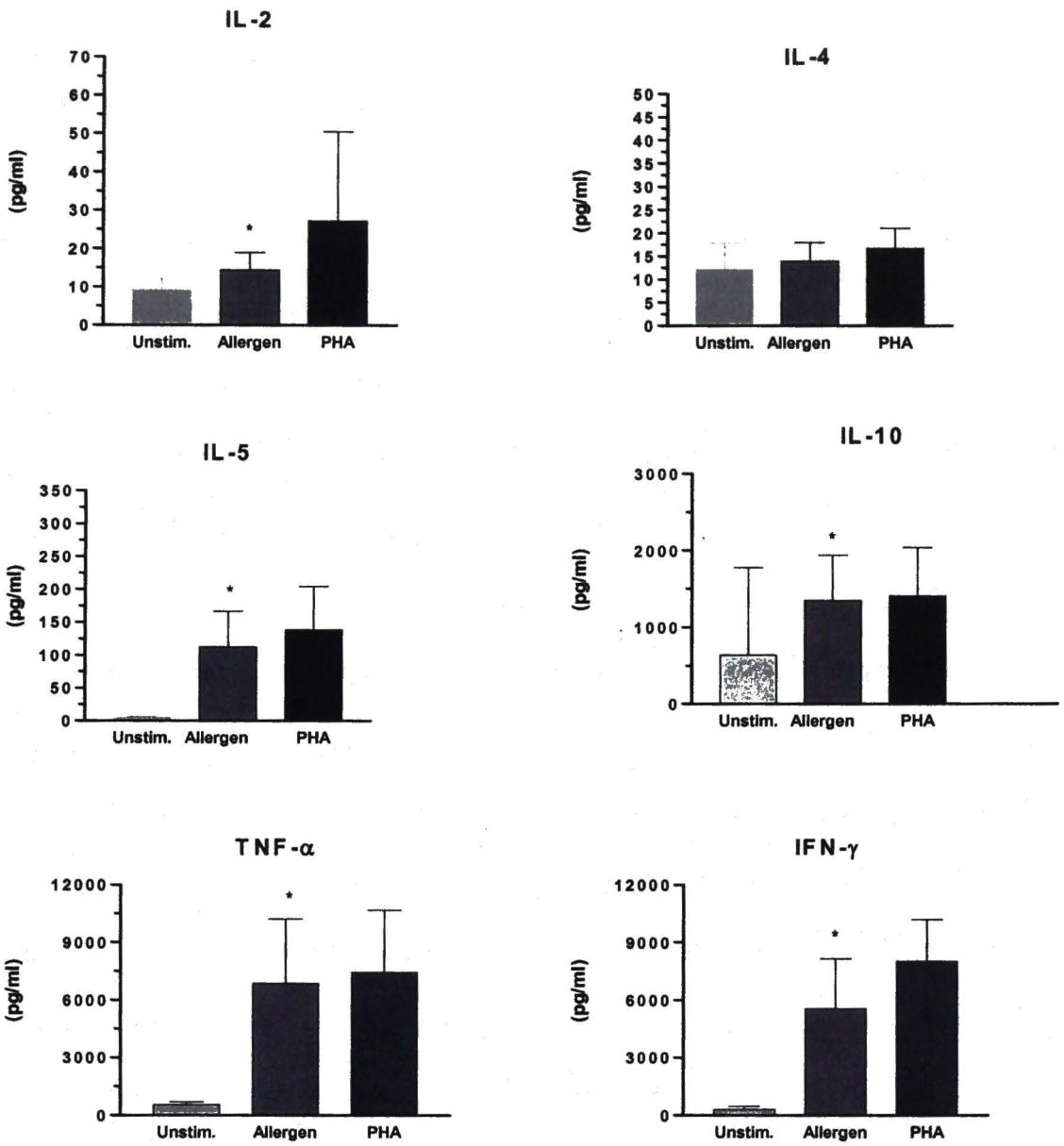

Figure. 2. Data represents results showing the varying levels of 6 cytokines in supernatant of cell cultures stimulated with a particular allergen, PHA and non-stimulated (Unstim). Cells were stimulated for four days then coated with capture antibodies that are specific for IL-2, IL-4, IL-6, IL-10, TNF-α and IFN-γ. Results are represented in pg/ml. Data suggests that the immune response is skewed towards a Th1 response (increase in IFN-γ). (* denotes statistical significance.)

Discussion: Tobacco smoke is linked to the increased susceptibility of adults and children to respiratory allergies, asthma and other forms of lung disease. Tobacco smoke damages the epithelium of the upper and lower respiratory tract, altering the immune response[11]. It is these alterations that are believed to be a major contributor to the pathological symptoms of these diseases, more specifically, T helper cell responses. The goal of this research was to determine if T helper cell responses against respiratory allergens are different between smokers and nonsmokers allergies. We were able to establish approaches to study human T helper cell responses when exposed to a known allergen. The atopic patients showed a strong Th2 response to the allergen stimulation, and a moderate Th1 response to the TT stimulation.

PHA was the polyclonal stimulator used to stimulate most lymphocytes, usually around 30-65% of T helper cells can be seen on the flow cytometer. Antigen specific stimulators, like the selected allergens that have a small, specific percentage of T helper cells, should be in the 5-10% percentile range. To our disappointment, the opposite effects transpired. The allergen was stimulating cells with almost the same efficacy as the polyclonal stimulator PHA in both the smoking and nonsmoking group. The allergen used was whole allergen extract that was commercially acquired. Therefore, there might have been a preservative or another impurity in the allergen preparation that was stimulating the cells in a non-specific manner. To test these observations, a subject's blood was stimulated with an appropriate allergen that was declared negative by their skin test results. The non-reactive allergen should have little to no stimulatory effects. The percentages should be comparable to that of the negative control.

The percentage of activated T cells for the non-reactive allergen however, was within 4% of the PHA stimulated cells (*Figure 3*). It was deduced that there was a protein, enzyme, or adjuvant within the allergen preparation activating T cells non-specifically. A dialysis technique was implemented in a hope to dialyze out any substance that might be stimulating the cells in an exaggerated manor. The samples were run on the flow cytometer and the results were the same (data not shown). It was determined that an allergen peptide, rather than a whole allergen extract was needed to stimulate the cells effectively.

Consistent with expectations, intracellular cytokine staining and staining of cell surface markers validates the protocol efficiency. Even though there was a non-specific activation of T cells, the actual staining protocol was successful. However, the results for cytokine levels in culture supernatants demonstrate that there is further need to validate both approaches to evaluate T_h cell cytokine responses against allergens. Overall, the experiment did allow the refinement of the techniques used in intracellular and cell surface staining, which will be utilized in the following project.

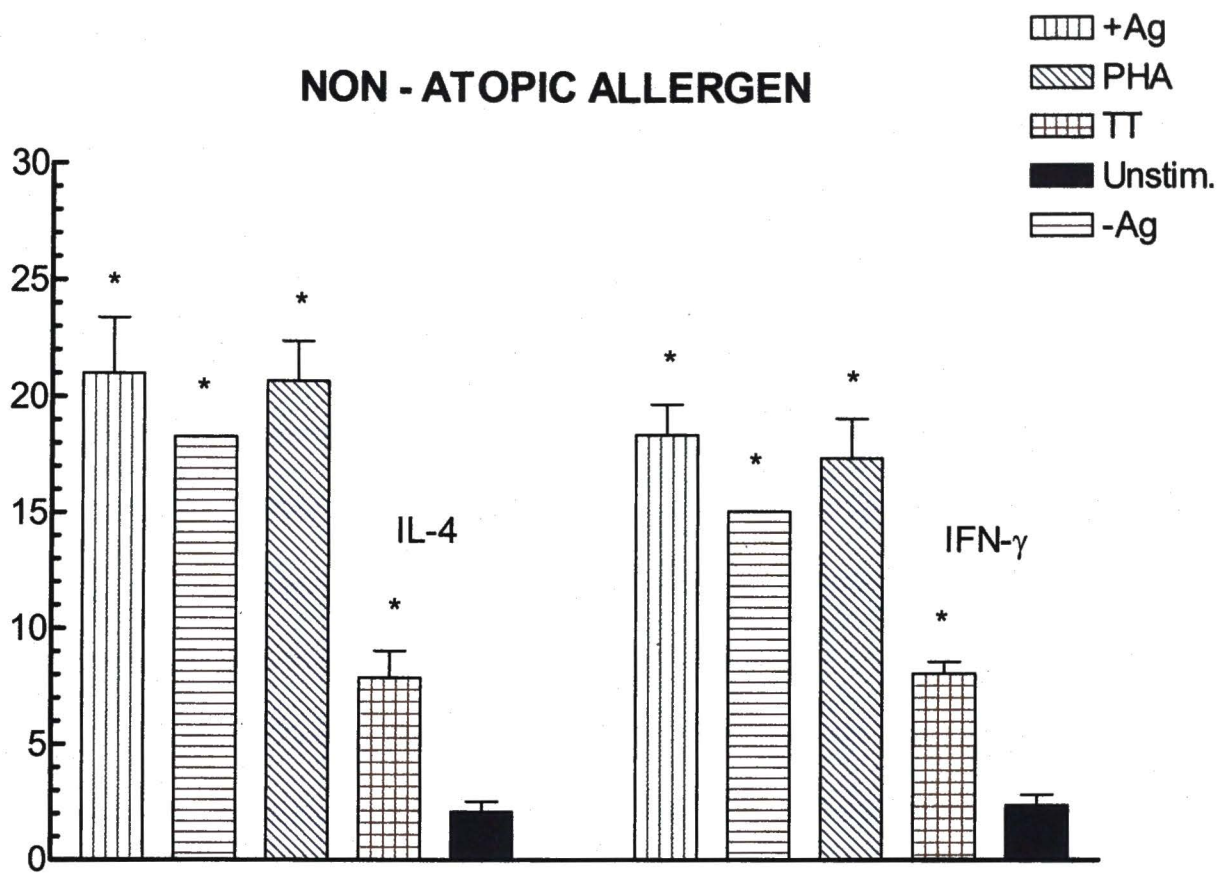

Figure 4. The results from the stimulation with an allergen that a subject tested negative. The allergen should have had no stimulatory effect. Intracellular staining after 24 hour stimulation with an allergen (Ag), a non-atopic allergen (-Ag), Tetanus toxoid (TT), PHA, or non-stimulated (Unstim.), showed that the non-atopic allergen stimulated cells non-specifically. Results are represented as the percentage of $CD3^+/CD4^+$ cell detected positive for either IL-4 or IFN-γ. (* denotes statistical significance.)

CHAPTER III

Response of T Cell Populations Along the Respiratory Tract of Calves Infected with *Mycoplasma bovis*

Summary: The objective of this research project was to study changes in T cell populations in calves when challenged with *Mycoplasma bovis*. *M. bovis* is responsible for a number of diseases in cattle including respiratory disease, mastitis and arthritis [23, 24]. Research done in these areas suggests that both the innate and adaptive immune systems are involved in the diseases caused by *M. bovis*.

Distinct T cell populations control both the innate and adaptive immune responses. Each T cell population, ($CD4^+$, $CD8^+$, and $\gamma\delta$-T cells), performs unique tasks that establish protection against foreign substances. Previous research in both mice and cattle suggest that numerous T cell populations play antagonizing roles in the progression of the disease [4, 25, 26]. This research characterized T cell subset responses against *M. bovis*. Calves were infected with *M. bovis* round their nine to eleventh day of life. Blood was drawn from the calves at days zero, three, seven, and fourteen post infection (p.i.). Necropsies were performed on the fourteenth day. Lymph nodes from the lower and upper respiratory tract were removed along with sections of the lung and spleen. Lymphocytes were isolated and stained for cell surface markers distinguishing the T cell populations. After staining, the samples were run on the flow cytometer and the data analyzed. The changes in T cell subset(s) that occur subsequent to mycoplasma infection indicated there are T cells responding to an infection in the respiratory tract and may be

playing the foremost role in the disease process. Improved understanding of the host immune response will help establish treatments and preventable measures against mycoplasma diseases. This information will also provide working knowledge of the relationship between mycoplasma and lung pathologies.

Problem/Hypothesis: Mycoplasmas are an amazing species of bacteria because until recently, they have eluded nearly every effort to establish a relationship with their host. Their biological properties are believed to challenge the host in "new" ways compared to other species of bacteria [27]. Mycoplasmal species are the etiological agents of many human, murine, and bovine respiratory diseases. New controversial research suggests mycoplasmas plays a role in diseases like SARS, and even AIDS pathogenesis [23]. The current research project investigated *M. bovis*, a pathogen of beef and dairy cattle. *M. bovis* causes pneumonia, arthritis, and other diseases in dairy calves [24]. With the ability of *M. bovis* to infect and cause serious disease in cattle and cause detrimental effects on the economic stability of the dairy and cattle industry, it is imperative a vaccine or treatment be developed to battle and prevent an infection with *M. bovis*. The predicament facing immunologists now is a gap in knowledge concerning the bovine immune response to mycoplasmal infection. As with most diseases, immunological responses have a major impact on the progression and severity of the disease.

We hypothesize that the principal population (s) involved in combating the disease along the respiratory tract are $\alpha\beta$- T cells, mainly the $CD4^+$ phenotype, but $\gamma\delta$-T cells are also a major component. Furthermore, the antigen specific antibody response is skewed towards the $CD4^+$ type.

Significance: *M. bovis* is a major contributor to the morbidity and mortality in dairy cattle in the United States [24, 28]. *M. bovis*, which can eventually lead to respiratory disease, causes significant economic loss in the dairy and cattle industry. In recent years, researchers have investigated the incidence of *M. bovis* among cattle herds. Outbreaks of respiratory disease caused by *M. bovis* can result in losses of 30% of calves and diminished health of the surviving calves [29]. The cost of replacing calves and heifers due to *M. bovis* outbreaks constitutes a significant economic loss to the industry. Therefore, improved measures to control the spread and infection of *M. bovis* infection would greatly benefit the dairy and cattle industry.

Understanding the immunological aspects of the infection will not only give insight to how *M. bovis* interacts with the calves immune system, but also may serve as a template for how the immune response within the lungs interacts in other respiratory diseases. As stated earlier, T cells are central in the regulation of an immune response. T cells determine a balance between beneficial and detrimental effects of the immune system in diseases like asthma, arthritis, allergies in human and animal models, and in this study, mycoplasma infection in cattle.

The two main populations of T cells are the $CD4^+$ T helper (T_h) cells and $CD8^+$ cytotoxic T lymphocytes (CTL). These populations are characterized by their function and by the cytokine arsenals predominantly produced. In many diseases, including mycoplasma diseases, the $CD4^+$ T_h and CTL populations play opposing roles. $CD4^+$ T_h cells increase the severity of the disease and $CD8^+$ T cells dampen the disease [4]. The balance between the groups is believed to be the cause of and the solution to many immune functions. There have been attempts to further characterize the roles of the innate and adaptive immune responses in respiratory diseases caused by mycoplasmas. However, the data is incomplete and replete with speculation.

Another group of T cells that has recently been discovered is the γδ-T cells. A number of functions have been accredited to this group of T cells. Early in life, γδ T cells are believed to play a protective role in immunity [2, 3]. Other studies have shown that these cells are pivotal in the inflammatory response to allergens and other bacterial and viral insults [30, 31]. In this study, we determined the response of γδ T cells to M. *bovis*. Characterization of the response of this elusive T cell subset will provide a better understanding of their specific role in the immune response within cattle.

This is the first study that defines the changes in lymphocyte population along the respiratory tract in calves infected with *M. bovis*. The investigation of T cell responses to a *M. bovis* infection will help to understand the features and direction of the immune response involved with a mycoplasma infection. Overall, this study facilitated our understanding of the activation and response of T cell populations.

Background: Mycoplasmas are unusual bacteria, and most of the time an overlooked species of bacteria [23]. Mycoplasmas are wall-less, gram positive, prokaryotes that colonize a diverse range of hosts and are considered the smallest self-replicating organisms known. Mycoplasmas have been grouped into the class Mollicutes (soft cutis, skin); because they lack a true cell wall, but most do have a sterol containing plasma membrane [27]. Since the earliest date in the 1930's when mycoplasma was identified as a human pathogen, mycoplasma have been implicated in the cause of pneumonia and other airway pathologies like bronchitis and pharyngitis [3, 4, 23-27, 32-34]. Mycoplasmas are also associated with disorders of the joints and central nervous system.

Mycoplasmas are probably the most under recognized pathogens of the infectious diseases. While widely recognized as important pathogens in veterinary medicine, mycoplasmas are entangled in a web of controversy over their pathogenic capabilities in humans. In humans, mycoplasmas are often considered a cross contaminant with other major disease causing organisms. Over recent years, research with *M. pulmonis* in mice and humans has given some insight into how the immune system and mycoplasma interact [4, 25, 26, 34, 35]. The research in this thesis not only adds to the existing theories about mycoplasma infections, but it also looks into the species *M. bovis*.

M. bovis is the etiological agent for pneumonia, otitis media, arthritis, mastitis, and even meningitis-like disorders in cattle. The economic impact on the beef and dairy industries is significant. Many studies have investigated the effects of mycoplasma on the immune system of mice and humans, but few have been carried out in cattle. There have been attempts to characterize the immune response in cattle with inoculations of *M.*

bovis, but most have been given in addition to bovine viral diarrhea or respiratory syncytial virus.

The immune response of cattle along the respiratory tract has not yet been studied in the detail needed to fully understand the effects and interactions of *M. bovis* with the T cell populations, and it is the immune response that has a critical role in the progression of mycoplasma diseases. There are vaccines that can be administered for *M. bovis* in which immunity is acquired, but for only a short time [13, 29]. Studies in mice, swine, and humans demonstrate that there is an antibody response, and the response is effective to a certain time point [4, 25, 26, 34, 37]. It is thought that the immune system has the ability to prevent the diseases caused by *M. bovis*, but lacks the ability to fully clear the organism [4, 34, 38]. In an organism that does not have a fully functional immune system, it has been shown that not only is the organism not cleared, the mycoplasma infection can cause secondary complications in other regions of the body, in the joints for instance. It is not a question of whether *M. bovis* is immunopathologic, but who is the culprit immune cell (s) involved in the pathologies. Some research suggests macrophages are a key player in the immune response [27, 37, 39, 40]. When *M. bovis* is incubated with alveolar macrophages, the macrophages produce TNF-α and nitrous oxide. It has also recently been determined that *M. bovis* induces lymphocyte apoptosis, but the study was performed with an *in vitro* infection model [33, 37].

While somewhat true, the evidence for such theories is not as exciting as studies that have shown lymphocytes, upon their activation with mycoplasmas, migrate to the tissues of the respiratory tract [4, 37, 39, 41]. This denotes that lymphocyte activation

and recruitment are necessary strategic events in the progression of the mycoplasma respiratory disease. Other evidence suggests an immunopathologic response in mycoplasma respiratory disease comes from studies that utilize T cell depleted and immune deficient mice [4, 26, 37]. The results from these studies revealed that the T cell depleted and immune deficient mice developed a less severe disease within the lungs when compared to immune competent mice. However, even though the disease severity is diminished, the numbers of organisms within the lung were comparable. The data from these studies suggests that both the organism itself and the immune system are both responsible for the lung pathologies seen in mycoplasma infections. The immune system is a key player in the disease process and it is most likely that T cell activation is a logical step to investigate when studying the host response to mycoplasma. T cells are regulators in the humoral, cell-mediated and inflammatory responses. However, the role of the different T cell populations in mycoplasma respiratory disease is for the most part unknown.

T cells are divided into two major populations, $CD4^+$ T_h and $CD8^+$ T cells. The T cell subsets play a role in the pathogenesis and elimination of most diseases. The different tasks of the T cell subsets in disease are involved not only in the manifestations of bacterial pathogens, but viruses, tumor, autoimmune, and allergic disease as well. Recent publications from our laboratory investigated the roles of lymphocyte populations within the lung of mice in response to *M. pulmonis* infections [4, 25, 26, 42, 43]. The results suggest that $CD4^+$ cells play a pro-inflammatory role while the $CD8^+$ cells tend to dampen the inflammatory response.

Other work from our laboratory studied the contributions of γδ-T cells in response to mycoplasma infection. The studies showed an increase in cell numbers at early time points, which suggests γδ-T cells are pro-inflammatory. Other research suggests γδ-T cells are regulators in inflammatory responses in infections with organisms like *Listeria monocytogenes* as well [2, 3, 32, 37, 39, 40, 44-52]. γδ-T cells are believed to play regulatory roles in mycoplasma bovis infections in cattle, making them a possible point of interest in this study. The precise balance of the immune systems protection and injurious responses are directly interrelated to the opposing T cell populations. To better understand the interaction of the immune system between *M. bovis* and its bovine host, the T cell subsets must be characterized. By comprehending the immune response, it could be become possible to alter the immune response in a mycoplasma infection to capitalize protection while at the same time diminishing the inflammatory and detrimental effects of the disease. This scheme will aid in the tremendous effects the dairy and cattle industry deal with in battling a disease that eludes researchers.

Methods and Materials:

Subjects: Male Holstein calves were obtained from the University of Florida Dairy Research Unit. This herd had a history of no clinical disease in the 2 years preceding the study. The pregnant heifers were screened for *M. bovis* infection, and only calves from culture negative heifers were used. The calves were removed from the herd prior to suckling, and received commercial colostrum supplement. They were transported from

the Dairy Research Unit to Animal Care Services at 1 to 4 days of age were they were supplemented with non-medicated milk replacer (10% of bodyweight/day). The calves were maintained in separate stalls at the collaborating institution (University of Florida in Gainesville) and had access to non-medicated calf starter pellets and fresh water at all times. When the calves reached seven to eleven days of age, they were infected with a strain of *M. bovis* isolated from a natural case. After infection, the calves were routinely examined for clinical signs of infection (weight loss, respiratory symptoms, etc.) *(Table 1)*.

Experimental infection: All calves that were experimentally infected by oral administration of *M. bovis* strain FA-1. *M. bovis* FA-1 is a field strain isolated from a lung abscess in a calf with severe fibrinous pneumonia and pleuritis. The mycoplasmal organisms were isolated and a pure culture was obtained. The farm that served as the source of the organism had a high incidence of mycoplasmal disease (middle ear and respiratory disease). The isolate was confirmed as *M. bovis* by PCR amplification of the 16S rRNA gene. Calves were inoculated between 7 and 11 days of age. The calves received an oral dose of *M. bovis* (infected group) or an identical volume of sterile Frey's broth (control group) at each of 3 consecutive feedings over a 24-hour period. Approximately 3 hours prior to inoculation, an aliquot of second passage culture in Frey's broth was thawed at room temperature. Immediately before inoculation, 19.9 ml of culture was mixed with 2 pints of milk replacer at 35-37°C. The mixture was then fed to the calf. The remaining volume of the calf's feeding (2 - 4 pints depending on bodyweight) was then added to the bucket and fed. A 0.1 ml sample of culture was

removed from the thawed aliquot for determination of CFU/ml (*Table2*). It was also imperative to see where the infection was throughout the infected calves to see if the T cell populations changed at those sites of infection. The distribution of infection was also observed to determine if the infection was predominantly in the upper respiratory tract (URT) or in both the upper and lower respiratory tracts (LRT) (*Table 2*).

Cell Isolation: Blood was drawn from the calves on days zero, three, seven, and fourteen (p.i.), and peripheral blood lymphocytes were isolated. Fifteen milliliters of blood were obtained by jugular venous puncture and collected in tubes containing heparin as anticoagulant. Blood was diluted 1:1 with wash medium (HBSS supplemented with 10% gamma-free equine serum, BioCell, Rancho Dominguez, CA). The blood was placed in Accuspin tubes (Sigma Diagnostics) containing Histopaque (specific gravity 1.077, Sigma) separating the cells of the blood sample by their density. After centrifugation at 800g for 20 min. at 20°C, the mononuclear layer was aspirated and placed in HBSS solution. If necessary, RBC's were lysed using BD FACS lysing solution. Total viable cells were counted on hemacytometer and adjusted to 4 X 10^5 cells per tube. Necropsies were performed on day fourteen after infection.

Level of clinical disease shown as the number of days clinical signs were observed.*

Clinical Signs	Calf # 275	Calf # 276	Calf # 277	Calf # 280	Control Calf # 278
Nasal Discharge	0	1	4	4	0
Ocular Discharge	6	10	12	0	2
Dyspnea	0	0	1	0	0
Abnormal Lung Sounds	2	0	3	1	0
Cough	0	0	2	0	0
RR > 60	2	0	2	1	0
Lethargy	0	0	0	3	0
Ear Droop	0	0	2	6	0

*Results are expressed as total number of days clinical signs observed during 14-day study period

Table 1: This table represents the clinical signs observed from the calves. While some calves received different doses than other calves, all inoculated calves had colonization in the upper respiratory tract. The calves received an oral dose of *M. bovis* (infected group) or an identical volume of sterile Frey's broth (control group) at each of 3 consecutive feedings over a 24-hour period.

Distribution of Infection: Isolation sites of *M. bovis* from tissues at day 14.

	Calf # 275	Calf # 276	Calf # 277	Calf # 280
Total Dose (CFU/ml)	1.38×10^{10}	1.02×10^{10}	1.53×10^{10}	7.14×10^{10}
Palatine tonsil	++++	+++	++++	++++
Pharyngeal tonsil	++++	+++	+++	++++
MRPLN	+	----	+	+
Eustachian tube	++	----	++	+++
Tympanic bulla	----	----	++++	++++
Trachea	----	----	+	----
Primary bronchus	+	----	+	----
Lung*	----	----	+	----

(----) =negative culture, (+) =light, (++) =moderate, (+++) =heavy, (++++) =very heavy.
*Isolation from left caudal lobe (3.5 CFU/g), right cranial lobe (3.8 CFU/g), right caudal lobe (20.1 CFU/g)

Table 2. Table represents the distribution and severity of infection. All infected calves had colonization in the upper respiratory tract and with in the inner ear. This is indicative of an upper respiratory tract infection. The sites of colonization correlated with an increase in T cells in the upper respiratory tract (URT).

The medial and lateral retrophrangeal (MRPLN, LRPLN), and the tracheobroncial (TBLN) lymph nodes from the upper and lower respiratory tract were removed along with sections of the lung, palatine tonsils, and spleen. The tissue was washed with Ca^{++} and Mg^{++} -free PBS to remove blood and debris. Lymph nodes and spleens were minced and filtered through 100-micron mesh boats. Lung tissue was minced in the same manner as the lymph nodes and spleen. 6 grams of lung was placed in 25 mls of collagenase type IV/DNAse for 1.5 hours to allow enzymatic degradation of the collagen matrix before further processing. Tissues were washed and the lymphocytes isolated with the same procedure as with the blood samples. The total number of viable cells were counted on a hemacytometer and adjusted to 4 X 10^5 per milliliter. HBSS was used until the staining began, then during the staining protocol; the cells were washed with staining buffer (PBS without Mg^{++} and Ca^{++} supplemented with 5% gamma-free equine serum, BioCell).

Immunofluorescent characterization of lymphocyte populations: Lymphocytes were isolated and two-colored stained for cell surface markers distinguishing the T cell populations, $CD3^+$ (T lymphocytes), $CD4^+$ (T_h cells), $CD8^+$ (CTL), and WC1 ($\gamma\delta^+$ T cells). $CD3^+$ cells were tagged with an anti-bovine CD3 (mouse IgG1 (mouse IgG1, SeroTec, Raleigh, NC)) and an anti-boyine CD4 (mouse IgG2a) or an anti-bovine CD8 (mouse IgG2a) antibody. $CD8^+$ ($CD8^+$ - $\gamma\delta^+$) and $CD4^+$ ($CD4^+$- $\gamma\delta^+$) $\gamma\delta^+$ T cells were stained with a similar method. All antibodies were diluted 1:50 with PBS, and 50 μl was then added to $4X10^5$ cells.

Negative controls for background staining included isotype controls for each of the fluorescent tags used in the experiment.

To help remove dead cells and non-bound antibodies, a cushion method was performed. Two milliliters of staining buffer was added to the cells. With a transfer pipette, one milliliter of equine serum (cushion) was placed at the bottom of the tube. The cells were centrifuged for 10 min. at 200 g at 4 °C. Dead cell debris and the unbound antibodies remained in the equine serum while the viable stained cell passed through the cushion and pelleted at the bottom of the tube. After staining, the samples were analyzed by flow cytometry. For each sample, 10,000 cells were analyzed using a Coulter EPICS flow cytometer, or a BD FACS SCAN at the Flow Cytometry Core Lab. at the University of Florida in Gainesville. All experiments were performed at least twice and the differences between the control and experimental groups were established by statistical analysis (ANOVA). The distinct T cell populations were expressed as a percentage of the number of stained cells. The total number of the specific lymphocyte phenotypes was determined by taking their percentage and multiplying it by the total number of lymphocytes isolated from the particular tissues.

Antigen specific antibody responses: The level of antigen specific antibody production was measured using the ELISPOT assay. Extracted *M. bovis* protein was used to coat multiscreen 96-well plates (Millipore, Billerica, MA) at 5 μg of protein per well. Plates were incubated overnight at 37°C, 5% CO_2, and then washed with PBS/Tween 20 (3X). Plates were blocked with RPMI-1640 supplemented with 10% gamma-free equine serum (BioCell) for at least 3 hours at room temperature. Plates were

washed with PBS/Tween 20 (3X) and samples were added to wells in triplicate. Plates were then incubated at 37°C, 5% CO_2 overnight. The plates were washed and 100 µl of biotin-conjugated antibodies for immunoglobulins IgG, IgG1, IgG2, IgM, and IgA were added (1:2000 from 1mg/ml stock). The plates were incubated overnight at 4°C. After incubation, plates were washed and 100 µl per well of avidin-peroxidase (diluted 1:1000 in PBS-Tween 20 containing 1% gamma-free equine serum) was added and incubated at room temperature for two hours.

After the 2-hour incubation, the plates were washed and 200 µl of substrate mixture was added to each well for the development of the spots. The plates incubated at room temperature for 10-15 min. and were washed with water to stop the reaction. The plates air-dried overnight to allow reduction of background color. The cells were counted at the highest possible dilution, and were expressed as the number of stained cells per tissue sample. The total number of the specific antibodies was determined by taking the number per sample and multiplying it by the total tissue weight. The goal of this research was to: 1) Establish a protocol for characterizing lymphocyte populations within cattle, and 2) elucidate the different immune responses of the upper and lower respiratory tract to mycoplasma infection.

Statistical analysis: The data was evaluated by ANOVA, followed by Fisher protected least significant difference multigroup comparison or Tukey's multigroup comparison. These analyses were performed using the StatView (SAS Institute, Cary, NC) computer program.

A p value of less than or equal to 0.05 ($p \leq 0.05$) was considered statistically significant. If the data was analyzed after logarithmic transformation, the standard error of the transformed data was used to present the data.

Results: The percentages of T cells from various tissues that stained with a panel of antibodies specific for lymphocyte differentiation antigens were analyzed to characterize the changes in T cell population in calves infected with *M. bovis*. To determine the changes in T cell populations after *M. bovis* infection, the lymphocytes from various lymphatic tissues of infected (N=3) and control (N=2) were collected at 14 days (p.i.) and were characterized by flow cytometry. The cells were stained with anti-bovine CD3 and anti-bovine CD4 or CD8 antibodies.

Blood was drawn from each animal on the day of infection, followed by days three, seven, and then on day 14, the day of necropsy. There were no changes in the blood T cell populations as a result of infection (*Figures 5- 8*). On day 0, there was no significant difference between control calves and those to be infected in any of the T cell populations. The percentage of both subsets of $\alpha\beta$-T cells increased on days 3 and 7, but began to decrease on day 14. The $\gamma\delta$-T cell subsets showed little to no change in percentages the infection. Overall, there were more $CD4^+$ $\alpha\beta$-T cells in the circulation than the other populations.

Percentage of T Cells in Circulation

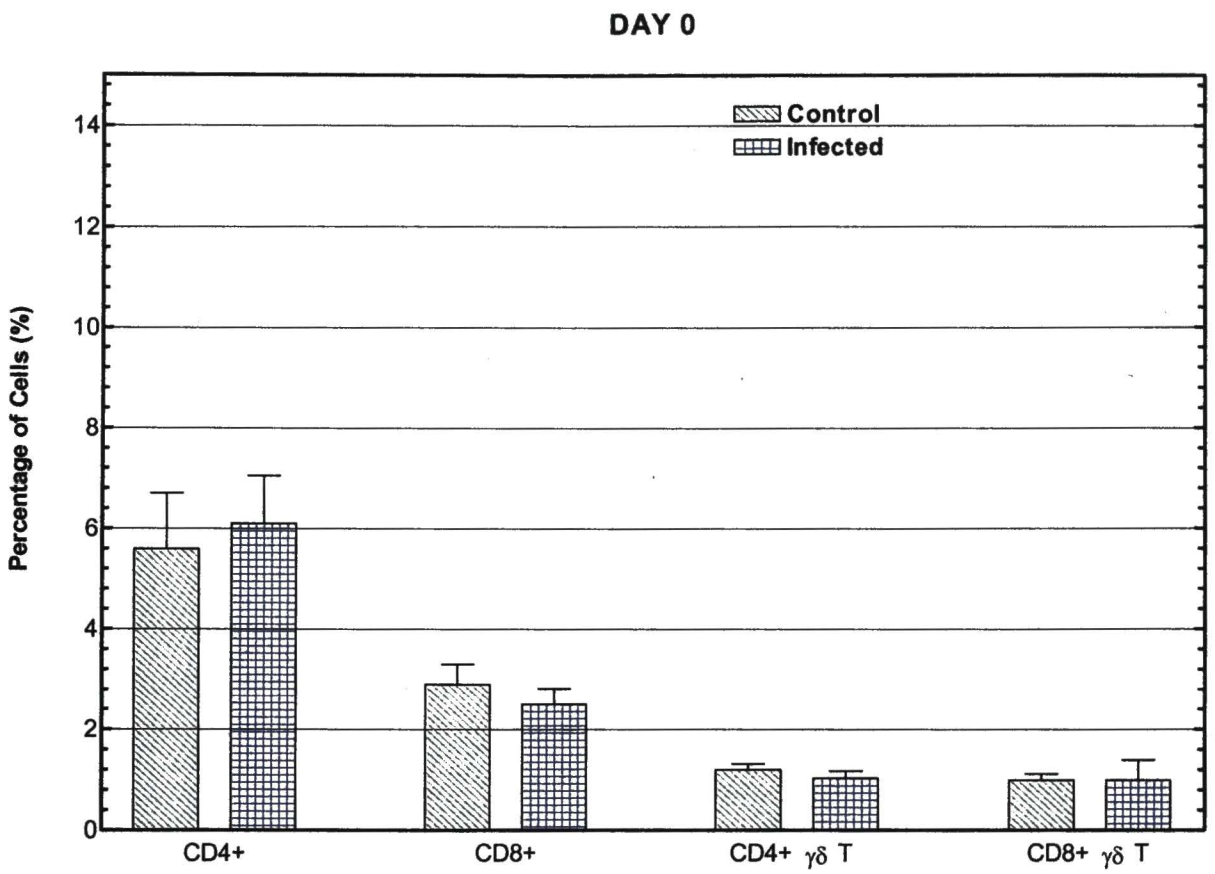

Figure 5. Blood was drawn from each animal on day 0 (day of infection), followed by days 3, 7, and day 14. Results are represented in percent of total lymphocytes isolated in 15 mls of heparinized blood. Results suggest that there is no change in the T cell populations as compared to the control animals. (* denotes statistical significance.)

Percentage T Cells in Circulation

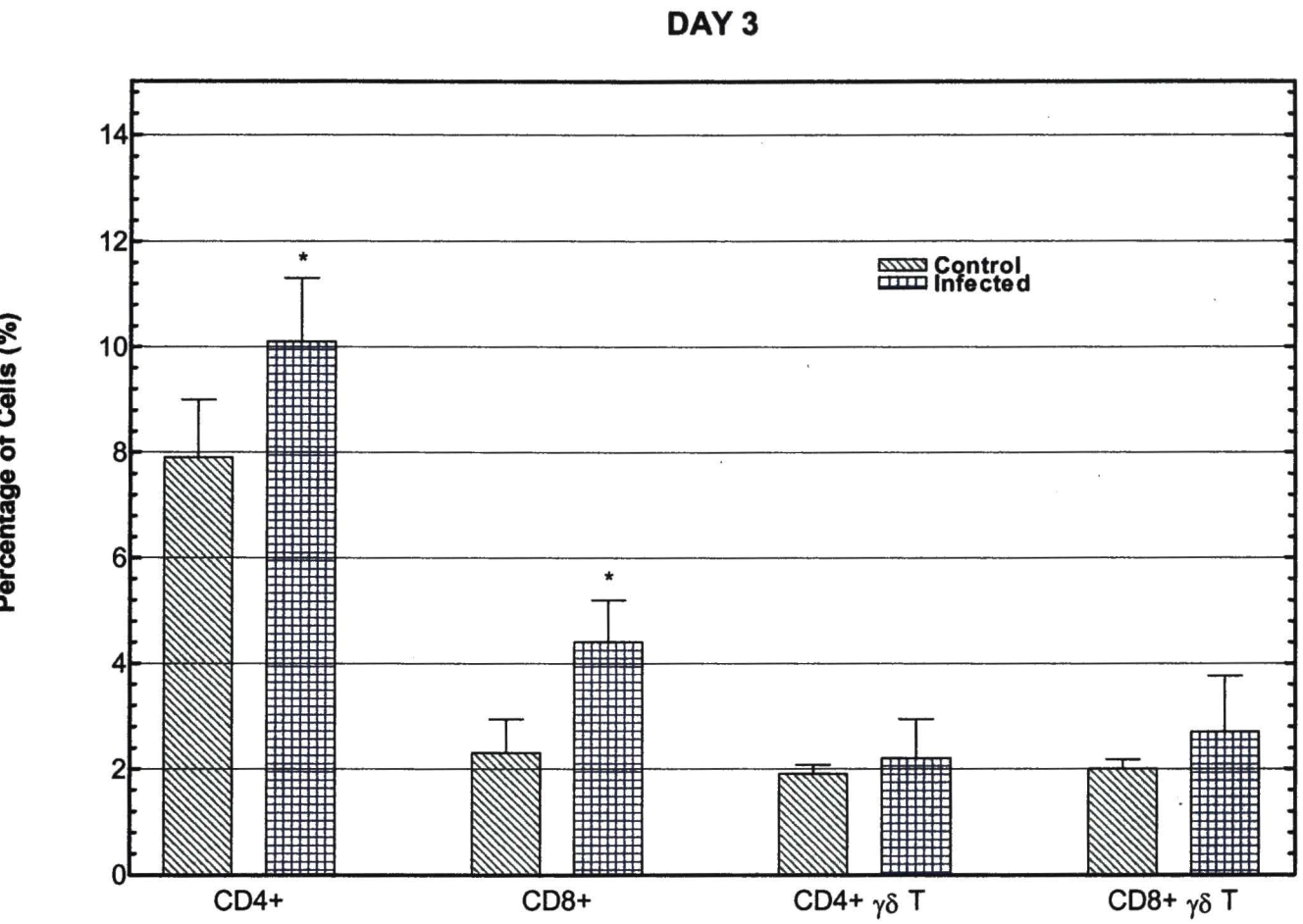

Figure 6. Blood was drawn from each animal on day 0 (day of infection), followed by days 3, 7, and day 14. Results are represented in percent of total lymphocytes isolated in 15 mls of heparinized blood. This data shows that there was a slight change in T cell populations at day 3 as compared to the non-infected animals. (* denotes statistical significance.)

Percentage T Cells in Circulation

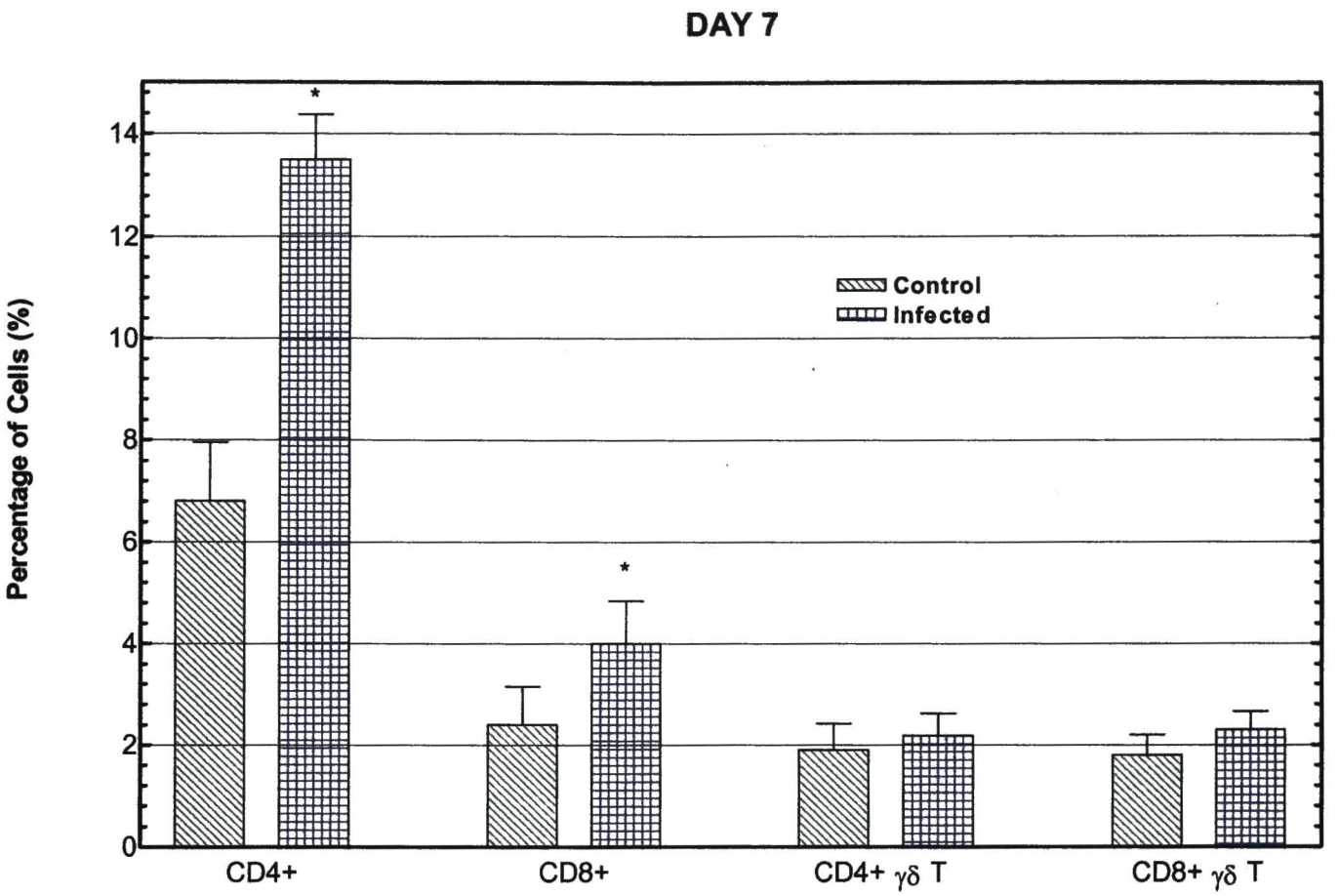

Figure 7. Blood was drawn from each animal on day 0 (day of infection), followed by days 3, 7, and day 14. Results are represented in percent of total lymphocytes isolated in 15 mls of heparinized blood. This data shows that there was a significant change in $\alpha\beta$-T cell populations at day 7 as compared to the non-infected animals. (* denotes statistical significance.)

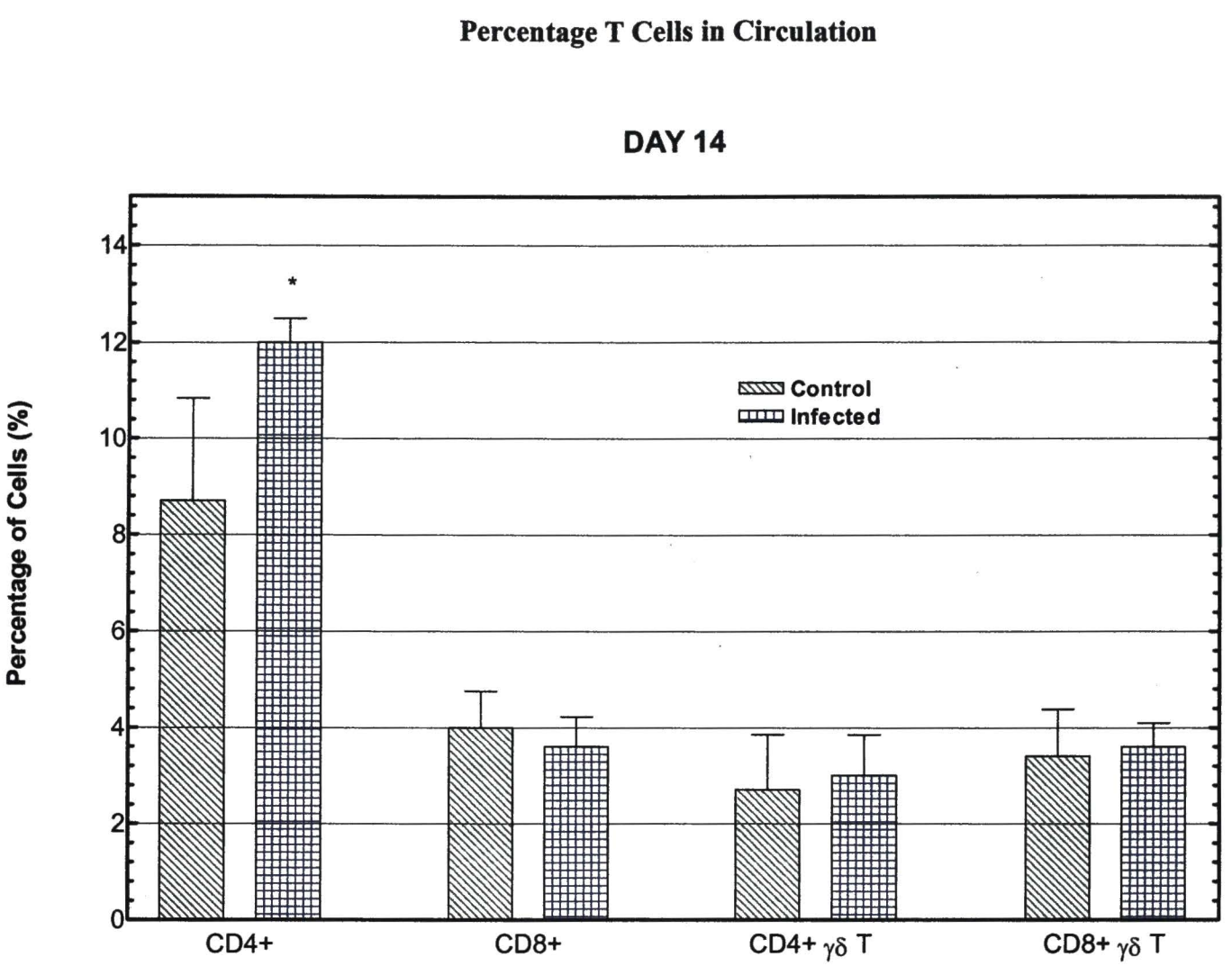

Figure 8. Blood was drawn from each animal on day 0 (day of infection), followed by days 3, 7, and day 14. Results are represented in percent of total lymphocytes isolated in 15 ml of heparinized blood. This data shows that there was no change in T cell populations at day 14 as compared to the non-infected animals. (* denotes statistical significance.)

To further characterize the immune response of calves infected with *M. bovis,* lymph nodes from the upper and lower respiratory tract, sections of tissue from each of the six lung lobes, and also from secondary lymphatic organs, the spleen and palatine tonsils, lymphocytes from each of the tissues were isolated and the T cell populations characterized.

The percentages of $\alpha\beta$-T Cells within most tissues were at much higher levels than the $\gamma\delta$-T cell subsets in control and infected calves. In the tissues from the upper respiratory tract (MRPLN, LRPLN, and tonsils), the number of both $CD4^+$ and $CD8^+$ $\alpha\beta$-T cells were at comparable percentages to those of control animals (*Figure 9-11*). The lungs showed no significant differences in either T cell populations between the two groups. There was a trend that $CD4^{+-}$ $\gamma\delta^+$- cells were elevated in the sites of infection, where as the $CD8^+$ $\gamma\delta^+$ cells had no significant change in percentages (*Figure 9-11*). The $CD8^+$ $\gamma\delta$-T cells in the spleen of infected calves showed a slight increase as compared to control animals (*Figure 12*).

Percentage of T Cells in Upper Respiratory Tract Tissues

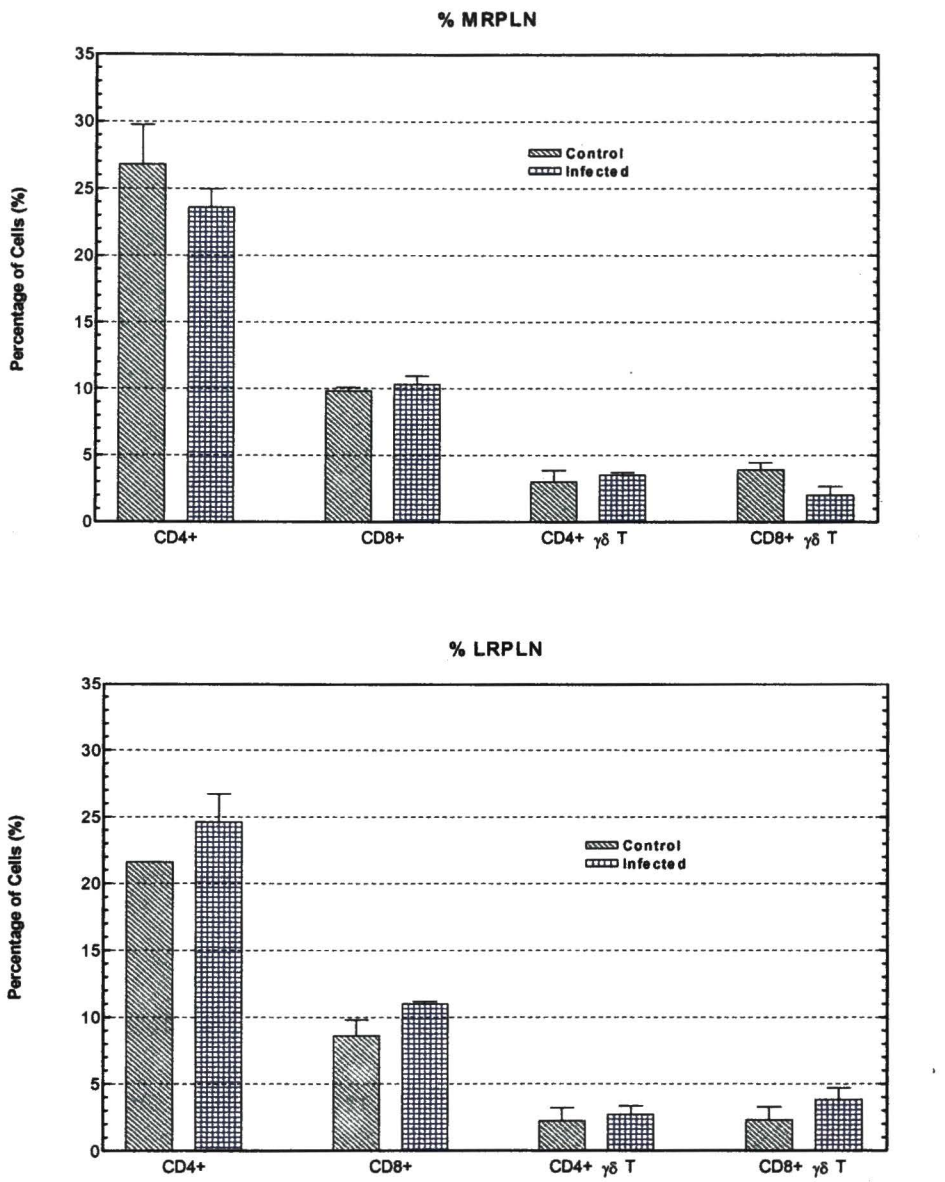

Figure 9. Results are represented in percentage of total lymphocyte population for particular tissues from the upper respiratory tract. The data suggests that there are no changes in T cell percentages in sites of colonization as compared to control calves. (* denotes statistical significance.)

Percentage of T Cells in Upper Respiratory Tract Tissues

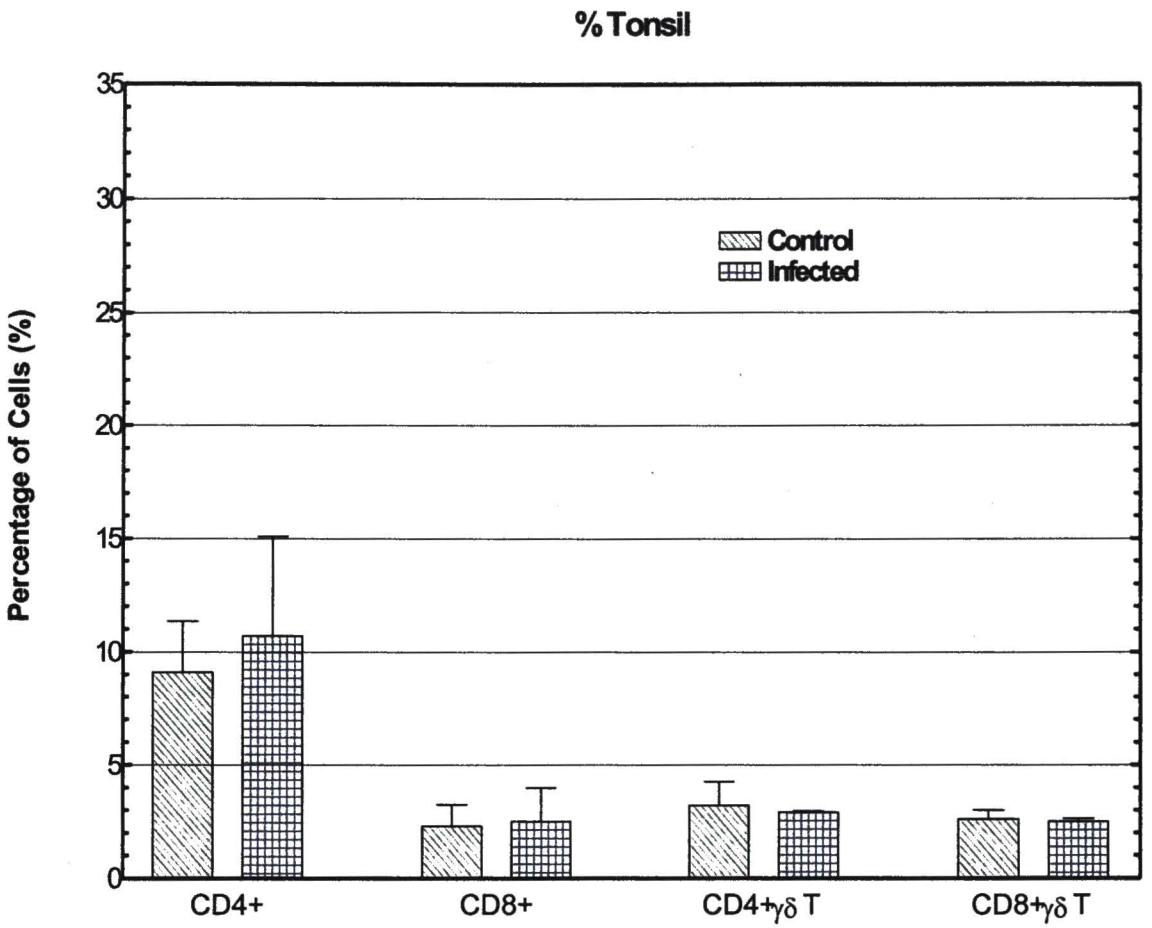

Figure 10. Results are represented in percentage of total lymphocyte population for particular tissues from the upper respiratory tract. The data suggests that there are no changes in T cell percentages in sites of colonization as compared to control calves. (* denotes statistical significance.)

Percentage of T Cells in Lower Respiratory Tract Tissues

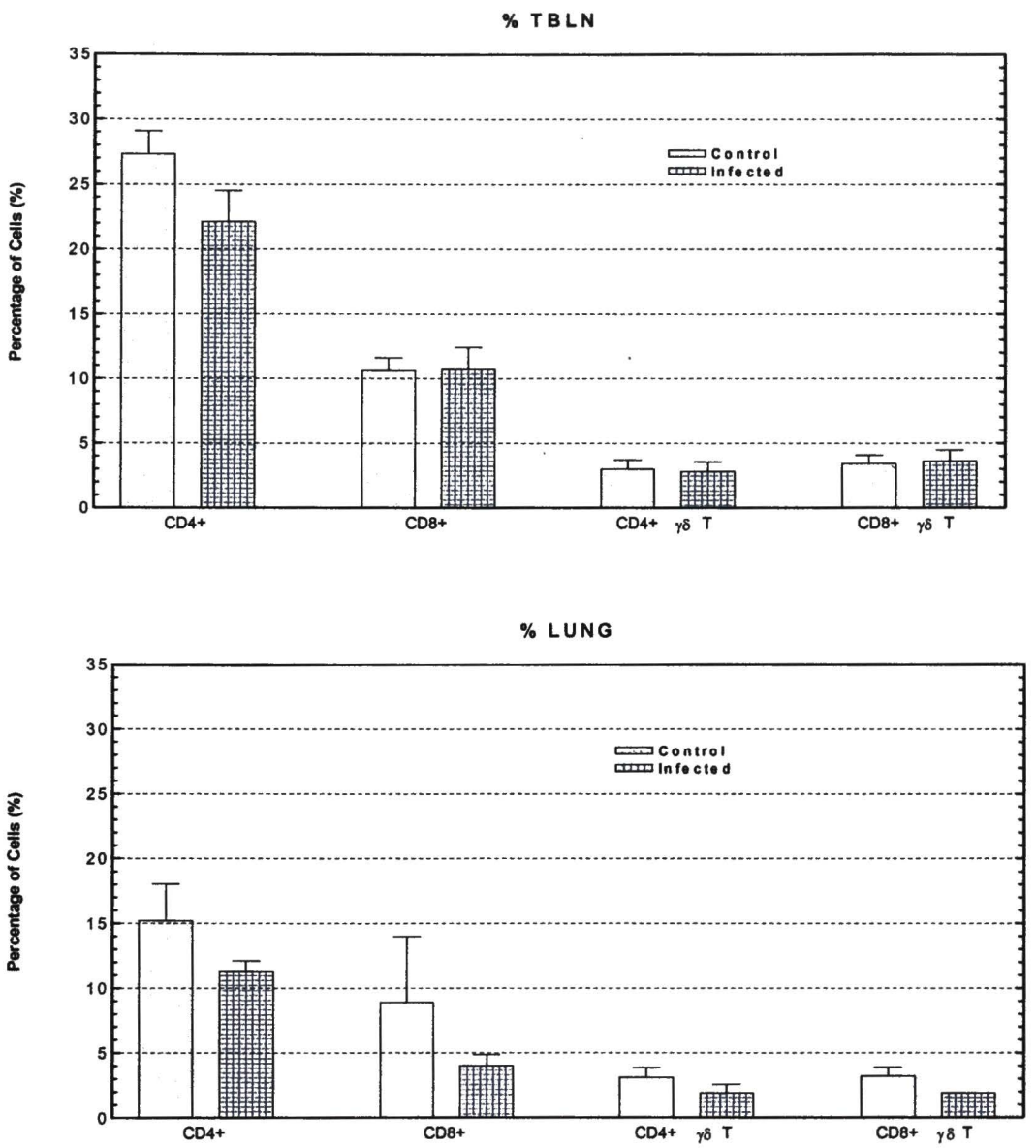

Figure 11. Results are represented in percentage of total lymphocyte population for particular tissues from the lower respiratory tract. The data suggests that $CD4^+$ $\alpha\beta$-T cell percentages do not increase in sites where no colonization was observed.

(* denotes statistical significance.)

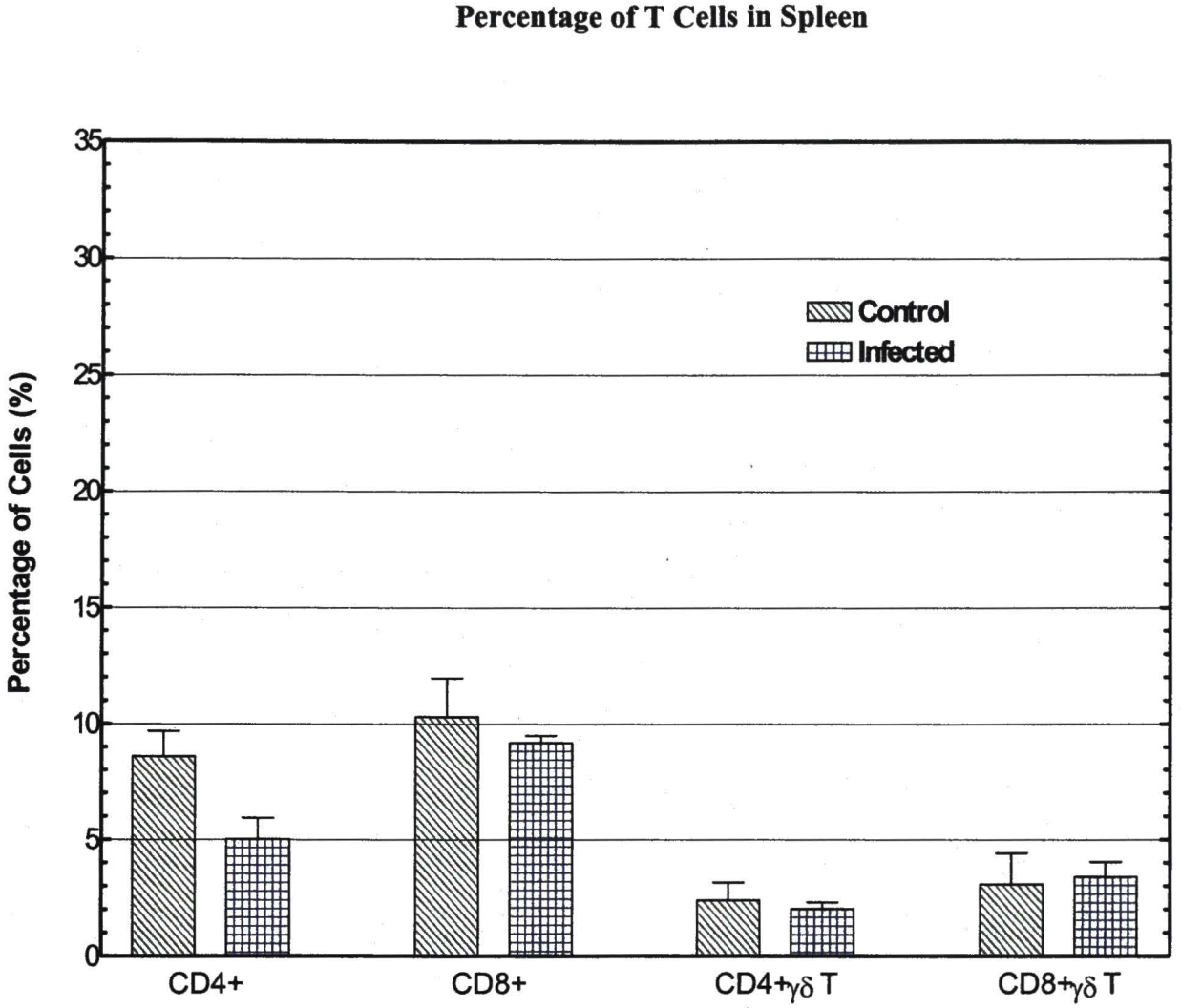

Figure 12. This data represents the percentage of total lymphocytes isolated from the spleen. Studies have shown that $CD8^+$ $\gamma\delta^+$ cells are in higher percentages within the spleen and that they do not aggregate at sites of inflammation. This data shows that the $CD8^+$ subset is in greater numbers than the $CD4^+$ subset within the spleen. However, there were not changes in any T cell population in the spleen during infection when compared to control calves. (* denotes statistical significance.)

To determine the overall contribution of each of the respiratory lymphoid tissues to the response to infection, we also examined the total numbers of T cell populations in each of these tissues. The results for the cell numbers were consistent with expectations. The numbers of $\alpha\beta$-T cells in the upper respiratory tract (URT) of infected calves were higher than that of control calves (*Figure 13, 14*). The $CD4^+$ population increased in all tissues in the URT where as the $CD8^+$ T cells increased in only select tissues. In the lower respiratory tract (LRT), the number of $\alpha\beta$-T cells in the lungs of infected calves was higher than the number in control calves, the $\gamma\delta^+$ T cell population increased as well (*Figure 15*). The numbers for both T cell populations in the TBLN were unchanged between the control group and the infected group. The number of $CD4^+$ $\alpha\beta$-T cells in the upper respiratory tract of infected animals increased nearly two fold compared to control animals. The number of $CD8^+$ $\alpha\beta$-T cells and both subsets of $\gamma\delta^+$ T cells also increased, though not as significant as the $CD4^+$ $\alpha\beta$-T cell population. There was a slight increase in the $CD8^+$ $\gamma\delta^+$ T cells within the spleen, but surprisingly, no significant changes in the $\alpha\beta$-T cell or $CD4^+$ $\gamma\delta^+$ T cell numbers occurred between the infected and control animals (*Figure 16*).

To look at antibody specific producing cells specific for *M. bovis*, IgG1, IgG2, IgM, and IgA were evaluated with in the circulation and various tissues. In all of the infected animals, there was a dramatic increase in cells secreting *M. bovis* specific IgG, IgG1, and IgM. IgA increased along the respiratory tract, especially in draining lymph nodes. IgG2 levels remained at very low levels in all tissues, except in the upper respiratory tract (*Figure 17-20).*

Number of T Cells in Upper Respiratory Tract Tissues

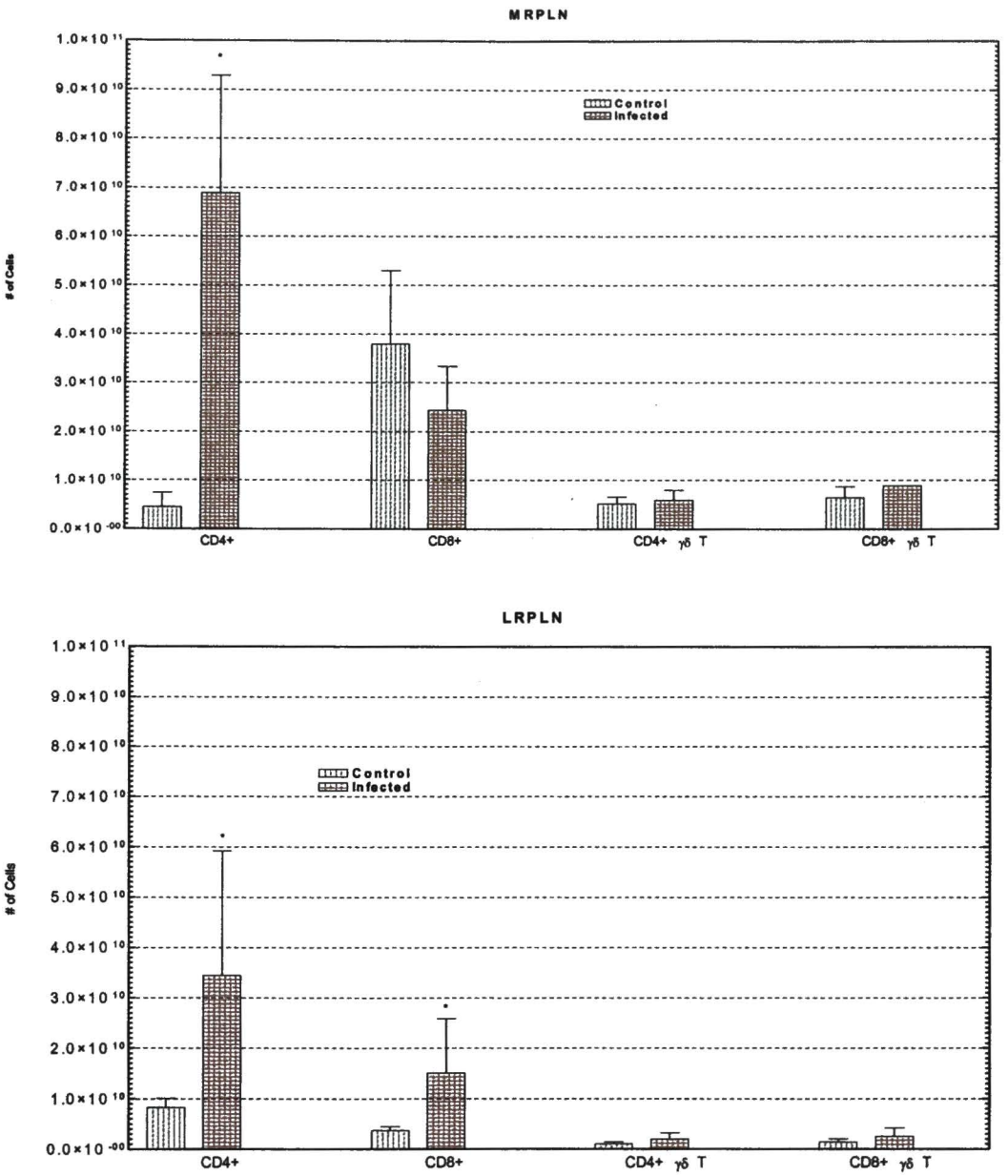

Figure 13. This figure represents the total number of lymphocytes isolated from tissues of the upper respiratory tract. The data suggests that while all the subsets increase in the upper respiratory tract, there was a preferential increase in the numbers of $CD4^+$ T cells. (* denotes statistical significance.)

Number of T Cells in Upper Respiratory Tract Tissues

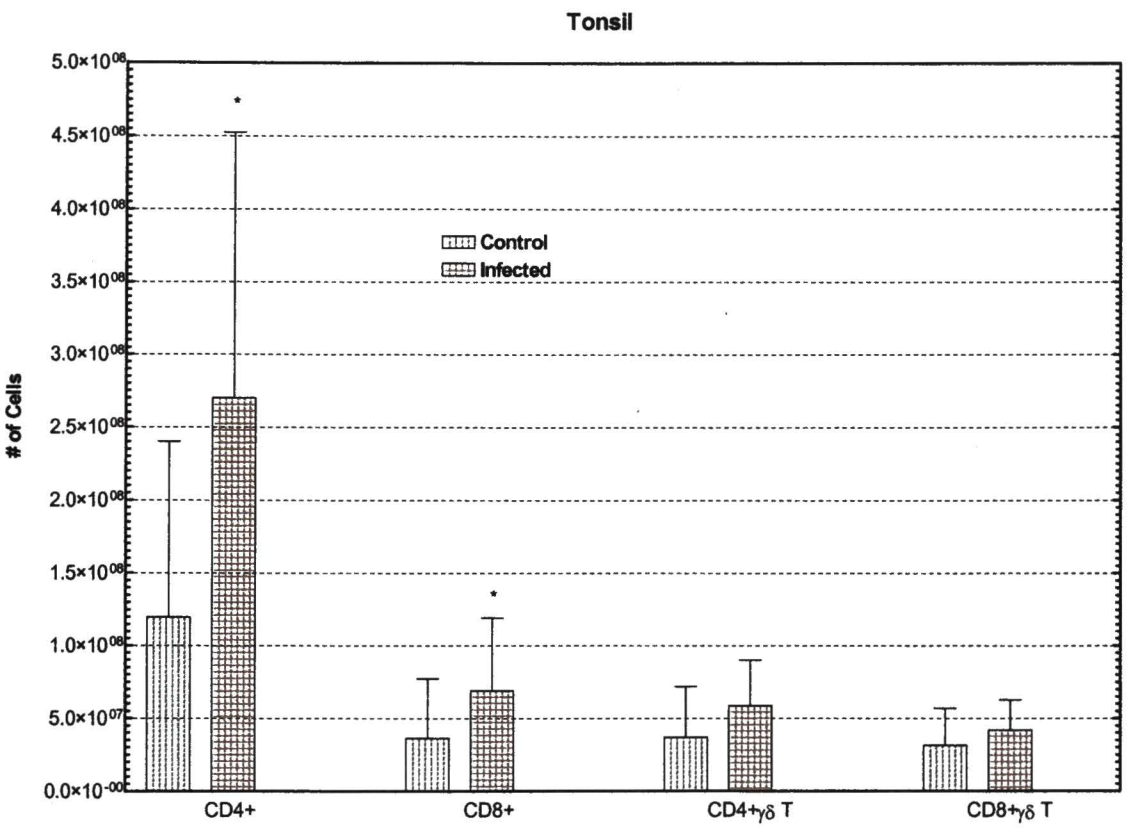

Figure 14. This figure represents the total number of lymphocytes isolated form the palatine tonsils. The results suggest that along the upper respiratory tract, there was a preferential increase in the numbers of $CD4^+$ T cells, and γδ+ T cells are increasing in number therefore may be playing key roles in the disease process. (* denotes statistical significance.)

Number of T cells in Lower Respiratory Tract Tissues

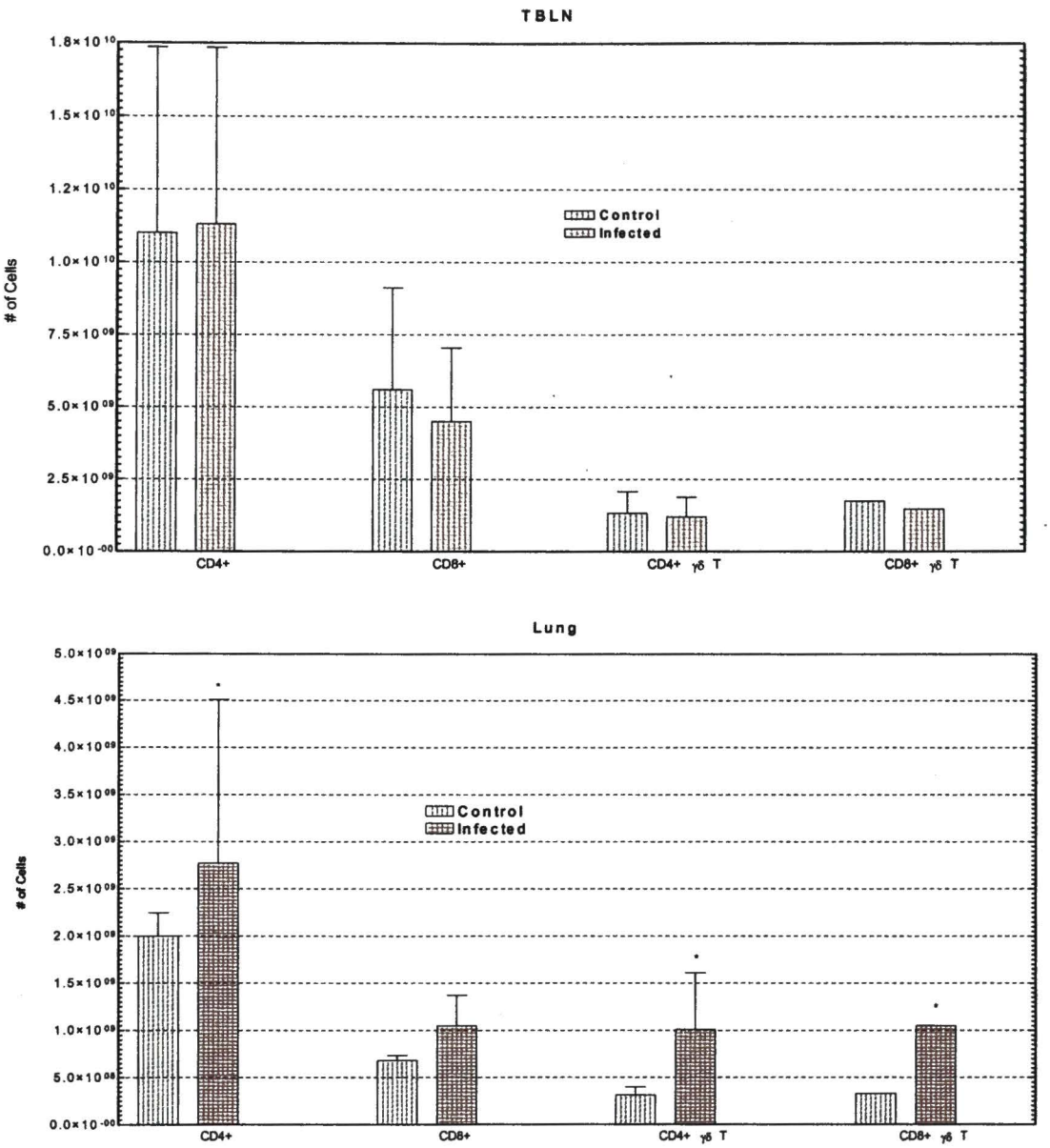

Figure 15. This figure represents the total number of lymphocytes isolated from tissues of the lower respiratory tract. The data suggests that while all the subsets increased in the lung, overall, the infection was localized in the upper respiratory tract. (* denotes statistical significance.)

Number of T Cells in the Spleen

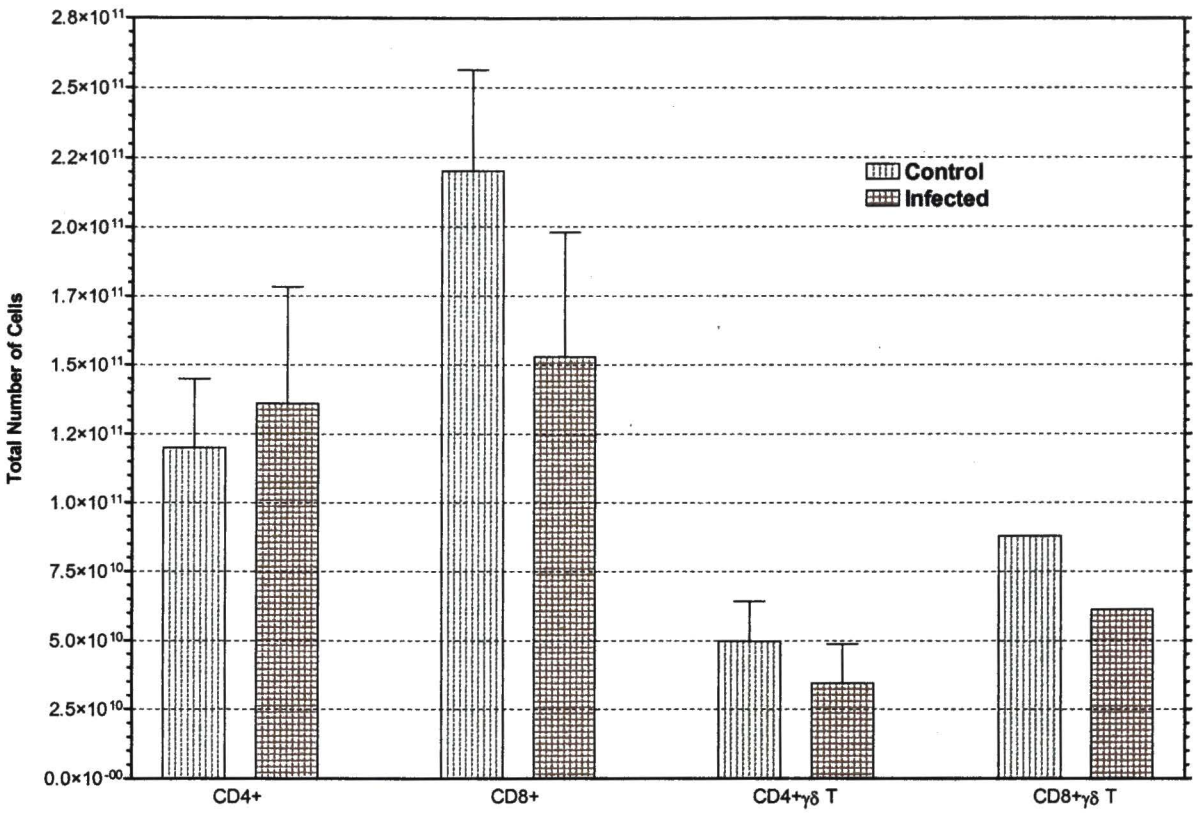

Figure 16. This figure represents the total number of lymphocytes isolated form the spleens of infected and control calves. To derive the total T cell number, the percentages of the isolated T cell populations were taken and used to derive the number of cells per T cell subset from tissues of infected and control calves. The spleen showed no significant changes in T cell populations. (* denotes statistical significance.)

Number of antibody secreting cells in Upper respiratory Tract Tissues

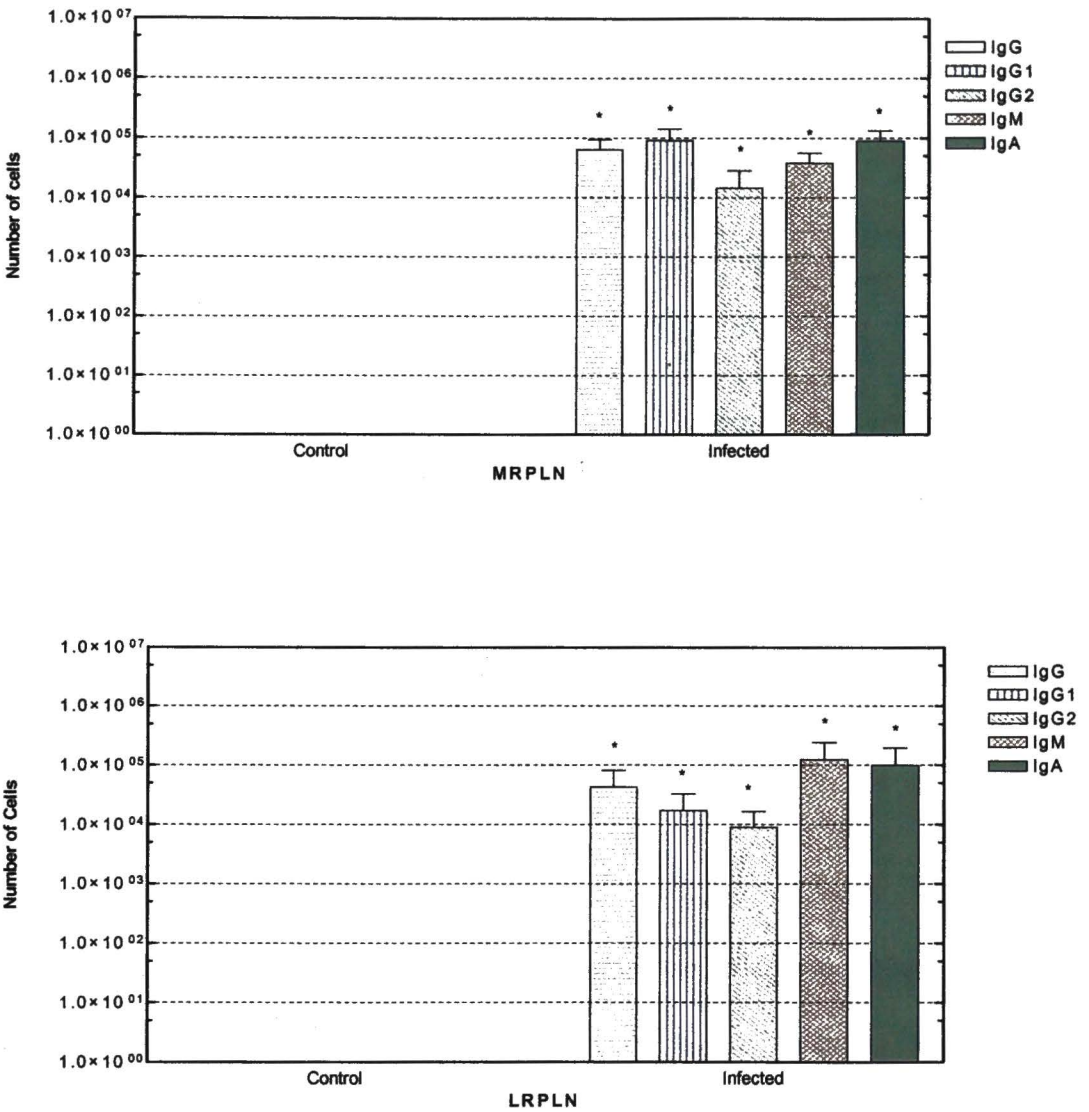

Figure 17. This data represents the antigen specific antibody production in response to *M. bovis*. Each graph represents the upper respiratory tract (medial and lateral retropharangeal lymph nodes (MRPLN/LRPLN) of five calves, three infected and two control animals. The data suggests a strong $CD4^+$ T cell dependent antibody response. (* denotes statistical significance.)

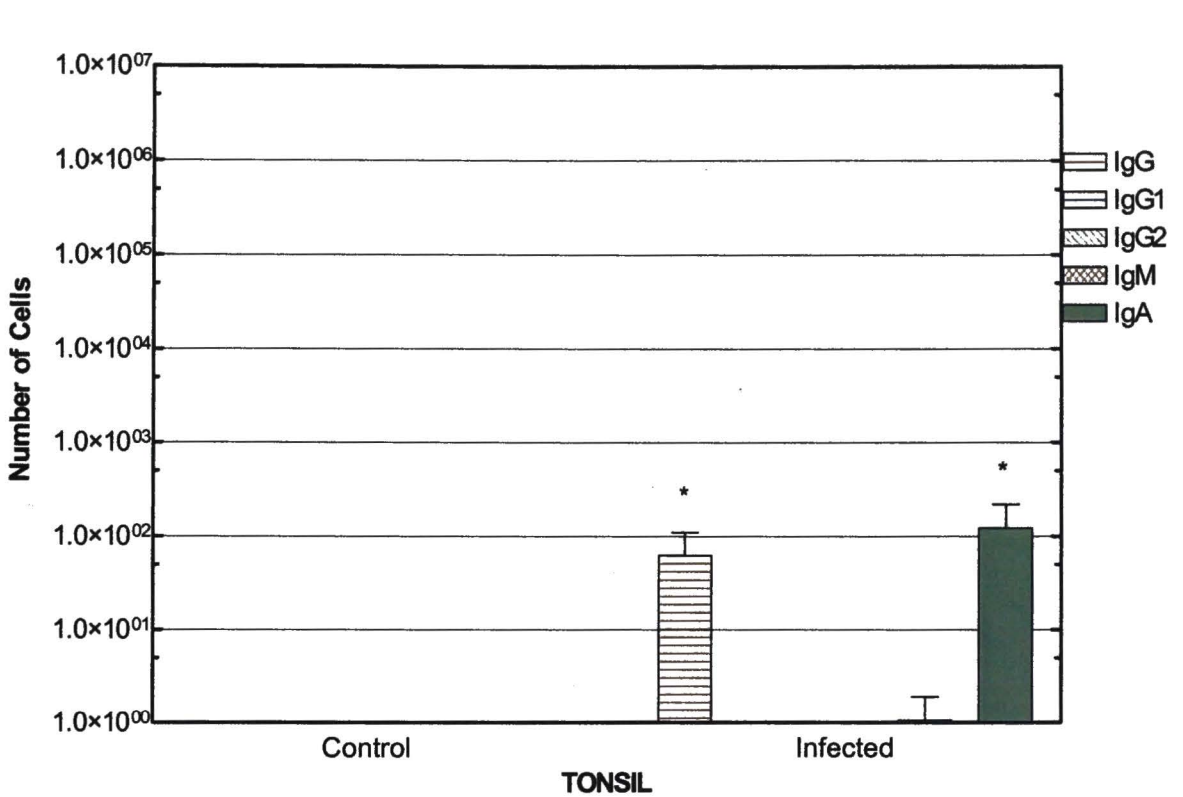

Figure 18. This data represents the antigen specific antibody production in response to *M. bovis*. The graph represents tissues in the upper respiratory tract (Palatine Tonsil) of five calves, three infected and two control animals. The data suggests a strong $CD4^+$ T cell dependent antibody response in the upper respiratory tract. (* denotes statistical significance.)

Number of antibody secreting cells in Lower respiratory Tract Tissues

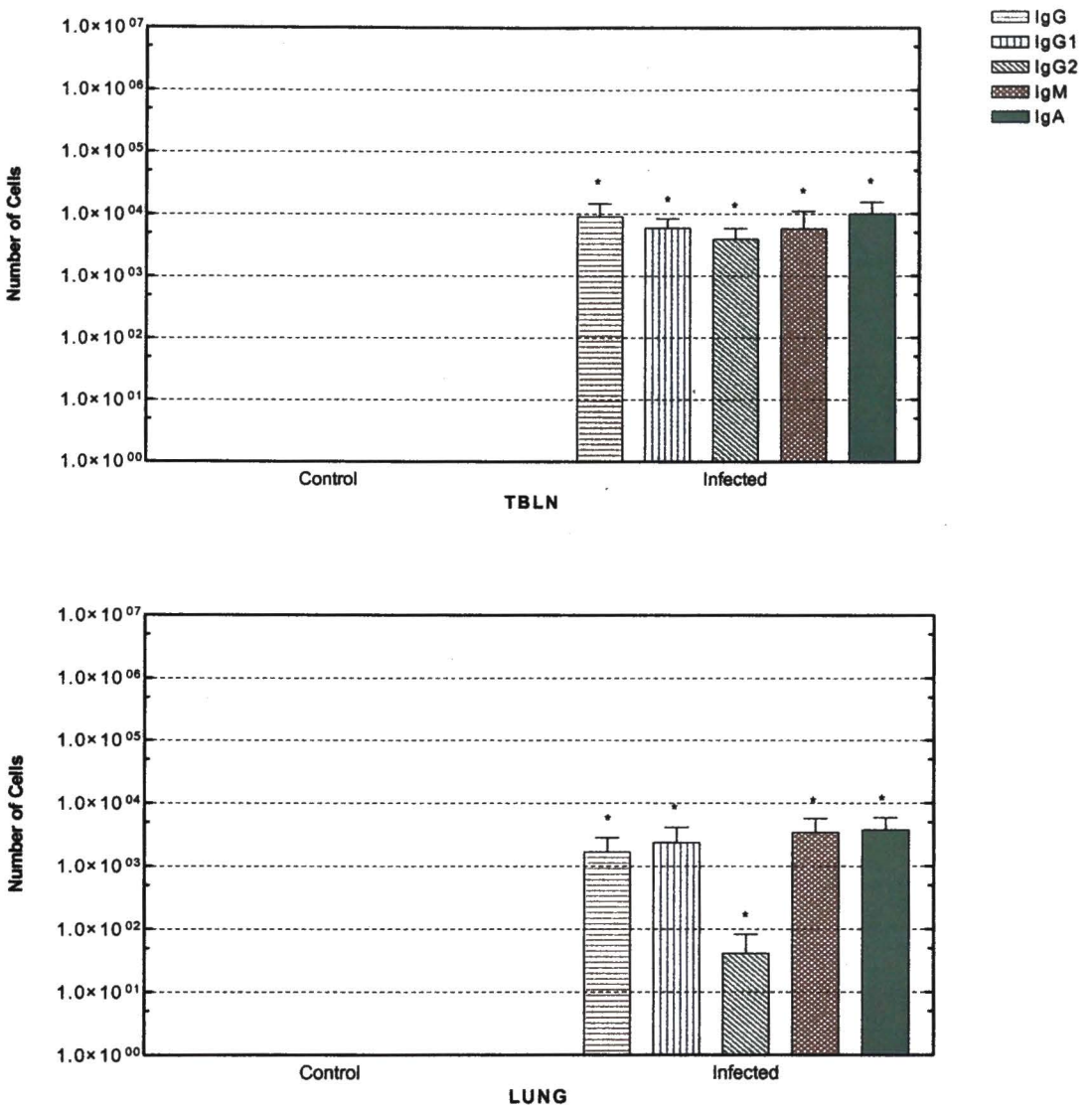

Figure 19. This data represents the antigen specific antibody production in response to *M. bovis* in the lower respiratory tract (tracheobronchial lymph node and lung). Each graph represents a total of five calves, three infected and two control animals. The data suggests a strong $CD4^+$ T cell dependent antibody response. (* denotes statistical significance.)

Number of antibody secreting cells in Spleen and Circulation

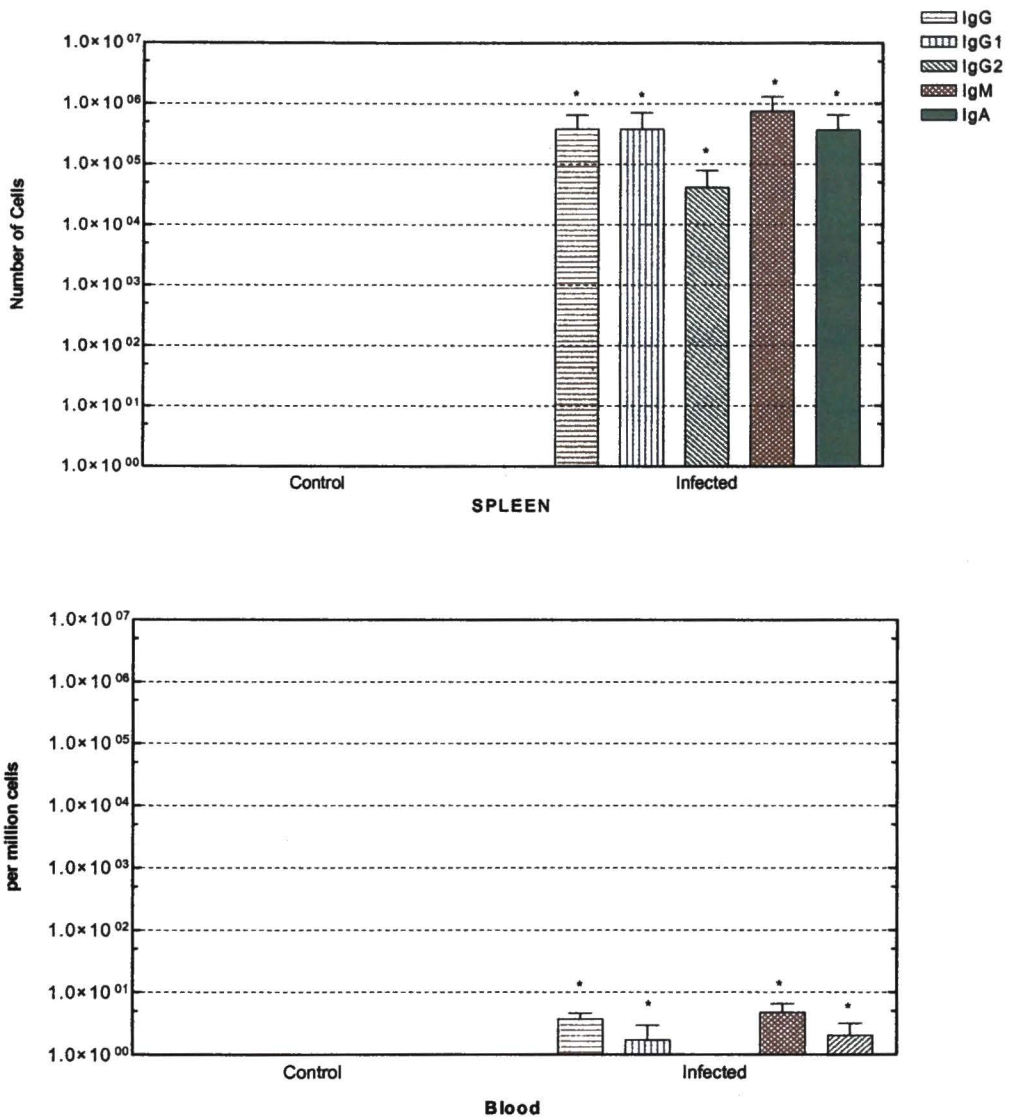

Figure 20. This data represents the antigen specific antibody production in response to *M. bovis* in the spleen and circulation. Each graph represents a total of five calves, three infected and two control animals. The data suggests a strong $CD4^+$ T cell dependent antibody response except within the blood, which indicates only trace levels of antibody producing cells. (* denotes statistical significance.)

Discussion: The purpose of this study was to determine the changes of T cell populations along the respiratory tract of calves infected with *M. bovis*. Previous studies have shown that T lymphocytes play pivotal roles in mycoplasma respiratory diseases [2-4, 26, 30, 32-34, 36, 37, 39, 40, 46-54]. A reliable characteristic of mycoplasma infections is the accumulation of lymphocytes along the respiratory tract. This suggests that lymphocytes are being activated and recruited to the sites of infection, depicting their role in the pathogenesis of mycoplasma respiratory disease. Studies using SCID mice (mice that lack functional B and T cells) and NUDE mice (mice that lack T cells) have demonstrated the importance of lymphocytes [4, 32, 34, 38, 42, 45]. When lymphocytes are removed from infected animals, the lung disease is less severe when compared to animal with functional immune systems. However, even though the lung disease was less severe, the number of organisms was unaffected.

Studies like these support the theory that lymphocytes, specifically T lymphocytes, play a significant role in the pathogenesis of mycoplasma diseases. Previous studies involving cattle suggest that the $\gamma\delta$-T cell subset may play important roles in bovine respiratory diseases [2, 3, 32, 45, 53, 55]. Studies that have investigated the immune response in cattle and observed that the predominant T cells populations that respond to mycoplasma infection are of the Th2 type as well. These studies however, have not looked at the total lymphocyte response along the respiratory tract. There were reports that T cells were the principal cell involved but no research to date had investigated the changes of the T cell populations along the respiratory tract of calves infected with *M. bovis*. Thus, the rationale behind this research was to determine the

principal population(s) involved in *M. bovis* infection along the respiratory tract. To investigate the changes in T cell populations after infection, the lymphocytes from various tissues of infected (N=3) and control (N=2) calves 14 days PI were characterized.

First, we can report that T cells within various tissues of infected calves are recruited or expand in response to *M. bovis*. Both the numbers and percentages of T cells increased in the tissues in and around the upper respiratory tract. The main population of T cells that responded to the infection was of the $CD4^+$-$\alpha\beta$-T cell subset. While the $\gamma\delta$-T cell subsets did show some increase, the $CD8^+$-$\alpha\beta$-T cell subset showed no significant increase. This suggests that CD4+ T cell populations dominate the URT environments of infected calves. This was reinforced in that humoral responses in the sites of infection are consistent with the generation of T dependent B cell responses. The increased numbers of cell secreting antigen-specific mucosal antibodies at the sites of infection are mainly IgA, IgG, and IgG1. IgG2 was at low levels in all the tissues investigated. This could indicate that the chronic nature of *M. bovis* is due to low numbers of IgG2 antibody secreting cells. The percentage of T cell subsets did not change within the spleen of infected calves, but there were antigen-antibody response with in spleen throughout all infected calves. Blood lymphocyte levels were monitored throughout the course of the infection. The percentages of T lymphocytes in infected calves did not change during the course of infection when compared to control calves. This was interesting because it was evident that there were T cells responding to the infection in the URT. This suggests that blood samples are not an accurate indicator of the type immune response that occurs within the respiratory tract of calves in response to an infection with *M. bovis*.

To determine the overall contribution of each of the respiratory lymphoid tissues to the response of an *M. bovis* infection, we also examined the total numbers of T cell populations in each tissue. The number of T cells along the respiratory tract was characteristic of mycoplasmal infections seen in both humans and mice. $CD4^+$ T cells were among the greatest in number, and in the lymph node that drains the mucosal surfaces, there were increases in $\gamma\delta$-T cell numbers. The spleen showed no changes in any of the T cell populations. However, there were larger numbers of resident $CD8^+ \gamma\delta$, and $CD8^+ \alpha\beta$-T cells in both control and infected calves. The lungs of infected calves showed slight increase in T cell populations. It was surprising to see an antibody response so large in the lungs. One possible reason for this is mucosal lymphocytes tend to recycle through-out other mucosal sites, so these antibodies may be re-circulating from the upper respiratory lymph nodes. Another reason for observing lymphocytes in tissues where no organisms were cultured is the model of infection used. The infection model used in the experiments was created to induce a URT infection only. Therefore, the immune cells seen at sites other than the URT may be due to the timing of the sampling during the infection process and the progression of the disease from the URT to the LRT. If the infection model effectively created an infection in both the URT and the LRT, the immune response could be different than that seen in the URT model.

Overall, there were changes in lymphocyte populations within the respiratory tract of calves infected with *M. bovis*. We were able to show and characterize the T cell populations within infected calves. This study set the stage to further study the immune response of calves and its interactions with *M. bovis*. More studies are needed however, to fully understand the T cell repertoire of the respiratory tract. Future studies will look at cytokine profiles to determine the cytokine environment of infected calves. This research provides the first kind of data that characterizes the T cell subsets and immune responses with in the respiratory tract of calves infected with *M. bovis*.

REFERENCES

1. Chaplin, D.D., *1. Overview of the immune response.* J Allergy Clin Immunol, 2003. **111**(2 Suppl): p. S442-59.
2. Born, W.K., et al., *Role of gammadelta T cells in protecting normal airway function.* Respir Res, 2000. **1**(3): p. 151-8.
3. Pollock, J.M. and M.D. Welsh, *The WC1(+) gammadelta T-cell population in cattle: a possible role in resistance to intracellular infection.* Vet Immunol Immunopathol, 2002. **89**(3-4): p. 105-14.
4. Jones, H.P. and J.W. Simecka, *T lymphocyte responses are critical determinants in the pathogenesis and resistance to mycoplasma respiratory disease.* Front Biosci, 2003. **8**: p. d930-45.
5. Magnan, A.O., et al., *Assessment of the Th1/Th2 paradigm in whole blood in atopy and asthma. Increased IFN-gamma-producing CD8(+) T cells in asthma.* Am J Respir Crit Care Med, 2000. **161**(6): p. 1790-6.
6. MacDorman, M.F., et al., *Annual summary of vital statistics--2001.* Pediatrics, 2002. **110**(6): p. 1037-52.
7. Kalra, R., et al., *Effects of cigarette smoke on immune response: chronic exposure to cigarette smoke impairs antigen-mediated signaling in T cells and depletes IP3-sensitive Ca(2+) stores.* J Pharmacol Exp Ther, 2000. **293**(1): p. 166-71.
8. Du, B.Y., *[Effects of smoking on human health].* Zhonghua Jie He He Hu Xi Za Zhi, 1989. **12**(5): p. 275-7, 318.
9. Moszczynski, P., et al., *Immunological findings in cigarette smokers.* Toxicol Lett, 2001. **118**(3): p. 121-7.
10. Scragg, R., M. Laugesen, and E. Robinson, *Cigarette smoking, pocket money and socioeconomic status: results from a national survey of 4th form students in 2000.* N Z Med J, 2002. **115**(1158): p. U108.
11. Mattoli, S., et al., *The role of CD8+ Th2 lymphocytes in the development of smoking-related lung damage.* Biochem Biophys Res Commun, 1997. **239**(1): p. 146-9.
12. Dow, L., et al., *The interaction between immunoglobulin E and smoking in airflow obstruction in the elderly.* Am Rev Respir Dis, 1992. **146**(2): p. 402-7.
13. Splettstosser, G., R. Schoneich, and W.D. Schneider, *[Effect of smoking on immunologic parameters].* Z Gesamte Hyg, 1990. **36**(12): p. 649-51.
14. Castro, M., et al., *Could asthma be worsened by stimulating the T-helper type 1 immune response?* Am J Respir Cell Mol Biol, 2000. **22**(2): p. 143-6.
15. Wahlgren, D.R., et al., *Involuntary smoking and asthma.* Curr Opin Pulm Med, 2000. **6**(1): p. 31-6.
16. Vinke, J.G., et al., *Passive smoking causes an 'allergic' cell infiltrate in the nasal mucosa of non-atopic children.* Int J Pediatr Otorhinolaryngol, 1999. **51**(2): p. 73-81.
17. Miescher, S.M. and M. Vogel, *Molecular aspects of allergy.* Mol Aspects Med, 2002. **23**(6): p. 413-62.

18. Singh, S.P., et al., *Prenatal cigarette smoke decreases lung cAMP and increases airway hyperresponsiveness.* Am J Respir Crit Care Med, 2003. **168**(3): p. 342-7.
19. Savage, S.M., et al., *Effects of cigarette smoke on the immune response. II. Chronic exposure to cigarette smoke inhibits surface immunoglobulin-mediated responses in B cells.* Toxicol Appl Pharmacol, 1991. **111**(3): p. 523-9.
20. Raghuram, A. and B.R. O'Driscoll, *Smoking and asthma.* Eur Respir J, 2000. **16**(3): p. 574-5.
21. Yamaguchi, E., Y. Kawakami, and N. Okazaki, *[Smoking and immunity allergy].* Kokyu To Junkan, 1990. **38**(1): p. 27-33.
22. Chung, F., *Anti-inflammatory cytokines in asthma and allergy: interleukin-10, interleukin-12, interferon-gamma.* Mediators Inflamm, 2001. **10**(2): p. 51-9.
23. Baseman, J.B. and J.G. Tully, *Mycoplasmas: sophisticated, reemerging, and burdened by their notoriety.* Emerg Infect Dis, 1997. **3**(1): p. 21-32.
24. Nicholas, R.A. and R.D. Ayling, *Mycoplasma bovis: disease, diagnosis, and control.* Res Vet Sci, 2003. **74**(2): p. 105-12.
25. Jones, H.P., et al., *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, 2001. **167**(8): p. 4518-26.
26. Hodge, L.M. and J.W. Simecka, *Role of upper and lower respiratory tract immunity in resistance to Mycoplasma respiratory disease.* J Infect Dis, 2002. **186**(2): p. 290-4.
27. Razin, S., D. Yogev, and Y. Naot, *Molecular biology and pathogenicity of mycoplasmas.* Microbiol Mol Biol Rev, 1998. **62**(4): p. 1094-156.
28. Thomas, A., et al., *Isolation of mycoplasma species from the lower respiratory tract of healthy cattle and cattle with respiratory disease in Belgium.* Vet Rec, 2002. **151**(16): p. 472-6.
29. Stipkovits, L., et al., *Clinical study of the disease of calves associated with Mycoplasma bovis infection.* Acta Vet Hung, 2000. **48**(4): p. 387-95.
30. Born, W.K., et al., *The role of gamma delta T lymphocytes in infection.* Curr Opin Immunol, 1991. **3**(4): p. 455-9.
31. Aoyagi, M., et al., *Respiratory syncytial virus infection suppresses IFN-gamma production of gammadelta T cells.* Clin Exp Immunol, 2003. **131**(2): p. 312-7.
32. Kennedy, H.E., et al., *Modulation of immune responses to Mycobacterium bovis in cattle depleted of WC1(+) gamma delta T cells.* Infect Immun, 2002. **70**(3): p. 1488-500.
33. Vanden Bush, T.J. and R.F. Rosenbusch, *Mycoplasma bovis induces apoptosis of bovine lymphocytes.* FEMS Immunol Med Microbiol, 2002. **32**(2): p. 97-103.
34. Cartner, S.C., et al., *Roles of innate and adaptive immunity in respiratory mycoplasmosis.* Infect Immun, 1998. **66**(8): p. 3485-91.
35. Cartner, S.C., et al., *Chronic respiratory mycoplasmosis in C3H/HeN and C57BL/6N mice: lesion severity and antibody response.* Infect Immun, 1995. **63**(10): p. 4138-42.

36. Mena, A., et al., *Th1/Th2 biasing effects of vaccination in cattle as determined by real-time PCR.* J Immunol Methods, 2002. **263**(1-2): p. 11-21.
37. Vanden Bush, T.J. and R.F. Rosenbusch, *Characterization of the immune response to Mycoplasma bovis lung infection.* Vet Immunol Immunopathol, 2003. **94**(1-2): p. 23-33.
38. Jones, H.P., et al., *Depletion of CD8+ T cells exacerbates CD4+ Th cell-associated inflammatory lesions during murine mycoplasma respiratory disease.* J Immunol, 2002. **168**(7): p. 3493-501.
39. Howard, C.J., R.N. Gourlay, and G. Taylor, *Immunity to Mycoplasma bovis infections of the respiratory tract of calves.* Res Vet Sci, 1980. **28**(2): p. 242-9.
40. Howard, C.J., L.H. Thomas, and K.R. Parsons, *Immune response of cattle to respiratory mycoplasmas.* Vet Immunol Immunopathol, 1987. **17**(1-4): p. 401-12.
41. Surovas, V.M. and V.I. Tamoshiunas, *[Immunological study of the T- and B-lymphocytes in normal cows and in chronic lympholeukemia].* Tsitologiia, 1986. **28**(4): p. 437-42.
42. Simecka, J.W., J.K. Davis, and G.H. Cassell, *Specific vs. nonspecific immune responses in murine respiratory mycoplasmosis.* Isr J Med Sci, 1987. **23**(5): p. 485-9.
43. Hodge, L.M., et al., *Immunoglobulin A (IgA) responses and IgE-associated inflammation along the respiratory tract after mucosal but not systemic immunization.* Infect Immun, 2001. **69**(4): p. 2328-38.
44. Yeung, V.P., et al., *Heat-killed Listeria monocytogenes as an adjuvant converts established murine Th2-dominated immune responses into Th1-dominated responses.* J Immunol, 1998. **161**(8): p. 4146-52.
45. Skeen, M.J., et al., *Exaggerated proinflammatory and Th1 responses in the absence of gamma/delta T cells after infection with Listeria monocytogenes.* Infect Immun, 2001. **69**(12): p. 7213-23.
46. Wilson, R.A., et al., *T-cell subsets in blood and lymphoid tissues obtained from fetal calves, maturing calves, and adult bovine.* Vet Immunol Immunopathol, 1996. **53**(1-2): p. 49-60.
47. Kennedy, H.E., et al., *The role of WC1(+) gamma delta T-cells in the delayed-type hypersensitivity (DTH) skin-test reaction of Mycobacterium bovis-infected cattle.* Vet Immunol Immunopathol, 2003. **93**(3-4): p. 169-76.
48. Born, W., et al., *Immunoregulatory functions of gamma delta T cells.* Adv Immunol, 1999. **71**: p. 77-144.
49. Sopp, P. and C.J. Howard, *IFN gamma and IL-4 production by CD4, CD8 and WC1 gamma delta TCR(+) T cells from cattle lymph nodes and blood.* Vet Immunol Immunopathol, 2001. **81**(1-2): p. 85-96.
50. Svensson, L., et al., *gammadelta T cells contribute to the systemic immunoglobulin E response and local B-cell reactivity in allergic eosinophilic airway inflammation.* Immunology, 2003. **108**(1): p. 98-108.
51. Wilson, E., M.K. Aydintug, and M.A. Jutila, *A circulating bovine gamma delta T cell subset, which is found in large numbers in the spleen, accumulates inefficiently in an artificial site of inflammation: correlation with lack of*

expression of E-selectin ligands and L-selectin. J Immunol, 1999. **162**(8): p. 4914-9.

52. Wilson, E., et al., *Bovine gamma delta T cell subsets express distinct patterns of chemokine responsiveness and adhesion molecules: a mechanism for tissue-specific gamma delta T cell subset accumulation.* J Immunol, 2002. **169**(9): p. 4970-5.
53. Baldwin, C.L., et al., *Activation of bovine peripheral blood gammadelta T cells for cell division and IFN-gamma production.* Vet Immunol Immunopathol, 2002. **87**(3-4): p. 251-9.
54. Welsh, M.D., et al., *Responses of bovine WC1(+) gammadelta T cells to protein and nonprotein antigens of Mycobacterium bovis.* Infect Immun, 2002. **70**(11): p. 6114-20.
55. Thomas, A., et al., *Adherence of Mycoplasma bovis to bovine bronchial epithelial cells.* Microb Pathog, 2003. **34**(3): p. 141-8.