
UNIVERSITY OF NORTH TEXAS
HEALTH SCIENCE CENTER AT FORT WORTH
1970

Senne, Jordan. Dendritic Cell and Macrophage Activation and Maturation in Response to Mycoplasma. Master of Science (Microbiology and Immunology), July 2003. 47 pp., 7 illustrations.

Murine respiratory mycoplasmosis, caused by *Mycoplasma pulmonis*, offers a model for studying the immunopathogenic mechanism of mycoplasma respiratory disease. My focus was to determine the effects of *M. pulmonis* on dendritic cells and macrophages. I demonstrated an increase in dendritic cell numbers and cell surface marker expression within the lungs of infected mice. However, there was no increase in the studied cytokine mRNA levels. *In vitro* studies determined increases in cytokine proteins and cytokine mRNA levels in dendritic cell and macrophages without a significant increase in cell surface marker expression by either cell line.

Thus, a mycoplasma infection is able to stimulate dendritic cells both *in vivo* and *in vitro*, while macrophages are stimulated *in vitro*. Such findings could be helpful towards the development of future mechanisms for therapy against mycoplasma respiratory diseases.

DENDRITIC CELL AND MACROPHAGE ACTIVATION AND MATURATION IN RESPONSE TO MYCOPLASMA

Jordan Elliott Senne, B.S.

APPROVED:

Major Professor

Committee Member

Committee Member

University Member

Chair, Department of Immunology and Microbiology

Dean, Graduate School of Biomedical Sciences

DENDRITIC CELL AND MACROPHAGE ACTIVATION AND MATURATION IN RESPONSE TO MYCOPLASMAS

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

Jordan Senne

Fort Worth, Texas

August of 2003

ACKNOWLEDGEMENTS

There are so many people that I have to thank. First, I would like to thank Dr. Simecka for allowing me to perform my graduate studies in his lab. I appreciate his guidance, patience and kindness. I would also like to thank Dr. Harlan Jones for his guidance and for taking me under his wing. To all my lab mates, thank you so much for your help and your encouraging presence. Drew, Xiangle, Doc "William", Harlan, Stephanie, and Greg you all made the lab such an enjoyable place to work, and prevented me from loosing my mind. I won't forget our laughter or our ping-pong matches.

Next I would like to thank my family. I am blessed with a family that is not only encouraging, but also excepting and that means so much to me. Mom-Dad thank you for always supporting me and for your guidence, but mainly for always showing your love. I love you all as Big As the World.

I would like to thank my friends. Sarah Swaim, thank you for showing me the potential that I had in myself, you opened my eyes to so many other possibilities in life and helped me keep my head on straight. Sarah Bubeck, thank you for always supporting me and lending a kind ear for me to rant and rave into. I would also like to thank all the wonderful friends I have made here in Ft. Worth. You all truly made my time here so enjoyable.

Finally, I think Winnie-the-Pooh said it best when he turned to Christopher Robin and told him, "I will never not remember you." That sums up how I feel about everyone I have met, I will never not remember you or your kindness. Thanks.

TABLE OF CONTENTS

LIST OF TABLES AND ILLUSTRATIONS

Figures: Page

Chapter 1

INTRODUCTION

Mycoplasmas, specifically *Mycoplasma pneumoniae*, are a major cause of respiratory pneumoniaes in humans worldwide (1-1,14,18-20,25). It is estimated that nearly 2 million cases of *M. pneumoniae* are reported each year, with almost 100,000 of those cases resulting in hospitalization in the United States alone (26). Murine respiratory mycoplasmosis (MRM), caused by *Mycoplasma pulmonis*, is a naturally occurring respiratory ailment of mice that offers a model for studying the pathogenic mechanism of mycoplasma respiratory disease (3,5). Our lab has demonstrated that a component of *M. pulmonis* disease is immunopathologic, and T cells play a central role in the severity of the disease (8,5,2,10). It has also been determined that T cell numbers are increasing and T cell sub-populations are changing in the lung environment in response to *M. pulmonis* (8,10). Due to these significant changes occurring in adaptive immunity, we believe that the next logical step is to look at changes that are occurring in innate immunity, specifically changes in dendritic cells and macrophages. Since macrophages and dendritic cells are major antigen presenting cells of innate immunity it would be prudent to examine variations in these cell populations and how those changes could in turn influence adaptive immunity in the lungs of infected mice (11-14,18-20). It has been suggested that these cells could have influential roles in determining not only the severity of mycoplasma and other diseases but also the pathway that the adaptive immune system takes in attempting to resolve the infection (8,10, 13-16,18-20).

The purpose of this study is to determine the effects that a *M. pulmonis* infection has on dendritic cell maturation and activation. We also examined effects of *M. pulmonis* on macrophages *in vitro*. Both dendritic cells and macrophages, which are cells of the innate immune system, are one of the first cells to encounter antigens upon entry into the lung (17,12). It is also known that both of these cell types are active antigen presenting cells, which implies that they both play a direct or indirect role in determining the manner by which the adaptive immune system initially comes into contact with antigens (11-21). It was suggested that dendritic cells and possibly macrophages can greatly influence T cell differentiation in dealing with the disease (13,14,16,12). Our lab has demonstrated that there is significant evidence of mixed populations of $CD4^+$ T helper cell sub-populations developing in lungs of *M. pulmonis* infected mice (8). Since T cells are presented antigens from various arms of the innate immune system the next logical step is to investigate dendritic cells and macrophages.

There are two other key factors that have made it worthwhile to examine the innate immune system. First, it has been demonstrated that in severe combined immunodeficient (SCID) mice infected with mycoplasma one there are no significant changes in the number of mycoplasma organisms located in the lung as compared with immuno-competent mice (2,4,5). Another interesting phenomenon seen in the SCID mice was a decrease in the severity of the disease (2,4,5). Both facts suggest that perhaps it is the innate immune system that is the key factor in determining the outcome of the severity of the disease (11,12,17,19,20).

Another task of this study is to lay down appropriate guidelines for future studies by determining the roles that dendritic cell and macrophage populations play during the pathogenesis of murine respiratory disease. In the remainder of this chapter we will further discuss murine respiratory disease, dendritic cells, macrophages and T cell responses.

Mycoplasma Respiratory Disease

Mycoplasma are characterized as prokaryotic, extra-cellular parasites lacking a cell wall (25). It is believed that mycoplasma are derived from *Bacillus* and *Lactobacillus* lineage and over time evolved to lose their cell wall (25). *M. pneumoniae* was first recognized in humans in the 1930's, though mycoplasma were known to cause disease in various animal species (25). As with many mycoplasma species, the level of cellular interaction is currently unknown, but most species are known to be extra cellular pathogens. (7,25). There is, however, close association of the organism with the host cells and this interaction has detrimental effects on the respiratory tract and the health of the host (1,7,25). Mycoplasmas cause cellular damage through various mechanism including nutrient deprivation, altering host cellular components and metabolites, producing hydrogen peroxide, and variance in phenotype which adds in immune evasion (1,7). Mycoplasmas contain a lipoprotein which can have effects analogous to LPS that may induce host cellular responses and induce inflammation and subsequent cellular damage (7). Combinations of these characteristics are not only detrimental to the host, but may also aid in stimulating the immune system in such a way as to cause further damage to the infected organism.

Specific clinical characteristics of a *M. pneumoniae* infection have been difficult to identify in the past simply due to the numerous similarities it shares with virally induced flu, and lack of an appropriate means of identification (25). However, an infection usually consists of airway inflammation, with a gradual to slow onset of coughing, chest pain and sputum production (25). Another important factor in dealing with a *M. pneumoniae* infection is its capability to exacerbate the effects of other ailment such as asthma and allergies (25). Further study and understanding of this organism with its relationship to the immune system is needed. *M. pulmonis* is a naturally occurring disease of mice and exhibits a similar pathology with the *M. pneumoniae* (18-20, 25). Murine respiratory disease, caused by *M. pulmonis*, offers an ideal model to examine and better understand the immune response occurring. Thus, it is prudent to study the interactions that are occurring between *M. pulmonis* and particular branches of the immune system, particularly those of innate immunity.

T cell Response

T cells are the major component used by the bodies' immune system to combat infection. They aid in determining how a pathogen will be dealt with. There are three major pathways in which T cells can be stimulated and/or differentiate into. These pathways are influenced by the manner in which the pathogen is presented to the T cell and also the type and level of cytokines produced at the time of presentation. One pathway occurs when the body is exposed to intracellular pathogens, such as a viral infection (24). When this type of infection occurs, portions of the pathogen are processed by the infected cell and presented on the cell surface in a MHC I molecule (24). This presentation of the

antigen on the MHC I will stimulate $CD8^+$ T cells also known as cytotoxic cells (24). The second major pathway deals with extra cellular pathogens. Components of the pathogen are internalized and processed by specific cells possessing MHC II and presented to $CD4^+$ T cell populations causing a Th2 response, which consists of the antigen presenting cell (APC) producing IL-10 (24). A Th2 response deals with B cell activation and antibody recognition of the pathogen and aids portions of the immune system in recognizing and dealing with the infection appropriately (24). The third major branch of T cell activation deals with differentiation and activation into a $CD4^+$ Th1 response. Th1 responses occur via intracellular pathogen infection and MHC II presentation with the APC producing IL-12 and TNF-α (24). Th1 responses help to effectively stimulate macrophages to fight the infection with increased specificity and vigor.

Previous studies done in our lab have proven that T cells are being activated and play a major role in determining the outcome and pathology of a *M. pulmonis* infection (8). Infected murine lungs were collected and showed an increase in T cell numbers (8). It was demonstrated that early in the infection, between day 0 and day 7, there was predominantly a Th2 T cell population located in the lung (9). However, by day 14 post infection, isolations revealed that the lung T cell environment had switched to a mixed population of Th2 and Th1 (9). It was also demonstrated that in $CD4^+$ T helper cell depleted mice the severity of the disease decreased, while depletion of $CD8^+$ cells exacerbated immunopathogenesis (8). These findings suggest that $CD8^+$ T cells may dampen inflammatory responses (8). Another example of immunopathology was

determined in C3H SCID mice which lack functional T and B cells (5). In these mice the severity of the disease decreased, suggesting that an aspect of the adaptive immune response to the infection is responsible in the development of pathology seen in murine respiratory disease (5). These results give insight into the fact that depending on the manner by which T cells, specifically $CD4^+$ T cells, are stimulated may determine how the pathogen is dealt with and the immunopathogenic characteristics of this infection. Therefore, further studies are needed to evaluate the manner by which $CD4^+$ T cells are becoming activated, specifically those interactions with antigen presenting cells of innate immunity.

Dendritic Cells

Dendritc cells play a central role in not only recognizing potential pathogens but also transporting and displaying their antigens to various branches of the immune system, including T cell populations (3,11,12). They are located throughout the body, including the lung, constantly patrolling tissue in search of pathogens and sampling self antigens (11,12,15). Another important aspect of dendritic cells is their ability, in an immature state, to promote tolerance among peripheral T cell populations (11,12).

Dendritic cells are the most potent antigen presenting cells of the innate immune system and even in an immature state, display a high level of receptors that allow for necessary uptake of antigen (3,11,12). It has been noted that different dendritic cell subpopulations can have varying effects on the type of T cell response, especially with regard to differentiation of these cells (11,12,14,15,13). As previously stated these cytokines can preferentially induce different T cells responses. Dendritic cells, during

respiratory disease, could be driving T cell responses that would contribute to immunopathogenesis, and studies in our lab have documented an increase in lung dendritic cell numbers after infection with *M. pulmonis*.

The current proposal is to determine the effects that *M. pulmonis* has on the cytokine and co-stimulatory molecules produced by dendritic cells. Co-stimulatory molecules such as CD11c, CD11b, CD80, CD86 and MHCII can all give insight into the possible activity occurring in dendritic cell populations. CD11c is a marker that is predominantly associated with dendritic cells and can infer maturation (24). $CD11b^+$ is associated with the ancestry of the dendritic cell which is that of a myeloid lineage that induces Th2 responses (11,24). Both CD80 and CD86 are co-stimulatory that are necessary to properly activate T cells. (24). Finally, MHCII is the major histocompatibility receptor responsible for presenting exogenous antigen to Th2 T cell populations (24). Both, cytokine and receptor levels, can have profound effects on T cell response and differentiation (13,16,15,14). The three main cytokines that will be monitored are IL-10, IL-12, and TNF-α. As stated above IL-10 will cause a Th2 shift in $CD4^+$ T cells, whereas IL-12 and TNF-α will initiate a Th1 shift.

Determining these interactions may help with future studies and an effective treatment in dealing with this type of *M. pulmonis* infection. One particular study demonstrated that $CD8^+$ dendritic cells presented antigen to T $CD8\alpha^+$ cells, while $CD8\alpha^-$ dendritic cells were able to present antigen to CD 4^+ T cells (14). These findings suggest that dendritic cells can have a significant role in not only the initiation of immunity but also the pathway that is taken during disease development. Specific $CD4^+$ Th cell

responses canbe driven by differential cytokines produced and co-stimulatory markers expressed by dendritic cells (27). Currently, little is known of the effect *M. pulmonis* has on dendritic cell maturation and activation. Since, as stated above, the differentiation of the T cell response can have significant effects on the outcome of the infection it is important to examine the interactions of *M. pulmonis* with this antigen presenting cells. This will aid in determining how a mycoplasma infection influences the ability of dendritic cells to generate a T cell, specifically $Cd4^+$ T helper cells, response during disease pathogenesis.

Macrophages

Macrophages, especially alveolar macrophages (AM,) are important cells of the innate immune system defense in the lung airways (28). They will most likely be the first cells to come into contact with pathogens entering the lung and are the main form of phagocytic clearance of lung contaminants (17). Reports have documented the importance of early macrophage engagement in decreasing instances of chronic mycoplasma infections and that their depletion has been shown to exacerbate disease severity (19,20).

Upon entry of a pathogen the alveolar macrophages will implement phagocytotic mechanisms along with nitric oxide or respiratory bursts to clear and kill the organism (19, 28). However, if the alveolar macrophages are unable to clear the pathogen one of two mechanisms ignite development of adaptive immunity. One mechanism is for the alveolar macrophages to begin antigen presentation via MHCII molecules to T cells directly (28). Another manner of adaptive activation is simply via cellular damage that is

caused by the pathogen itself. Macrophages are capable of releasing specific cytokines, such as TNF-α, that will help in recruiting additional cells of the immune system, such as dendritic cells, to the sight of the infection (28). We hypothesize that macrophages are, on some level, being activated and maturing upon contact with *M. pulmonis in vitro.* Since macrophages are most likely the first cells to come into contact with respiratory antigens, we believe it beneficial to look at possible changes occurring in these cells. Macrophages could possibly play a role in not only T cell differentiation but perhaps in dendritic cell activation as well (17). As stated above, we will monitor specific cytokine production of IL-12, IL-10 and TNF-α. Cytokine production and increased receptor expression by these cells could be influential factor in not only the immune response taken, but perhaps the severity of the infection itself. Furthermore, we believe that macrophage interactions with *M. pulmonis* could have profound effects on the T cell response and contribute to subsequent severity of the disease.

We believe that dendritic cells are being activated both *in vitro* and *in vivo* by *M. pulmonis* and macrophages are being activated *in vitro*. We want to better specify this activation through monitoring of cytokine production, mRNA levels, and cell surface marker levels. Through these studies we will be better able to understand the influence that these antigen presenting cells could be having on the adaptive immunity, specifically T cells in combating mycoplasma disease.

In vitro studies with the dendritic (JAW) cell line and macrophage (J774) cell line will be used to study cytokine production levels post stimulation with *M. pulmonis* and *M.pulmonis* inactivated membrane antigen. Cytokine protein levels will be

determined using ELISA analysis while cytokine mRNA levels will be determined via Real Time PCR. Also, specific cell surface markers such as CD11c, CD11b, CD80, CD 86 and MHC II will be monitored to aid in the determination of the maturation level of the cells.

In vivo studies included infection of C3H mice and determination of dendritic cell number changes by day 14-post infection. Dendritic cells were also isolated from the lung and cell surface marker changes were determined between day 0 and day 14. Specific cell surface markers monitored included CD11c, CD11b, CD80, CD86, and MHC II. Finally, $CD11c^+$ dendritic cells were collected from the lungs of infected mice and real time PCR was performed to determine specific mRNA cytokine levels, IL-10, IL12, and TNF-α of these cells.

These studies will help us to gain a better idea of the factors that lead up to the immune system fighting a mycoplasma infection. It will also aid in determining the differences between particular innate branches of the immune system with those of the adaptive immunity and how they may be influential towards one another. In all, these findings will aid in understanding mycoplasma disease and possibilities for appropriate treatment of the disease.

Chapter 2

MATERIALS AND METHODS

Mycoplasma culture

The UAB CT strain of *M. pulmonis* was used in all experiments. Stock cultures were grown, as previously described (6) in mycoplasma broth and frozen in 1-mL aliquots at -80°C. For inoculation, thawed aliquots containing 4.9 x 10^9 colony forming units/mL of *M. pulmonis* were diluted to various concentrations as needed.

Animals

7-8 week old uninfected C3H mice were purchased from Jackson laboratory. One week was given to allow the mice to become acclimated to the environment. Mice were first anesthetized with a Ketamine/Xylene and then infected with 1x10^5 CFU/ 20 μL of *M. pulmonis* intra-nasally. Mice were then sacrificed with a lethal dose of Ketamine/Xylene 14 days post infection.

Lung Monocyte Cell Isolation

5-7 mice were sacrificed and lungs were taken. Lungs were perfused with PBS (no C^+ or Mg^+) which will minimize contamination by blood cells. Lung lobes were then separated and cut into fine pieces. The tissue was then suspended in RPMI 1640 (Hyclone Laboratories, Logan, UT) medium which contains 300 U/mL *Clostridium histolyticum* type I collagenase (Worthington Biomedical, Freehold NJ), 50 U/mL DNase (Sigma-Aldrich, St. Louis, MO), HEPES, 10% FBS (Hyclone Fetal Bovine Serum) and

antibiotic/antimyotic solution (Life Technologies, Grand Island NY). Lungs were incubated with this solution at 37°C while being mixed with a Nutator (Fisher Scientific, Pittsburgh, PA) for 2 hrs. During the 2 hour incubation, the tissue was shaken every 20 minutes. Once incubation was complete the remaining tissue was filtered through 250 μm nylon mesh to remove any unwanted product.

Density Gradient Purification of Lung Dendritic Cells

Dendritic cells were then isolated using OptiPrep™ separation. Cells were isolated into a 15 mL conical tube and washed 2-3 times with wash media to remove any residual collagenase. The first, and bottom layer, of the density gradient consisted of a 3:1 mixture of suspension fluid (Hank's Balanced Salt 1x Solution without Ca^{2+} and Mg^{2+}) and 60% iodixanol or OptiPrep™ . The cells and the mixture were thoroughly mixed. The next, middle layer, consisted of an 11.5% mixture (1:4.2) of OptiPrep and dilutent (0.88% (w/v) NaCl, 1 mM EDTA, 0.5% (w/v) bovine serum albumin, 10 mM, Hepes-NaOH, ph 7.4.). The mixture was carefully added in to ensure that there was no mixing of the two layers as this would be detrimental to cell isolation. The final top layer was 3 mL of Hank's 1x salt solution. The cells were then centrifuged at 600 x g for 20 minutes. Dendritic cells were isolated from the top gradient between the 11.5% solution and Hanks 1x Salt solution. Wash media was then added and the cells were counted for subsequent purification.

Purification CD11c$^+$ of Lung DC

DYNAL CELLection™ biotin binder kit with strepavidin coated magnetic beads (Dynal Inc. Lake Success, NY) and biotinylated anti-CD11c+ antibodies (BD Bioscience) were used to isolate CD11c$^+$ DC from lung tissue. Prior to use the magnetic beads were washed in 100 µL of PBS-1% tween 20 (Polyoxyethylene-Sorbitan, Monolaurarte, Sigma Chemical Company) 2-3 times. The beads and PBS-tween were mixed and placed on the Dynal MPS magnet (Dynal Inc. Lake Success, NY), and once the beads were pulled to the side the remaining PBS-tween was removed.

Next, the magnetic beads were coated with the biotinylated anti-CD11c$^+$ antibody (BD Bioscience). For every 100 µL suspension of magnetic beads 1 µL of anti-CD11c$^+$ antibody was added. The beads and the anitbodies were rotated at room temperature for 30 minutes to ensure proper binding. The bead and bound antibody were then placed onto the MPS magnet and wash 3-4 times with 1 mL PBS-1% Tween 20. Once washing was complete the beads and antibody were placed in PBS with 0.1% BSA (bovine serum albumin).

Cell samples were re-suspended in PBS-tween to a concentration of 5-20 x10^6 cells/mL. The beads, with attached antibodies, were added to the cell suspension and gently mixed, constantly at a temperature of 2-8°C for 15 minutes. Once the incubation was complete, the beads were placed back onto the magnet and the supernatant removed. Next the beads, with attached cells, were washed 2 times with 500 µL of RPMI 1640 with 1% FBS (fetal bovine serum). After the final wash the cells were placed into 200 µL of RPMI with

1% FBS that had been warmed to 37°C and 4 μL of releasing buffer was added which would facilitate the removal of the beads from the cells. It was necessary to remove the beads since the cell samples were to be used for real time PCR. The beads may produce a luminescence that could interfere with the PCR readings. Releasing buffer was incubated with the cells for 15 minutes at room temperature while being slowly rotated. The cells were then pipetted with force to completely mix the cells and the beads and the tube was placed back onto the magnet. The supernatant, now containing the released cells, was removed and placed into a new tube with RPMI containing 10% FBS (fetal bovine serum). The isolated cells were centrifuged at 200 x g for 10 minutes, supernatant decanted off, and 1 mL of Trizol RNA (Invitrogen/Life Technologies Carlsbad, CA) was added for RNA isolation.

Cell culture

Dendritic (JAW) cell line and macrophage (J774) cell line were both purchased from the American Tissue Collection Center. Cells were thawed and cultured in 10 mL of cell media. For the dendritic cell line, culture medium consisted of 32 mL of sterile filtered Minimal Medium Alpha Medium (Gibco), 400 μL of 100mM sodium pyruvate (Hyclone Logan, UT), 8 mL FBS (Hyclone Logan, UT), and 5 ng/mL of GM-CSF (Biosource). Cells were allowed to grow for four days in a Greiner 200 mL flask, upon which media was changed. The dendritic JAW cell line consists of cells that both attach to the flask and that are free floating. After four days of incubation, medium was collected and put on ice. Remaining attached cells were scraped off the surface of the bottom of the cell flask. After cell detachment, the cell mixture was added to original medium taken off.

This was then taken and centrifuged for 10 min x 200 g. Excess medium was poured off and the pellet of cells was re-suspended in fresh medium as described above. Cells were either re-cultured or used for experiments.

J774 macrophage cell line was cultured in 10 mL RPMI (Dulbecco's Eagle Medium) with 10% FBS (Hyclone Logan, UT). Cells were allowed to grow for two days upon which medium was changed. The J774 macrophage cell line also consists of cells that both attach and are free floating cells that were removed by being scraped off the bottom of the Greiner 200 mL flask. The flask was then rinsed with the original medium to remove scraped cells. Cells were then combined and spun down for 10 min x 200 g. Excess media was then poured off and the pellet of cells was re-suspended in fresh medium.

In vitro cell line stimulation

Once cells numbers reached appropriate levels, 4.0-6.0 x 10^7, they were collected, scraped off the flask, and centrifuged. The cell pellet was re-suspended in media for cell count. The desired concentration of cells per plate well was 2.5 x 10^5. Once cells were counted, they were again centrifuged and the appropriate amount of media was added to elicit 2.5 X 10^5 cells per 500 μL of media. In preliminary experiments, varying concentrations of *M. pulmonis* were used. To our knowledge, there are no previous papers documenting appropriate levels of *M. pulmonis* to use in dendritic cell or macrophage cell line stimulation. After several cell stimulation experiments with dendritic cells, it was determined that a 1 x 10^6 colony forming units/ mL concentration

of *M. pulmonis* was sufficient in stimulating the cells. *M. pulmonis* concentrations were diluted in cell culture medium to defer variability. Cells were also stimulated with 50 μg/mL of crude, inactivated *M. pulmonis* membrane. LPS was used as a positive control at a concentration of 100 ng/ml. Medium alone was used as a negative control. All wells were completed with a total volume of 1mL with stimulant and cells. Both dendritic cells and macrophages were stimulated for approximately 24 hours and the supernatants (SN) were collected for cytokine determination by ELISA assays. The remaining cells were either treated with 1 mL of Trizol RNA (Invitro/Life Technologies Carlsbad, CA) for RNA isolation and cytokine detection or collected and stained for maturation marker developments through FLOW cytometry.

Cytokine ELISA

ELISA kits for IL-10, IL-12 (p-40), and TNF-α (mono-mono) detection were purchased from BD PharMingen. Assays were carried out according to manufactor's protocol. Supernatants from stimulated dendritic and macrophage cells were collected. Specific antibodies, IL-10, IL-12, and TNF-α were diluted in coating buffer to concentrations of 1:250. Coating buffer consisted of 50 mL of DiH_2O with 0.59 grams of Na_2HPO_4 and 0.80 grams of NaH_2PO_4 and pH to 6.5. Microtiter plates (Falcon Pro-Bind ELISA Assay Plates) were coated with cytokine/coating buffer dilutions and sealed over night at 4^0C. Plates were washed 3 times with PBS 0.5% Tween 20 excess liquid was tapped off onto towels. To prevent any non-specific binding plates, were coated with PBS 10% FBS (Hyclone Logan, UT), sealed and allowed to incubate for an hour at room temperature. Plates were washed 3 times with PBS 0.5% Tween 20 excess liquid was tapped off onto

towels. 100 μL supernatants samples were collected and placed into appropriate well along with specific cytokine standards. Standards were diluted as follows IL-12 (1000 pg/ml), TNF-α (1000 pg/ml), IL-10 (2000 pg/ml). A serial dilution by two was done until 10 standard dilutions were completed. 100 μL of each standard was placed in appropriate wells. Both standards and samples were left in cellophane wrapped plates at room temperature for two hours. Plates were washed 5 times with PBS 0.05% Tween 20 and excess PBS was tapped off onto a towel. Working detector was prepared by making a 1:250 of detection antibody and adding an additional 1:250 dilution of Avidin HRP into blocking buffer. The solution was then mixed and 100 μL was added to each well of the plate. The plate was sealed and set at room temperature for 1 hour. Plates were washed 7 times with 30-second soaks with PBS 0.05% Tween. Excess solution was tapped off. Substrate solution tetramethylbenzidine (TMB) Peroxidase Solution was added at 100 μL per well and incubated in sealed plates at room temperature for 30 minutes. Plates were then read using MX-80 microplate reader and Dynatech Revelation 2.0 Program (Dynatech Laboratories, Inc). Initial readings were taken at a wavelength of 630. Stop solution made of 2 N H_2SO_4 and 50 μL was applied to the plates. Plates were immediately read by Dynatech Revelation 2.0 Program at a wavelength of 450.

Flow Cytometry Staining

In vitro cell surface molecules of dendritic cells and macrophages were characterized by two color staining. PE (BD PharMingen, San Diego, Ca) labeled anti-CDllc$^+$ was used to stain all cells and identify the appropriate dendritic cell populations. Cells were then stained with FITC-labeled anti-CDllb$^+$, MHC II, CD80$^+$ or CD86$^+$ antibodies (BD

PharMingen, San Diego, Ca). Cells were stained for 30 minutes at a 1:200 dilution at 4°C. Cells were then centrifuged and washed with staining buffer (Mg^{++} and Ca^{++} free PBS plus 0.05% Na_2 Azide, 1% FBS) and re-suspended in 500 mL of staining buffer and until analysis.

In vivo dendritic cells were stained as described above. However, and additional stain of PerCb labeled anti-CD8 (BD PharMingen, San Diego, Ca) was also included. Cells were again stained for 30 minutes at a 1:200 dilution at 4°C. Cells were then centrifuged and washed with staining buffer (Mg^{++} and Ca^{++} free PBS plus 0.05% Na_2 Azide, 1% FBS) and re-suspended in 500 mL of staining buffer and until analysis.

Cells were examined by EPICS XL-MCL flow cytometer (Beckman-Coulter). Cells were analyzed using System 2 software, and specific data was collected using Expo 2 software analysis (Beckman Coulter).

Mycoplasma pulmonis membrane culture

For mycoplasma culture medium, 21 grams of PPLO Broth (without Crystal Violet), 0.2 grams of Deoxribonucleic Acid (Sigma) and 4 mL of a 1% Phenol red were added to 780 mL of DiH_2O. The medium was fully mixed, and the pH was set to 7.5. The broth was then autoclaved for 20-25 minutes for sterilization. Once autoclaving was complete, the broth was then put into 50°C water bath and allowed to cool. Equine Serum (Hyclone Logan, UT) was thawed and 200 mL was filtered with a 0.22 μm Steritop GP Express PLUS membrane. A 50% dextrose (Fisher Scientific) solution was prepared (25 grams of dextrose to 50 mL of DiH_2O). For the 1 liter medium preparation, 200 mL of equine

serum was added, 10 mL of the 50% dextrose and 2 vials of *M. pulmonis* at a concentration of $1x10^7$. The culture was allowed to grow for 5-7 days at 37°C.

Crude preparations of *M. pulmonis* membrane were isolated as previously described (4). The 1 liter culture of *M. pulmonis* was evenly distributed into four sterile 250 mL centrifuge bottles and centrifuged for 20 minutes at 10,000 rpm (20°C). The mycoplasma was re-suspended in 20 mL of sterile 0.25 M NaCl (OmniPur EM Science) and placed in evenly into 4 sterile 30 mL centrifuge tubes. The tubes were then centrifuged for 20 minutes at 9,000 rpm (20°C). The supernatant was removed, and the pellet was re-suspended into 20 mL of 2M gycerol (FisherBiotech, Enzyme Grade), 5 mL going into each 30 mL tube. Each tube was then sonicated using a Vibra cell sonicator (Sonic and Materials/Vibrio Cell, Newton, CT) at the highest setting for 15-30 seconds, and then placed into the incubator (37°C) for 10 minutes. 25 mL of DiH20 was placed into 4 sterile 30 mL centrifuge tubes and 5 mL of the glycerol/pellet mixture was added. To ensure proper lysing of the cells, the glycerol mixture was forcibly added, using a sterile syringe and a 27-28 gauge needle, into the chilled,sterile DiH_20. To remove intact membrane this mixture was then centrifuged at 10,000 rpm for 20 minutes. Once centrifugation was complete the supernatant, containing the membrane, was carefully removed and placed into sterile 30 mL centrifuge tubes along with 30 mL of fluid. The crude membrane preparation was then centrifuged for 1 hour at 20,000 rpm, and supernatant was removed while maintaining the membrane pellet integrity. The membrane pellets were re-suspended into the appropriate cell culture medium excluding FBS.

Protein Assay

The 2 mL samples of membrane were serially diluted in the same medium that the membrane was suspended in. The membrane was serially diluted at a 1:2 ratio six times, adding 100 ul of the membrane solution and 100 ul of media. A standard protein (Bio-Rad Protien Assay Standard II) was also serially diluted at a 1:2 ratio six times as a standard control. Once all the dilutions were complete, 10 μL of each dilution were placed in triplicates onto Microtiter plates (Falcon Pro-Bind ELISA Assay Plates). Once all the dilutions were in the appropriate wells 200 ul of Bradford Reagent (Bio-Rad Protein Assay 1:4 dilution) was added. At this point, a color change would be observed between the various protein concentrations. Ten minutes later the plates were read by SpectraMAX 340 (Molecular Devices Sunnyvale, CA) on Soft Pro (version 2.2.1) at a wavelength of 595. The concentration of the membrane samples was determined by reference to the standard curve.

RNA Preparation

Stimulated cells were placed into 1 mL of Trizol Reagent (InVitrogen Technologies Carlsbad, CA) and frozen for RNA isolation.. 200 mL of chloroform was added to each thawed sample with the Trizol reagent, shaken vigorously for 15 seconds and allowed to sit for 2-3 minutes at 5-30°C. Samples were then centrifuged for 30 minutes at a speed of 12,000 x g at a temperature of 2 to 8°C. After centrifugation the top, clear aqueous phase was carefully removed, which contains the RNA, and transferred to another tube. At this point, 500 mL of isopropyl alcohol was added to each sample along with 1 μL of

glycogen (Invitrogen Technologies). The samples were gently vortexed to ensure complete mixing, incubated for 10 minutes at a temperature of 15-30°C and then centrifuged for 30 minutes at 12,000 x g at a temperature of 2-8°C. After the centrifugation was complete, the supernatant was poured off and the remaining pellet was washed with 1 mL of 75% ethanol. Each sample was vortexed and centrifuged at 12,000 x g for 10 minutes at a temperature between 2-8°C. Again after centrifugation, the supernatant was completely removed by inverting the tube and tapping off as much liquid as possible. At this point, the tubes remained inverted and were allowed to dry at room temperature for 30-45 minutes. After drying, the pellet was then re-suspended in 15-30 uL of DiH_2O. The samples are now ready for Real Time or can be stored at $^{-}80°C$ until a later use. Each RNA sample concentration was determined using a spectrophotometerically (Gene Quant; Amersham Pharmacia Biotech, Piscataway, NJ).

RNA Real Time Preparation

Reverse Transcriptase (RT) was performed by preparing the following RT mix: 5x MLV Buffer 2 μL/10 μL, dNTP's (10Mm) 2 μL/10μL, RNase Inhibitor 0.25 μL/10μL, Oligo dT 0.5 μL/10μL, and reverse transcriptase 0.125 μL/μL. One uL of the 100 ng of RNA dilution was added to the RT mix and an additional 4.125 μL of DiH_20 was added to make a total volume of 10 μL. Before the RNA was added to the final RT mix, it was placed into the PTC-100 Programmable Thermal Controller (MJ Research Waltman, MA) and heated to 65^0C for two minutes and then cooled to 4°C. The RT reaction was performed in the PTC-100 Programmable Thermal Controller with the following

program: 15 minutes at 42°C, 45 minutes at 37°C, 15 minutes at 99°C, and finally the temperature is held at 4°C.

PCR Protocol

The PCR reaction consisted of using a specific protocol from BD Bioscience. For each cytokine that we wanted to observe a separate PCR mixture had to be made. The PCR mixture for each cytokine was as follows: Taq Man Mix (Applied Bioscience) 12.5μ L, 10x Primer Pair solution 2.5 μL, 10x cytokine specific Fret Probe 0.5 uL, and finally 7 uL of DiH20 for a total volume of 22.5 μL. This PCR mix was then placed into specific 25 μL PCR tubes along with 2.5 μL of the desired cDNA sample. The tubes were spun prior to placing into the SmartCycler (Fisher Scientific Houston, TX) to ensure that the sample was at the bottom of the tube with no air bubbles. The samples were then run using a 50 cycle Fret Probe PCR protocol which consisted of the following parameters: Stage 1-Hold at 95^{0}C for 600 seconds and Stage 2-Temperature cycle repeat of 95°C for 15 seconds and 60°C for 60 seconds with optics on. Cytokine samples were read on FAM wavelength, while our housekeeping gene GAPDH was read by TET wavelength. To normalize cytokine levels between samples, the cycling threshold (CT) of GAPDH controls were subtracted from the cytokine thresholds. The medium controls for each cytokine were averaged together. This control average was then subtracted from the subsequent stimulant for each particular cytokine (Experiment – Control = x). Finally, to determine fold increases in cytokine production the number (x) that was calculated was raised to the power of 2 (2^X).

Statistical Analysis

Data results were analyzed using ANOVA, with group comparisons. Analysis was performed using StatView (SAS Institute, Cary, NC) program with a p value ≤ 0.05 considered statistically significant.

Chapter 3

RESULTS

M. pulmonis infection induces an increase in the number of lung dendritic cells

To determine if dendritic cells in the lung were increasing, mononuclear cells were isolated from C3H mice at day 0,7, and 14 post infection. To enhance identification of the population, dendritic cells were initially purified using a density gradient centrifugation. Cells were then counted, and observed for appropriate dendritic cell morphology. Finally, cells were stained with fluorescently labeled with anti-$CD11c^+$ antibody. Cell numbers were then counted and analyzed by flow cytometer. Using this protocol we determined that there was no significant increase in $CD11c^+$ dendritic cells between day 0 and day 7 post infection as compared with uninfected mice. However, between day 7 and day 14 post infection, there was a 7-10 fold increase in $CD11c^+$ dendritic cells numbers found in the lungs of infected mice as compared with naïve mice. (Figure 1)

M. pulmonis infection stimulates changes in maturation marker expression on lung dendritic cells

To examine whether *M. pulmonis* infection induced maturation, pulmonary cell suspensions were isolated from infected mice on day 0,7 and 14. Dendritic cells were purified as described above. Cells were then immuno-fluorescently stained with anti-$CD11c^+$ antibody and with anti-$CD11b^+$,-$CD80^+$,-$CD86^+$, -MHCII and -CD8 antibodies.

Maturation marker levels were then analyzed using flow cytometer. As shown in figure 2, all of the above listed maturation markers did increase in $CD11c^+$ lung dendritic cell populations 14 days post infection as compared with naïve mice.

Mycoplasma infection can directly stimulate dendritic and macrophage cell lines.

Based on the results stated above the direct effects of mycoplasma infection on both dendritic cells and macrophages were examined. Denditic JAW cells and macrophage J774 cells were stimulated for 24 hours with viable *M. pulmonis* and cytokine production and changes in surface markers were monitored.

First, cytokines released into the supernatant were determined using ELISA techniques. The dendritic JAW cell line had higher levels of TNF- α in culture supernatants after infection as compared to unstimulated cells. However, there were no significant increases in IL-10 or IL-12 levels. In contrast, the macrophage cell line showed increases IL-10, IL-12, and TNF-α levels in response to mycoplasma infection (Figure 3a & 3b). Cytokine mRNA levels in the cell lines 24 hours after in vitro infection were determined using real time-PCR and compared to control medium only cell cultures. In response to mycoplasma infection, the dendritic JAW cell line showed no significant increases in mRNA levels for IL-10, IL-12, or TNF-α as compared with the medium control cultures. Macrophages mRNA levels, however, did exhibit an increases in IL-12 production, but not IL-10 or TNF- α. (Figure 4a &4b)

Finally, changes in cell surface markers were observed in response to a 24 hour *M. pulmonis* infection. Neither dendritic cell nor macrophage cell lines showed

percentage increases in the cell expressing markers. Further more, neither cell line displayed increased fluorescence intensity for these markers after 24 *in vitro* mycoplasma infection. (Figure 5a, 5b, 6a & 6b)

Crude mycoplasma membrane can directly stimulate dendritic and macrophage cell lines

To determine if mycoplasma membranes had stimulatory effects, dendritic cells and macrophages cell lines were also stimulated with crude mycoplasma membrane preparations. After 24 hour stimulation, cytokine, cytokine mRNA expression, and cell surface markers were monitored, similar to above.

Cytokine protein levels, determined by ELISA, were increased in both cell lines in response to membrane interactions. Dendritic cells showed an increase in TNF-α, while macrophage showed increases in IL-10, IL-12, and TNF-α cytokine protein levels as compared with media controls. (Figure 3a & 3b)

Next, mRNA levels were again determined by real time-PCR. Dendritic cells displayed no significant increases in mRNA levels for listed cytokines. The macrophage J774 cell line exhibited increased mRNA levels for IL-12 as compared with the media control cultures. (Figure 4a & 4b)

Finally, maturation marker levels were monitored in response to membrane stimulation. Again, cells were stained with anti-Cd11c^{+} antibody along with the above listed maturation marker and levels were then analyze. Neither dendritic cells nor macrophages showed increases in either percentage changes or in mean fluorescence in any of these markers as compared with the media control. (Figure 5a, 5b, 6a & 6b)

In vivo CD11c$^+$ lung dendritic cell cytokine levels post infection with mycoplasma

Preliminary studies were performed to determine lung dendritic cell cytokine responses to a mycoplasma infection, *in vivo*. Specifically, CD11c$^+$ dendritic cells were isolated on day 14 from the lungs of mycoplasma infected as well as naïve C3H mice. As established earlier, dendritic cells were initially purified using density gradient centrifugation. CD11c$^+$ cells were then isolated using strepavidin coated magnetic and biotinylated anti-CD11c$^+$ antibodies using techniques previously described. Total RNA was isolated from these cells and real-time PCR was done to determine cytokine mRNA levels. There was no significant increases in IL-10, IL-12, or TNF-α mRNA expression by CD11c$^+$ cells from mycoplasma infected mice as compared with cells from uninfected control mice. (Figure 7a, 7b, & 7c)

Chapter 4

ILLUSTRATIONS

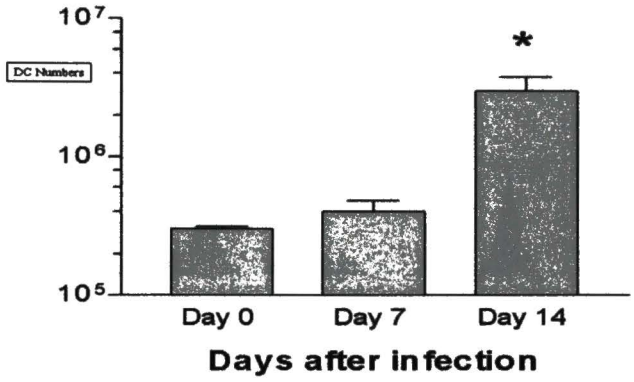

Figure 1. $CD11c^+$ Dendritic cell Numbers Within the Lungs of Infected Mice. CD11c+ dendritic cells were isolated from the lungs of *M. pulmonis* infected mice at listed time points. Significant differences ($p \leq 0.05$) were seen between day 0 and day 7 as compared with day 14.

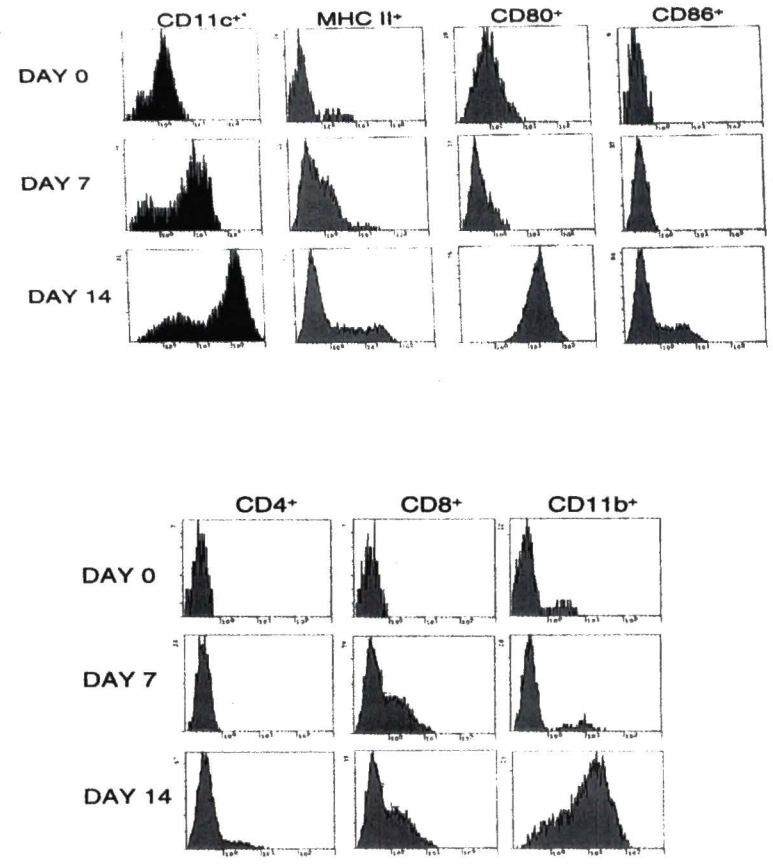

Figure 2. Lung dendritic cell surface marker Expression. $CD11c^+$ lung dendritic cells were isolated over a 14 day time course after infection with *M. pulmonis*. Shifts in all of the above discussed cell surface markers occurred.

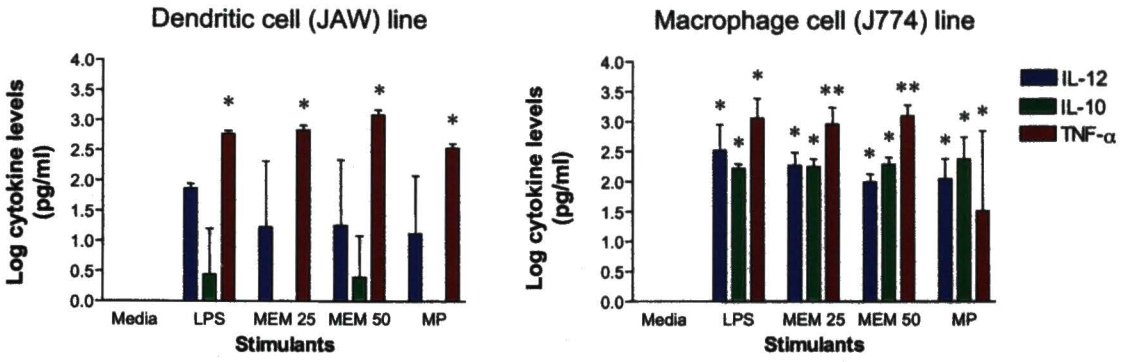

Figure 3. Effects of Stimulants on Cytokine Release by Cell Lines.
A) Dendritic JAW cell isolated and exposed for 24 hours to LPS, *M. pulmonis* (MP), membrane 25 μg/mL, membrane 50 μg/mL and media control. Asterisks signify significance ($p \leq 0.05$) differences as compared with media control.
B) Macrophage (J774) cells were isolated and exposed for 24 hours LPS, *M. pulmonis* (MP), membrane 25 μg/mL, membrane 50 μg/mL and media control. Asterisks signify significance ($p \leq 0.05$) differences as compared with media control.

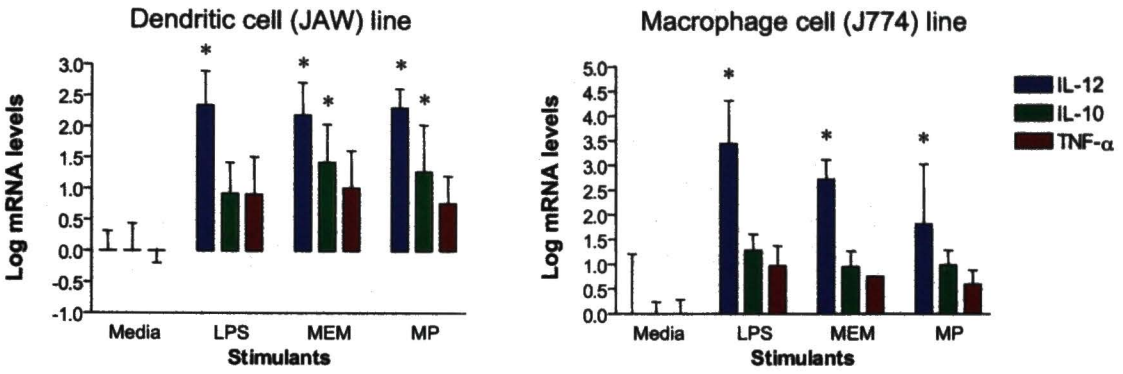

Figure 4. Effects of Stimulants on Cytokine mRNA Production by the Cell Lines.
A) Dendritic (JAW) cells were isolated after 24 hour stimulation with LPS, *M. pulmonis*, membrane 25 μg/mL, membrane 50 μg/mL, and media control. Cytokine mRNA levels were determined using real-time PCR. Asterisks denote significant ($p \leq 0.05$) differences between stimulants and the media control.
B) Macrophage (J774) Cells were isolated after 24 hour stimulation with LPS, *M. pulmonis*, membrane 25 μg/mL, membrane 50 μg/mL, and media control.. Cytokine mRNA levels were determined using real-time PCR. Asterisks denote significant ($p \leq 0.05$) differences between stimulants and the media control.

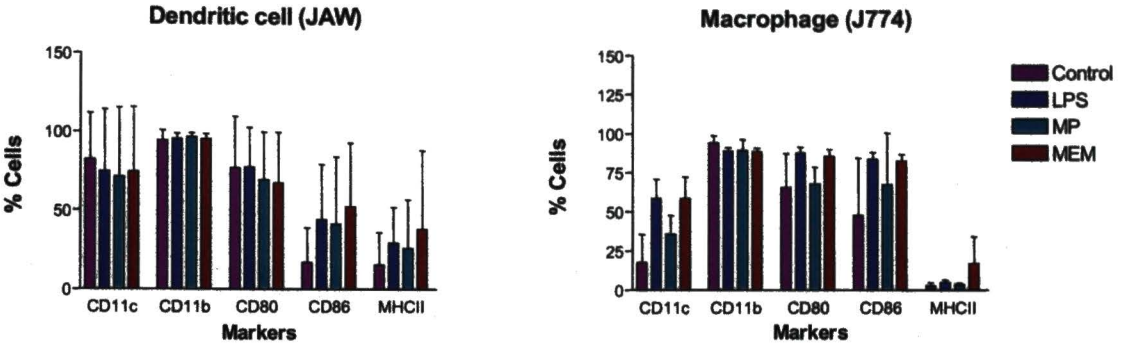

Figure 5. Percentage Changes in Cell Surface Marker Expression.
A) Dendritic (JAW) Cells. Cells were isolated after 24 hour incubation with LPS, *M. pulmonis*, crude mycoplasma membrane, and media control then stained for markers. No statistical ($p \leq 0.05$) shift change was seen in any of the markers after stimulation.
B) Macrophage (J774) Cells. Cells were isolated after 24 hour incubation with LPS, *M. pulmonis*, crude mycoplasma membrane, and media control then stained for markers. No statistical ($p \leq 0.05$) shift change was seen in any of the markers after stimulation.

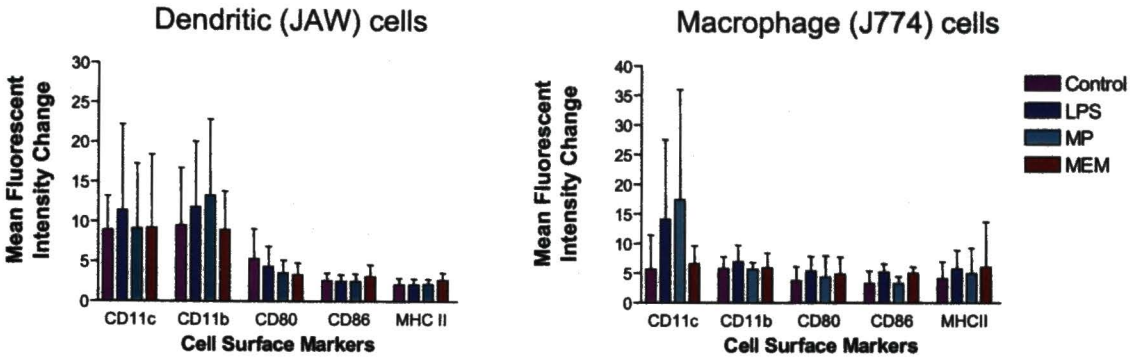

Figure 6. Fluorescent Intensity Changes in Cell lines.

A) Dendritic (JAW) Cell Surface Markers. Cells were stimulated for 24 hours with LPS, *M. pulmonis*, crude mycoplasma membrane and media control. After stimulation cells were stained for the listed cell surface markers. No significant ($p \leq 0.05$) changes in fluorescent intensity was seen in any of the cell surface markers in response to the stimulants.

B) Macrophage (J774) Cell Surface Markers. Cells were stimulated for 24 hours with LPS, *M. pulmonis*, crude mycoplasma membrane and media control. After stimulation cells were stained for the listed cell surface markers. No significant ($p \leq 0.05$) changes in fluorescent intensity was seen in any of the cell surface markers in response to the stimulants.

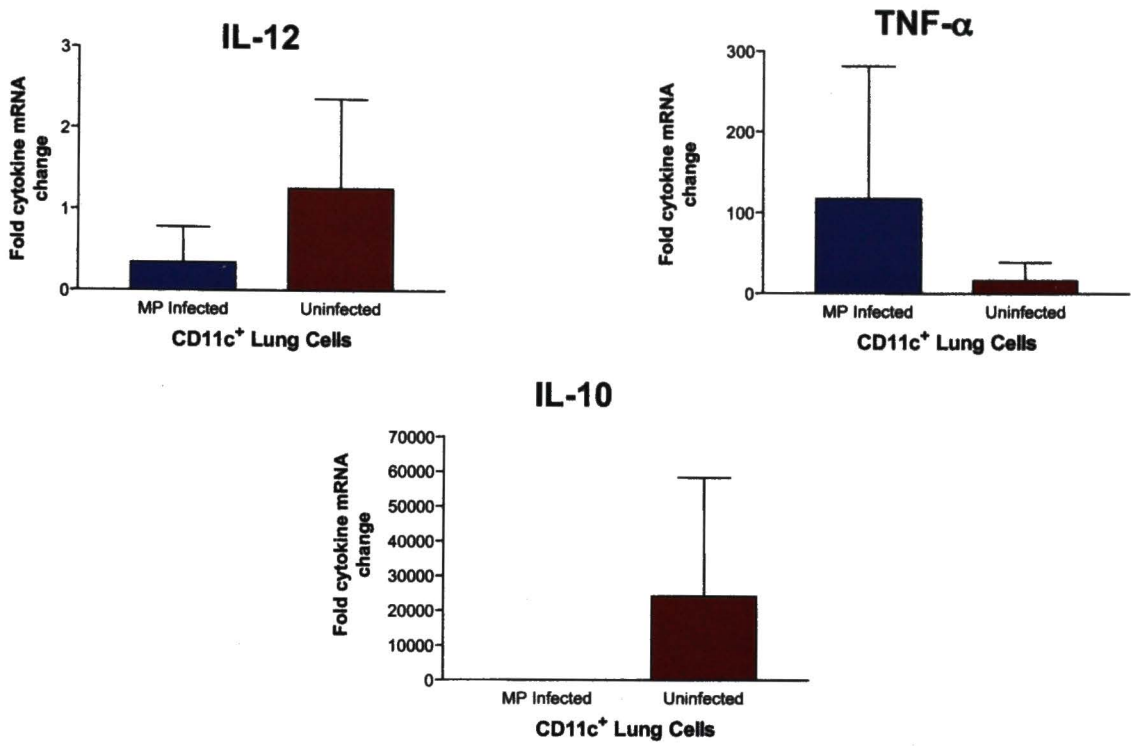

Figure 7. Lung Dendritic Cell Cytokine mRNA Levels. *In vivo* cytokine mRNA levels of *M. pulmonis* (MP) infected mice as compared with mRNA levels of uninfected mice.
A) $CD11c^+$ lung dendritic cells were isolated 14 days post infection and IL-12 cytokine mRNA levels were determined.
B) $CD11c^+$ lung dendritic cells were isolated 14 days post infection and TNF-α cytokine mRNA levels were determined.
C) $CD11c^+$ lung dendritic cells were isolated 14 days post infection and IL-10 mRNA levels were determined. No were no significant ($p \leq 0.05$) differences in any of the cytokine levels as compared with the uninfected controls.

Chapter 5

DISCUSSION

Previous studies have demonstrated that activation of $CD4^+$ and $CD8^+$ T cell populations could have profound effects of the immunopathology of mycoplasma disease (8,9,10). As dendritic cells are critical in the development of both $CD4^+$ and $CD8^+$ T cells, it is likely the dendritic cells contribute and influence the T cell responses against *M. pulmonis* respiratory disease. We hypothesize that dendritic cells are being activated both *in vitro* and *in vivo* by *M. pulmonis* and macrophages are being activated *in vitro*. To begin to address this hypothesis, we have analyzed $CD11c^+$ lung dendritic cells from mycoplasma infected mice, along with effects that mycoplasma has on both a dendritic and macrophage cell line.

We demonstrated that there were increases in $CD11c^+$ lung dendritic cell numbers in response to mycoplasma infection. The increase in dendritic cell numbers occurred between day 7 and day 14 post infection. Importantly, T cell responses develop during this time frame as well (8,10). This suggests that dendritic cells may not only contribute to mycoplasma specific T cell response but T cell activation may in turn stimulate increases in dendritic cell numbers. However, the mechanism by which these interactions take place is still undefined. Dendritic cells could be recruited to the lung from the circulation or other tissue in response to chemotactic signals, such as chemokines and cytokines released in response to a mycoplasma infection. Another possibility could be that resident lung dendritic cells are expanding or differentiating within the lung. Most

likely both processes are occurring. It would be beneficial to determine the location of dendritic cells in regard to mycoplasmas and inflammatory lesions within the lung. This would aid in determining the relationship of dendritic cells in an infection as well as with T cells and their responses. Cell cycle analysis or BRDU incorporation to determine if lung dendritic cells are actively dividing is response to some aspect of the infection would also be valuable. Thus, there are significant increases in dendritic cell numbers within the lung of mycoplasma infected mice,however further determination of the maturation levels of these cells is needed.

In addition to increased dendritic cell numbers, there was a concurrent change in cell surface markers on dendritic cells found with in the lung of mycoplasma infected mice. As described earlier in the text, increases in $CD11b^+$ and a lack of CD8α- suggest a cell of myeloid origin with a preference for inducing Th2 responses (11). This would suggest that dendritic cells were being recruited to the lung from the blood. The other cell surface markers, CD80, CD86, and MHCII, signify that the dendritic cell is being induced into a mature state and likely can stimulate T cells responses. Studies are however needed to confirm the ability to support T cell activation. Dendritic cells inducing a Th2 response may be attempting to counter some aspect of the mycoplasma infection that has induced the $CD8^+$ T cell response. However, this Th2 response could potentially be a contributing factor to the severity of mycoplasma disease as described earlier with SCID mice. Though surface markers are beneficial in determining maturation and origin, further studies are still needed to determine their activation, such as cytokine production.

To our surprise, there were no significant increases in IL-10, IL-12, or TNF-α mRNA levels from $CD11c^+$ dendritic cells isolated from the lungs of infected mice. Since these cytokines cover the two extremes for $CD4^+$ differentiation, it was expected that at least one of the cytokines would have increased. However, IL-10, IL-12, and TNF-α are just a few of a gambit of cytokines that can influence the differentiation of T cell populations. Further studies are needed to expand the type of cytokines to be analyzed. One potential approach is to use a SuperArray technique which monitors expression of a large number of mRNA cytokines.

Another possibility for the lack of increased mRNA production of these cytokines could again be a matter of the time point at which we observed the cells. By day 14 the cells may have already gone through their stage of cytokine production and moved into another phase in fighting the infection. To determine whether this occurs, time course studies could be implemented. Cytokine mRNA levels could be determined from the lung dendritic cells at various stages after the infection, and cytokine could be compared to pulmonary dendritic cells from uninfected mice. Thus, it is unclear whether lung dendritic cells are actively producing cytokines in response to mycoplasma disease. Our studies, however demonstrate that mycoplasma and crude membrane are capable of directly activating dendritic cells as well as macrophages. A dendritic cell line was induced by both *M. pulmonis* and membrane to secrete TNF-α protein. There was no change seen in mRNA levels in any of the three cytokines IL-10, IL-12, and TNF-α. TNF-α is a pro-inflammatory cytokine with the task of sequestering infectious agents to a localized area (24). It is also known to polarize CD4+ Th cells to differentiate into a Th1

response (24). Although TNF-α production by dendritic cells is consistent with *in vivo* studies where mixed Th cell subset responses occur, there are differences between populations of dendritic cells and these results from a cell line may not reflect normal dendritic cell function in vivo. Furthermore, our *in vivo* studies suggest that myeloid dendritic cells are present in the lungs of mycoplasma infected mice, and myeloid dendritic cells are thought to promote Th2-type responses (11,24). However, our recent evidence suggest that these activities may be dependent on the type of activation that occurs. Production of TNF-α could also aid in recruiting additional T cells to the sight of the infection. The lack of increase TNF-α mRNA levels despite the presence of protein in cell culture supernatants could be due to several points. Cells can have vacuoles of cytokine stores that are immediately released upon contact with a pathogen without additional mRNA expression. Also, we may have missed transient increases in the amounts of TNF-α mRNA expressed. Furthermore, if the cell had initially released stores of TNF-α and had just begun to increase mRNA expression it would have been undetectable. Thus, mycoplasma infection and components of mycoplasma membrane can stimulate dendritic cells to produce cytokines. The apparent lack of increase in mRNA expression indicates future studies are needed to examine protein production by pulmonary dendritic cells. In addition to mRNA levels may be consistent with our studies of cytokine mRNA expression by dendritic cells in response to *in vivo* mycoplasma infection.

In addition increased levels of IL-10, IL-12, and TNF-α cytokine production by macrophages was exhibited in response to mycoplasma and mycoplasma membrane.

This is in contrast to stimulation of dendritic cell line where only TNF-α was produced. These results suggest that dendritic cells and macrophages may respond differently to mycoplasma and may have differing roles in disease pathogenesis (36,37).

Typically, one would expect to see production of either Th1 (IL-12, TNF-α) or Th2 (IL-10) promoting cytokine production, not both. One factor that may be responsible for the cytokine response is that a component or components of the *M. pulmonis* membrane interacts with multiple receptors on macrophages of which dendritic cells only express as a subset (30-35). The production of this mixture of cytokines could be a mechanism by which the organism could initially evade immune clearance, by inducing mixed signaling. Future studies are needed to determine if macrophages during mycoplasma disease produce these cytokines and whether they support both Th1 and Th2 response.

It should also be noted that IL-10, IL-12, and TNF-α were the focus of our study because of their characteristic ability to polarize T cell differentiation. However, there are several other cytokines and factors that can be just as influential in inducing polarization of T cells. Though cytokine production is important in determining the activity of dendritic cells and macrophages in response to mycoplasma infection, it is also beneficial to monitor cell surface markers that aid determining such characteristics as cell lineage and maturation levels of the cell.

Although we found changes in cell surface markers on pulmonary dendritic cells for mycoplasma infected mice, there was no increase on any of the previously listed markers in both cell lines after exposure to *M. pulmonis* or crude membrane, as compared

with media controls. This may be due to the fact that these markers are already expressed well on these cell lines, or a difference between "normal" cells and the cell line. Finally, it should be noted that with these *in vitro* stimulations there was little to no difference in cytokine, mRNA, or marker levels in either cell line with regard to intact *M. pulmonis* versus the inactivated membrane isolate. This may have important implications in developing treatments against mycoplasma. Many vaccines take advantage of the different immune responses that occur when a subject is treated with inactivated surface antigens of the pathogen to prevent disease. Though we are dealing with a cell line, it is worth noting that this ability of membrane alone to activate dendritic cells and macrophages may be a potential problem in vaccine development for murine respiratory disease and human forms of the infection. A vaccine containing membrane may elicit inflammatory response or skew the type of immune response that develops. Other avenues may have to be explored to induce immunity while not causing detrimental side effects to the host. Although the *in vitro* studies are beneficial in determining potential responses of the immune system, future studies directly stimulating dendritic cells and macrophages isolated from the lungs of naïve mice may be more useful in determining the potential effects of mycoplasma.

Chapter 6

REFERENCES:

1- Cartner, S.C., Simecka, J.W., Briles, D.E., Cassell, G.H., and Lindsey, J.R. 1996. Resistance to Mycoplasma Lung Disease in Mice Is a Complex Genetic Trait. Infec. Immun. 64:5326-5331.

2- Cartner, S.C., Lindsey, J.R., Gibbs-Erwin, J., Cassell, G.H., Cassell, G.H., and Simecka, J.W. 1998. Roles of Innate and Adaptive Immunity in Respiratory Mycoplasmosis. Infec. Immun. 66:3485-3591.

3- Cassell, G.H., Lindsey, J.R, Overcash, R.G., and Baker, H.J. 1973. Murine Mycoplasma Respiratory Disease. Ann. N Y Acad Sci. 225:395-412.

4- Simecka, J.W, Davis, J,K., Cassell, G.H. 1989.Serum Antibody Does Not Account for Differneces in the Severity of Chronis Respiratory Disease Caused by *Mycoplasma pulmonis* in LEW and F344 Rats. Infec. Immun. 57: 3570-3575.

5- Cartner, S.C., Simecka, J.W., Lindsey, J.R., Cassell, G.H., and Davis, J.K. 1995. Chronis Respiratory Mycoplasmosis in C3H/HeN and C57BL/6N Mice: Lesion Severity and Antibody Response. Infec. Immun. 63:4138-4142.

6- Stadlander, C.T.K.H., Watson, H.L., Simecka, J.W., and Cassell, G.H. 1991. Cytopathic Effects of *Mycoplasma pulmonis* In Vivo and In Vitro. Infec. Immun. 59:4201-4211.

7- Salyers, A.A., Whitt, D.D. 2002. Baterial Pathogenesis- A molecular Approach,2nd Edition Chap. 21. Mycoplasma pneumoniae and Chlamydia pneumoniae. p- 322-329. ASM Press.

8- Jones, H.P., Tabor, L., Sun, X., Woolard, M.D., Simecka, J.W. 2002. Depletion of CD8+ T Cells Exacerbates CD4+ Th Cell-Associated Inflammatory Lesions During Murine Mycoplamsa Respiratory Disease. The Jour. of Immun. 168:3493-3501.

9- Jones, H.P., Hodge, L.M., Fujihashi, K., kiyono, H., McGhee, J.R., Simecka, J.W. 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. Jour. of Immun. 167: 4518-4526.

10- Jones, H.P., Simecka, J.W. 2003. T Lymphocyte Responses are Critical Determinants in the Pathogenesis and Resistance to Mycoplasma Respiratory Disease. Front. In Biosc. 8:930-945.

11- Banchereau, J., Steinaman, R.M., 1998. Dendritic cells and the control of immunity. Nature. 392:245-251.

12- Upham, J.W. 2003. The Role of Dendritic Cells in Immune Regulation and Allergic Airway Inflammation. Respirology. 8:140-148.

13- Pulendran, B., Smith, J.L., Caspary, G., Brasel, K., Pettit, D., Maraskovsky, E., Maliszewski, C.R. 1999. Distinct dendritic cell subsets differentially regulate the class of immune response *in vivo*. Proc. Natl. Acad. Sci. 96:1036-1041.

14- Pooley, J.L., Heath, W.R., Shortman, K. 2001. Cutting Edge: Intravenous Soluble Antigen Is Presented to CD4 T Cells by $CD8^-$ Dendritic Cells, but Cross-Presented to CD8 T Cells by $CD8^+$ Dendritic Cells. Journ, of Immun.166:5327-5330.

15- Roncarolo, M.G., Levings, M.K., Trversari, C. 2001. Differentiation of T Regulatory Cells by Immature Dendritic Cells. J. Exp. Med. 193:F5-F9.

16- Maldonado-Lopez, R., De Smedt, T., Michel, P., Godfroid, J., Pajak, B., Heirman, C., Thielemans, K., Leo, O., Urbain, J., Moser, M. 1999. $CD8\alpha^+$ and CD8α- Subclasses of Dendritic Cells Direct the Development of Distinct T Helper Cells In Vivo. J. Exp. Med. 189:587-592.

17- Geiser, M.2002. Morphological Aspects of Particle Uptake by Lung Phagocytes. Microscopy Res. And Tech. 57:512-522.

18- Howard, C.J., Taylor, G. 1979. Variation in the Virulence of Strains of *Mycoplasma pulmonis* Related to Susceptibility to Killing by Macrophages *in vivo*. Jour. of Gen. Microbiology. 114:289-294.

19- Hickman-Davis, J.M., Lindsey-Russel, J., Zhu, and S., Matalon. 1998. Surfactant protein A mediates mycoplasmacidal activity of alveolar macrophages. Am. J. Physiol. Lung Cell Mol. Physiol. 274:270-277.

20- Hickman-Davis, J.M., Michalek, S.M., Gibbs-Erwin, J., and Lindsey-Russel, J. 1997. Depletion of Alveolar Macrophages Exacerbates Respiratory Mycoplasmosis in Mycoplasma-Resistant C57BL Mice but Not Mycoplasma-Susceptible C3H Mice. Infec. And Immun. 65:2278:2282.

21- Bradley, L.M., Habertson, J., Biederman, E., Zhang, Y., Bradley, S.M., Linton, P.J. 2002. Availability of antigen-presenting cells can determine the extent of CD4 effector expansion and priming for secretion of Th2 cytokines *in vivo*. Eur. J. Immunol. 32:2338-2346.

22- Dabbagh, K., Dahl, M.E., Stepick-Biek, P., Lewis, D.B. 2002. Toll-like Receptor 4 Is Required for Optimal Development of Th2 Immune Responses: Role of Dendritic Cells. The Journ. of Immun. 168:4524-4530.

23- Gonzalez-Juarrero, M., Orme, I.M. 2002. Characterization of Murine Lung Dendritic Cells Infected with *Mycobacterium tuberculosis*. The Journ. of Immun. 69:1127-1133.

24- Janeway, C.A., Travers, P., Walport, M., Capra, J.D. 1999. *Immunobiology. The Immune System in Health and Disease.* 4:263-310,365-386, 580-584.

25- Maniloff, J., McElhaney, R. N., Finch, L.R., Baseman, J.B., 1992. *Mycoplasmas Molecular Biology and Pathogenesis.* Chapter 24- Mycoplasma Disease in Animals. Simecka, J. pg 391-415. Chapter 25-Mycoplasmas Which Infect Humans. Krause, D.C., and Taylor-Robinson, D. pg 417-444.

26- Mycoplasma statistic:

www.edc.gov/ncidod/dbmd/diseaseinfo/mycoplasmapneum_t.htm

27- Lagranderie, M., Nahori, M., Balazuc, A., Kiefer-Biasizzo, H., Lapa E Silva, J., Milon, G., Marchal, G., Vargaftig, B.B. 2002. Dendritic cells recruited to the lung

shortly after intranasal delivery of Mycobacterium bovis BCG drive the primary immune response towards a type 1 cytokine production. Immun. 108: 352-365.

28- Goldsby, R.A., Kindt, T.J., Osborne, B.A. 2002. *Immunology*. Chapter 2- Cells and Organs of the Immune System. p. 41-48. W.H. Freeman and Company, New York.

29- Qi, H., Denning, T.L., Soong L. 2003. Differential induction of interleukin-10 and interleukin-12 in dendritic cells by microbial toll-like receptor activators and skewing of T-cell cytokine profiles. Infect. Immun. 71(6):3337-42.

30- Iwasaki, A., Kelsall, B., 1999. Freshly Isolated Peyer's Patch, But Not Spleen, Dendritic Cells Produce Interleukin 10 and Induce the Differentiation of T Helper Type 2 Cells. The Jour. of Exp. Med. 190:229-239.

31- Ohteki, T., Fukao, T., Suzue, K., Maki, C., Ito, M., Nakamura, N.,and Koyasu, S. 1999. Interleukin 12-dependent Interferon γ Production by $CD8\alpha^+$ Lymphoid Dendritic Cells. J. Exp. Med. 189:1981-1986.

32- Fehrenbach, H., Zissel, G., Goldman, T., Tschernig T., Vollmer, E., Pabst, R.,and Muller-Quernheim, J. 2003. Alveolar macrophage are the main source for tumor nucrosis factor-alpha in patients with sarcoidosis. Eur. Respir. J. 21(3):421-8.

33- Oltmanns, U., Scmidt, B., Hoernig, S., Witt, C.,and John, M. 2003. Increased spontaneous interleukin-10 release from alveolar macrophages in active pulmonary sarcoidosis. Exp. Lung Res. 29(5):315-28.

34- Hubeau, C., Singer, M., Lagranderie, M., Marchal, G., and Vargaftig, B. 2003. Extended freeze-dried *Mycobacterium bovis* Baciilus Calmette-Guerin induces the

release of interleukin-12 but not tumour necrosis factor-α by alveolar macrophages, both *in vitro* and *in vivo*. Clin. Exp. Allergy 33:386-393.

35- Lang, R., Divyen, P., Morris, J.J., Rutschman, R.L., and Murray, P.J. 2002. Shaping Gene Expression in Activated and Resting Primary Macrophages by IL-10. 169: 2253-2263.

36- Niang, M., Rosenbusch, R.F., Lopez-Virella, J., Kaeberle, M.L. 1997. Expression of functions by normal sheep alveolar macrophages and their alteration by interaction with Mycoplasma ovipneumoniae. Vet. Microbiology 58(1): 31-43.

37-Kirby, A.C., Yrlid, U., Svensson, M., Wick, M.J. 2001. Differential involvement of dendritic cell subsets during acute Salmonella infection. J. Immunol. 166(11): 6802-11.