Interactions between Mycoplasma bovis and bovine lymphocytes: characterization of a lympho-inhibitory peptide produced by Mycoplasma bovis

Anthony J. Vanden Bush
Iowa State University

Follow this and additional works at: http://lib.dr.iastate.edu/rtd
Part of the Immunology and Infectious Disease Commons, Medical Immunology Commons, Microbiology Commons, and the Veterinary Medicine Commons

Recommended Citation
http://lib.dr.iastate.edu/rtd/748

This Dissertation is brought to you for free and open access by Iowa State University Digital Repository. It has been accepted for inclusion in Retrospective Theses and Dissertations by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.
Interactions between Mycoplasma bovis and bovine lymphocytes: characterization of a lympho-inhibitory peptide produced by Mycoplasma bovis

by

Anthony J. Vanden Bush

A dissertation submitted to the graduate faculty in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY

Major: Immunobiology

Program of Study Committee:
Ricardo Rosenbusch, Major Professor
Joan Cunnick
Douglas Jones
Allen Myers
Michael Wannemuehler
Robert Thornberg

Iowa State University
Ames, Iowa
2003
INFORMATION TO USERS

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleed-through, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

UMI Microform 3118262
Copyright 2004 by ProQuest Information and Learning Company. All rights reserved. This microform edition is protected against unauthorized copying under Title 17, United States Code.
This is to certify that the doctoral dissertation of

Anthony J. Vanden Bush

has met the dissertation requirements of Iowa State University

Signature was redacted for privacy.

Major Professor

Signature was redacted for privacy.

For the Major Program
# TABLE OF CONTENTS

**ABSTRACT**  

**CHAPTER 1. GENERAL INTRODUCTION**  
Introduction  1  
Dissertation Organization  2

**CHAPTER 2. LITERATURE REVIEW**  
Mycoplasmas and Disease  4  
*Mycoplasma bovis*  9  
Lymphocyte Function and Suppression  15  
References  28

**CHAPTER 3. MYCOPLASMA BOVIS INDUCES APOPTOSIS OF BOVINE LYMPHOCYTES**  
Abstract  56  
Introduction  56  
Materials and Methods  57  
Results and Discussion  60  
Acknowledgements  72  
References  72

**CHAPTER 4. CHARACTERIZATION OF AN IMMUNOSUPPRESSIVE PEPTIDE PRODUCED BY MYCOPLASMA BOVIS**  
Abstract  75  
Introduction  75  
Materials and Methods  76  
Results  80  
Discussion  85  
References  89

**CHAPTER 5. GENERAL CONCLUSIONS**  
Summary  94  
Recommendations for Future Research  94

**ACKNOWLEDGEMENTS**  96
APPENDIX A. CHARACTERIZATION OF THE IMMUNE RESPONSE TO Mycoplasma bovis LUNG INFECTION

Abstract 97
Introduction 97
Materials and Methods 98
Results 103
Discussion 107
Acknowledgements 115
References 116

APPENDIX B. ACTIVATION OF WC1+ γδT CELLS BY Mycoplasma bovis IS CONSISTENT WITH INNATE IMMUNITY

Abstract 121
Introduction 121
Results and Discussion 122
References 128
ABSTRACT

The effects of Mycoplasma bovis on bovine lympho-proliferation and viability were studied. Incubation of bovine peripheral blood mononuclear cells (PBMC) in vitro with M. bovis induced lymphocyte apoptosis as monitored by Annexin V binding, propidium iodide incorporation, and DNA fragmentation. The induction of lymphocyte death was abrogated by chloramphenicol, indicating that lymphocyte death was dependent upon prokaryotic protein production.

In attempts to better define the lymphotoxic factors associated with M. bovis, the task of isolating the M. bovis lymphocyte inhibitory or cytotoxic factor was undertaken. Using size exclusion chromatography a fraction able to suppress bovine lympho-proliferative responses to ConA was isolated. MALDI-TOF analysis of the suppressive fraction revealed one dominant peak (M.W. of 2,970 daltons) unique to the suppressive fraction. N-terminal sequencing of the suppressive fraction revealed a 19 amino acid sequence homologous to the C-terminus of the M. bovis theoretical gene encoding Vsp-L (variable surface protein-L) as determined by a BLAST search against the NCBI national protein database. The sequence data was used to construct primers for PCR amplification of the vspL gene domain corresponding to the last 26 amino acids of the VspL's protein C-terminus. This PCR product was cloned and inserted into an expression plasmid as a GST-fusion protein engineered with a thrombin recognition cleavage site between GST and the recombinant peptide (to facilitate purification of the peptide). Upon purification, the peptide Mb-LIP (M. bovis lymphocyte inhibitory peptide) was tested for lympho-proliferative inhibition. The recombinant peptide inhibited bovine lymphocyte proliferation responses to ConA and CD3 stimulation in vitro. Further study indicated that incubation of PBMCs with the recombinant peptide decreased the number of viable lymphocytes in culture. The recombinant peptide Mb-LIP is therefore considered a lymphotoxic peptide. This marks the first described characterization of a mycoplasmal product able to inhibit naive lymphocyte function.
CHAPTER 1. GENERAL INTRODUCTION

*Mycoplasma bovis* is a wall-less prokaryotic (class Mollicutes) pathogen of cattle causing multiple diseases, including mastitis, pneumonia, and arthritis. As with other mycoplasma infectious agents *M. bovis* infections are known to be chronic in nature, being isolated from animals for months or even years after the initial infection. While the mechanism used by *M. bovis* to maintain a presence within its bovine host is unknown, two general mechanisms have been proposed, antigenic variation and immune response modulation.

Antigenic variation is accomplished through the variable expression of surface proteins or altering the primary structure of surface antigens. The *M. bovis* genome codes for a family of surface proteins known as VspS (variable surface proteins) that are variable in expression, size, and antigenicity. The plasticity within the expression of these highly antigenic surface proteins is believed to be one mechanism of immune evasion. While these proteins appear to have a passive role in *M. bovis* pathogenicity, some of them play an active role in cellular adhesion. Despite years of research, products of some *vsp* genes have not been identified. In addition, roles of individual Vsp proteins in *M. bovis* pathogenicity are as of yet unknown. In addition, roles of individual Vsp proteins in *M. bovis* pathogenicity are as of yet unknown.

Immune response modulation is accomplished by down-regulation or nonspecific activation of host immune cells. Through these modulations Mycoplasma species can evade immune detection. Prior to the discovery of VspS, *M. bovis* was shown to inhibit lymphocyte proliferation in response to lectin mitogens. This lympho-suppression could be used to help explain the chronicity of *M. bovis* infections. Presumably by limiting adaptive responses, *M. bovis* can persist within its host. The research described herein was undertaken with the intention of better understanding *M. bovis* and bovine lymphocyte interactions by isolating the *M. bovis* produced factor(s) able to inhibit lympho-proliferation.

The lympho-suppressive factor(s) produced by *M. bovis* was determined to be heat labile and non-toxic as lymphocyte viability was not affected by incubation with *M. bovis*. However, with the use of modern techniques, *M. bovis* was shown to decrease lymphocyte viability in vitro thru the induction of apoptosis. In addition to the observation of *M. bovis*'s
ability to induce bovine lymphocyte apoptosis, the isolation and characterization of a lympho-inhibitory peptide produced by *M. bovis* was also accomplished. The peptide, referred to as *M. bovis* lymphocyte inhibitory peptide (Mb-LIP), is homologous to the C-terminus of VspL. This discovery, therefore, defines a pathogenic role for an individual Vsp gene product.

**Dissertation Organization**

This dissertation is comprised of a literature review, two manuscripts, a brief summary of the work, and two appendices. The literature review is divided into three sections; 1) a general background of mycoplasmas in relation to disease and immunity, 2) a introduction to *Mycoplasma bovis* emphasizing immunopathology, and 3) a section serving as an overview of T-lymphocyte activation and suppression (including mycoplasma-mediated immune suppression). The central portion of this thesis contains two manuscripts. The first (chapter 3), *Mycoplasma bovis induces apoptosis of bovine lymphocytes*, has been published in *FEMS Immunology and Medical Microbiology*. The second manuscript (chapter 4), *Isolation and characterization of a lymphosuppressive peptide produced by Mycoplasma bovis*, will be submitted to *Biochemical and Biophysical Research Communications* for publication. A brief summary section discussing the implications of the work and possible directions for continued studies follows these chapters. The central text of the dissertation is immediately followed by acknowledgements.

The dissertation is concluded with two appendices highlighting cellular and humoral immune responses to *M. bovis* antigens. Appendix A, *Characterization of the immune response to Mycoplasma bovis lung infection*, is a manuscript published in the journal *Veterinary Immunology and Immunopathology*. Appendix B, *Activation of WC1+ γδT cells by Mycoplasma bovis is consistent with innate immunity*, was derived from work presented as a poster at the 14th International Congress of the International Organization of

---

Mycoplasmology (IOM). Figures for each chapter are placed within each respective chapter. References for each chapter directly follow their respective chapter.

---

3 Poster #152, 14th International Congress of the IOM, July 7-12, 2002, Vienna, Austria
CHAPTER 2. LITERATURE REVIEW

1. Mycoplasmas and Disease

1.1 Introduction to mycoplasmas

Mycoplasma species belong to the class Mollicutes, the smallest, self-replicating organisms known (1, 2). The class Mollicutes is phenotypically differentiated from other prokaryotes by the lack of a rigid peptidoglycan cellular wall, hence the class moniker Mollicutes (molli Latin for soft; and cutis for skin) (2). Due to their limited metabolic capabilities, assumed to be a consequence of their small genomes, mycoplasmas exist in nature as obligatory parasites, relying on their hosts for growth potential (2). Mycoplasmas species are known to colonize humans and animals with extreme host and even tissue specificity (2). While some species are commensal organisms, others mycoplasma are infectious agents, causing disease in their respective hosts (1, 2).

Mycoplasma species maintain genomes rich in adenine and thymidine. In fact, one of the phenotypic characteristics of the genus is a G+C molar percent genomic content of 23 to 41 (3). In addition, the mycoplasmal genome is relatively small in size (580 to 1,300 kbp) compared to other prokaryotes (Escherichia coli genome is 4,640 kbp) (3-5). Mycoplasmal genomes have a coding density of approximately 90% (6, 7), in comparison to 85% for Haemophilus influenza and 88% for E. coli (4, 8). The slight increase in coding density by mycoplasmas is most likely due to the increased utilization of operon systems, limiting the need for regulatory elements (2, 9). Because of their size and small genomes, it is believed that the majority of ORFs code for necessary housekeeping genes, such as Hsp70. Even still, a relatively high percent of proteins (approximately 7% of the ORFs) are expressed as lipoproteins on the surface of the mycoplasma (based on membrane targeting sequence homologies) ((6), Dr. Chris Minion, Iowa State University – personal communication). Along with the lipoproteins, mycoplasmas also express non-lipid modified membrane-associated proteins. These proteins are presumably expressed in high copy numbers as membrane-associated protein amounts of some mycoplasma species approaches 44% of the total protein content (10). In addition to proteinacious surface molecules, numerous mycoplasmas, including the swine, avian, human, and bovine respiratory pathogens, M.
hyopneumoniae, M. gallisepticum, M. pneumoniae and M. dispar respectively, are encapsulated by highly antigenic polysaccharides (11-15).

In terms of infectious agents, J.W. Simecka refers to mycoplasmas as, “the most under recognized pathogens known today (16)”. This is in no way indicative of their importance to human and animal health, as up to 30% of all human pneumonia cases are reportedly caused by M. pneumoniae (17), and multiple domesticated animals harbor infectious mycoplasmas affecting agricultural economics (1,18, 19). In addition to their ability to cause disease, mycoplasma infections have also been associated with ailments generally considered non-infectious, such as asthma, rheumatoid arthritis, gulf war syndrome, tumor formation, and Crohn’s disease (20).

1.2 Innate immunity in mycoplasmal respiratory disease (MRD)

Innate immune defenses are comprised of physical barriers (mucus), molecular defenses (surfactant proteins, lysozyme, and antimicrobial peptides – defensins), and cellular defenses (macrophages, neutrophils, and dendritic cells). The role of physical and molecular defenses in inhibiting and combating mycoplasmal infections is largely unknown; however, the importance of cellular innate immunity in defense against mycoplasmas has repeatedly been demonstrated (21). Pathogen loads in SCID (severe combined immunodeficiency) mice (mice lacking lymphoid cells) during a M. pulmonis infection are no different than those in immunocompetent mice (22). These findings are consistent with lymphoid depleted humans infected with M. pneumoniae (23, 24). Corroborating evidence supporting the importance of innate immunity in fighting mycoplasmal infections is that the negative manipulation of alveolar macrophages in M. pulmonis resistant C57BL/6 mice results in a level of infection comparable to susceptible C3H/He mice, linking resistance to macrophage activity (25, 26). In addition, reduction of mycoplasmal killing is witnessed in nitrogen dioxide exposed mice; this has been directly related to the specific damage of alveolar macrophages caused by NO₂ (27, 28). Surfactant proteins, SP-A and SP-D, molecular components of innate immunity, are also important in mycoplasmal infections as these proteins increase alveolar macrophages’ mycoplasmal killing capacity through induction of nitric oxide and ROS (reactive oxygen species) production (29, 30). The induction of ROS
and nitric oxide by innate immune cells may affect lymphoid cell activity (discussed in section 3.4, endogenous T lymphocyte suppressors).

Interestingly, alveolar macrophages are the predominant immune cell in combating mycoplasmal infection as the increase of PMNs (polymorphonuclear cells – neutrophils) has no effect on mycoplasmal clearance (31, 32). The lack of effective clearance by PMNs may contribute to the chronic inflammatory state of mycoplasmal infected tissue as PMN accumulations are evident during infection (21). The interaction between mycoplasma species and innate immune cells generally leads to the production of pro-inflammatory cytokines – interleukin-1 (IL-1), IL-6, and TNF-alpha (33-36). In addition to the production of IL-1, IL-6, and TNF-alpha, these and other cells (epithelial, fibroblasts, and endothelial – Dr. Xaodong Lu, Iowa State University, personal communication) also produce chemokines (such as MIP-1α, MIP1-β, and ENA-78) in an effort to elicit the help of other innate cells and adaptive immune cells (37-44). The chemokines result in the characteristic perivascular and peribronchiolar accumulation of mononuclear cells during mycoplasmal respiratory infections (discussed later). Other cytokines known to effect adaptive immune responses (such as IL-13) can also be produced by cells in contact with mycoplasmas, potentially determining the type of adaptive immune response generated during an infection (38).

Note that the role of dendritic cells (DCs) in combating mycoplasma infections is unknown. However, because of immature DC’s importance in lymphocyte activation and regulation (45, 46), these cells may play important roles in lymphocyte directed inflammation, respiratory damage, and adaptive immune progression (16, 46, 47).

1.3 Adaptive immunity in MRD

Adaptive immunity, immune reactivity related to lymphoid cells (largely T and B cells), can be further broken down into cellular and humoral immunity. These two arms of the adaptive immune response work concomitantly to clear pathogens while also establishing memory cells to better combat similar or related pathogens if ever re-infected. Humoral immunity is traditionally thought of as antibody, or immunoglobulin mediated immunity (B cell driven) while cellular responses are traditionally cytotoxic T cell mediated. However, since the discovery that activated T helper cells (CD4+ T cells) differentiate into at least 2
phenotypically different effector populations (defined by differential cytokine production profiles) adaptive immune responses have been re-evaluated in terms of activated T helper cell (Th cell) phenotypes (48-50). The two Th effector populations are Th1 (T helper type 1) cells and Th2 (T helper type 2) cells. The defining differences between the two Th populations are the characteristic cytokines produced by each population. Th1 cells produce cytokines that are indicative of a cellular response such as IL2, a positive regulator of T cell development, and IFN-γ, a positive regulator of antigen processing. Th1 cells are therefore associated with cellular immune responses (49, 50). The Th2 phenotype cells produce IL4 (interleukin 4), IL5, IL10, and IL13, cytokines that influence B cell development and immunoglobulin isotype switching (49, 51, 52); hence the association between Th2 cells and humoral immunity.

The importance of a properly phenotyped response to the invading pathogen has been documented in multiple infectious agent models (51, 53-56), the most recognized of which is \textit{Leishmania major} infection. In brief, mice resistant to \textit{L. major} infection respond to the parasite with a Th1 type response while susceptible mice generate a Th2 response upon infection (55, 57-59). The production of antigen-specific antibodies via reactive B cells occurs in both Th1 and Th2 characteristic immune responses. The difference in Ig-production between these two types of responses is the isotype of antibody produced (52). The differences in isotype can have a substantial effect on disease progression as some antibody isotypes are more proficient at complement activation, opsonization, or innate immune cell activation (60, 61). While the phenotype of a protective response to mycoplasmal infection is not known, multiple studies suggest that mycoplasmal infection induces a Th2 type response (62-68). This connection has been suggested as a role for mycoplasmal agents in increasing the incidence of asthma or airway allergies (63, 66), as Th2 responses in the respiratory tract are known to contribute to these types of diseases (69-75).

In terms of T and B cell responsiveness, both are activated during mycoplasmal infection (16, 67, 76, 77). Early studies indicated an influx of mononuclear cells, which included T and B cells, into the lungs and lung draining lymph nodes of mycoplasma infected animals (78-81). These immunohistochemical observations indicate an activation of adaptive
immune cells based on cellular trafficking and active antibody production by B cells located in infected lungs (79, 82). The importance of adaptive immunity has been documented in the protection against select mycoplasmal infectious diseases through vaccination (83-86). The contribution of each type of response (cellular vs. humoral) in protection however, may vary from species to species as humoral responses are sufficient to protect mice against *M. pulmonis* infection (87), while passive transfer of immune cells but not sera is able to protect rats against *M. pulmonis* infection (88). As discussed earlier, the innate immune system is important for the clearance of respiratory mycoplasmas, however, the infection of T cell deficient mice with *M. pulmonis* leads to an increase in arthritis severity compared to mice with normal T cell numbers (16, 89). Experimental lung infection of SCID mice (mice lacking both T and B cells) with *M. pulmonis* leads to severe, disseminating disease causing both MRD and arthritis (22, 90) while not effecting the pathogen load (discussed earlier). The passive transfer of immune sera to infected SCID mice is sufficient to limit the systemic spread of the mycoplasma, therefore limiting clinical symptoms of arthritis (22). This suggests that the observations in T cell deficient mice were possibly due to the lack of B cell activation by T helper cells. The findings demonstrate the importance of antibody responses in limiting the dissemination of infectious mycoplasmas and are consistent with clinical observations of hypogammaglobulinemic patients infected with *M. pneumoniae*, who show persistence of clinical symptoms as well as increased incidence of multi-organ infection, despite having normal T cell counts (91, 92). Individuals suffering from T cell deficiencies do not experience increased susceptibility to *M. pneumoniae* infection as do hypogammaglobulinemic patients (21, 23). These observations highlight the importance of humoral responses in combating mycoplasma infections.

Cellular responses, however, are also important in protection against some mycoplasmal disease conditions as passive transfer of mycoplasma specific mononuclear cells into mycoplasma infected rats decreases severity of disease while passive antibody transfer has no effect (88). Despite the apparent effectiveness of immune responses in combating mycoplasmal infections, very few mycoplasmal vaccines are licensed for use. The lack of success in creating mycoplasmal vaccines can be attributed to duration of immune protection (acquired immunity to mycoplasmas is short-lived (16)), and that many
vaccine attempts have simply decreased infectious agent loads and did not deter colonization or infection (16, 86, 93, 94).

It is well accepted that immunological responses to respiratory mycoplasmal infection play a role in disease pathogenesis through induction, production, and maintenance of inflammatory mediators (16). The most incriminating studies consistently show a decrease in lung lesion size and severity in animals treated with immunosuppressive therapies (22, 89, 90, 95). Reconstitution of T cells in infected, immunocompromised animals results in an increase in lung lesion size and severity (22, 96). Delineating the importance of individual immune cells and regulators in disease progression is currently an active area of mycoplasmal research (96). Despite the damage induced by an immune reaction to mycoplasmal infection, however, it has been demonstrated that immunological responses are important in protecting against or reducing the severity of clinical disease (16). The complexity of host immune cells and mycoplasma interactions is compounded by the fact that multiple mycoplasmal species are able to directly or indirectly modulate immune cell responses (discussed later).

2. *Mycoplasma bovis*

2.1 Introduction to *Mycoplasma bovis*

The bovine pathogen *Mycoplasma bovis* is one of more than twenty mycoplasmas known to colonize cattle (97); and is responsible for multiple diseases in cattle, including pneumonia, mastitis, arthritis, otitis, conjunctivitis, decubital abscesses, and urogenital tract infections (97-102). In addition, *M. bovis* has been cultured from brain, spinal cord, nerve endings, bull semen, and from the abomasal (4th stomach) contents of an aborted calf (99, 103, 104). While predominantly considered an agent of mastitis, pneumonia, and arthritis, isolation of *M. bovis* from other, non-related, organs (as described) has been cited as proof of systemic capabilities (97).

*Mycoplasma bovis* was first described under the name *Mycoplasma agalactiae mastitidis* isolated in 1961 from a bovine mastitis infection (105). Between 1962 and 1976 the pathogen was referred to as either *M. agalactiae* subspecies *bovis* (97) (owing to its antigenic and pathological similarities to the goat pathogen *M. agalactiae* (97), or
*Mycoplasma bovima*stitidis (106), until an official species assignment of *M. bovis* in 1976 (107). Since its discovery, *M. bovis* has been isolated from animals in a number of countries (108); and, while not ubiquitous, is considered to have world-wide distribution (97, 108). In addition to its distribution, the number of outbreaks and/or infected herds in endemic areas appears to be on the rise (109, 110).

As a pathogen of both respiratory tract and mammary glands, *M. bovis* is responsible for substantial economic losses in both beef and dairy industries (18, 19). The severity of a herd infection is compounded by a non-responsiveness of infected animals to antibiotic treatment (108). Lacking a cell wall, mycoplasmas are inherently resistant to antibiotics that target cell wall construction (e.g. penicillin, ampicillin, oxacillin). Other antibiotics targeting prokaryotic ribosome function or DNA gyrase, such as tylocin, lincomycin, oxytetracycline, and enrofloxacin, have shown inhibitory effects on *M. bovis* growth in vitro; however, these therapeutic agents are not effective in combating infections (97). Due to the ineffectiveness of current treatment strategies, developing an effective vaccine, as a preventive measure against *M. bovis*, is an active area of research.

Compared to other mycoplasma species, *M. bovis* has an average sized genome 1,080 kbp (*Mycoplasma* spp. genome size range 600kbp – 1350kbp) maintaining a G + C ratio of 27.8-32.9 mol% (*Mycoplasma* spp. G + C ratio range 23-40%) (108, 111). Due to its limited metabolic capabilities (2), *M. bovis* is commonly grown in highly enriched media based on Friis formulations (112). This media contains a high level of serum, necessary to fulfill *M. bovis*’s requirement of cholesterol for growth, as serum lipoproteins have been shown to be a cholesterol source for mycoplasmas (113-115). This requirement may have implications in understanding changes in cell physiology and/or function upon interaction with mycoplasma species, as cholesterol is an important component of cellular lipid bilayers.

Like other mycoplasmas, *M. bovis* is extremely host-specific being isolated primarily from domesticated bovine species. This concept of a limited host range may be a consequence of limited research into other large ruminants as *M. bovis* has been isolated from respiratory tract of pneumonic American bison (Dr. Ricardo F. Rosenbusch, Iowa State University – personal communication)(108), and has been demonstrated to colonize sheep that are in contact with infected cattle (97). Infected sheep however, do not show clinical
signs of disease and are therefore considered a possible reservoir (97). There has also been one published case of \textit{M. bovis} being isolated from a woman suffering from bronchopneumonia (116). While the natural susceptibility of non-bovine hosts to \textit{M. bovis} is unclear, experimental models of \textit{M. bovis} disease have been successful in sheep, mice, rabbits, and goats (97, 117-120).

2.2 Variable surface proteins (Vsp)

Chronicity is a common characteristic of \textit{M. bovis} infections, shared by most infectious mycoplasmas (97, 121). \textit{M. bovis} has been isolated from animals for months to years following clinical disease (97). While the factors responsible for mycoplasmal chronicity may vary depending upon species and encompass a number of mechanisms, evading the immune system is paramount for mycoplasmal persistence within the host (122). Phenotypic plasticity is one immune evasion mechanism used by mycoplasmas that has received attention (122). The plasticity in phenotype is a result of altering surface antigens on the mycoplasma. This phenomenon of antigenic variation, also seen in other bacteria and parasites, presumably renders adaptive immune responses ineffective against heterogeneous populations of mycoplasmas during an infection. The basis of mycoplasmal antigenic variation is the maintenance of surface proteins that can be altered in structure or expression (123-129). Families of these variable proteins have been identified in multiple mycoplasma species (2, 130, 131) including the respiratory pathogens \textit{M. pulmonis}, \textit{M. agalactiae}, \textit{M. gallisepticum}, and \textit{M. bovis} (125, 127, 132). The family of alterable surface proteins encoded for by the \textit{M. bovis} genome are called variable surface proteins or Vsp's (133). The Vsp family of proteins contains 13 members (133). While this group is the most studied of the \textit{M. bovis} variable proteins, others do exist (134) (Anthony Vanden Bush, Dr. Rosenbusch's laboratory, Iowa State University - lab observation).

The Vsp proteins of \textit{M. bovis} share 2 basic characteristics: 1) a homologous N-terminus highly conserved as a lipoprotein signal peptide (133) and 2) a unique internal sequence characterized by multiple amino acid repeat regions. The first 25 amino acids of the N-terminus are highly conserved (99%) between Vsp family members as this region encodes the lipoprotein signal peptide, therefore, targeting the mature protein to the cell membrane.
post-modification (133, 135). The 25th amino acid residue, Cys, is 100% conserved (125, 133). This Cys residue is acylated and subsequently responsible for anchorage of the mature lipoprotein to the cell membrane (133).

The variability between family members is within repeat domains contained in the internal portion of the protein. These domains are generally comprised of amino acid sequence units repeated in tandem (125, 133). These repetitive units vary in amino acid sequence and length, and may occur in more than one Vsp family member. For example, the first repetitive unit found in VspA, the hexamer -PGENKT (denoted R₁, for repeat #1 of VspA protein), is present as 10 tandem repeats in VspA, 5 tandem repeats in VspG, and 8 tandem repeats in VspH. In contrast, VspO contains 12 repeats of R₁ distributed in two distinct locations (11 tandem repeats and one hexamer unit that stands alone). In all, 18 distinct repeat regions have been identified, comprised of 6, 8, 10, 11, 12, 26, 84, or 87 amino acids (133).

The variability in the expression pattern of Vsp family members can occur via three recombinatory mechanisms, 1) the expression of one gene is ‘turned off’ while another is ‘turned on’, 2) one gene is ‘turned off’ without inducing expression of a second gene, and 3) recombination of two genes leading to the expression of an unique gene product (136). These events can occur through high frequency, site specific inversions or recombinations (possibly driven by site specific recombinases or through spontaneous mutations of regulatory 5 nucleotide regions such as promoters (127, 136-139). Interestingly, in addition to the identification of a *M. bovis* site specific recombinase (138), there exist 2 regions of the vsp loci that encode sequences homologous to known ‘moveable genetic elements’ (133).

The hypothesis that Vsp modulation is a mechanism of immune evasion by *M. bovis* is validated by the high antigenicity of Vsp family members (140) (if these proteins were not antigenic they would not make good decoys) and that incubation of VspA+ *M. bovis* with anti-VspA antibodies triggers phenotypic changes in Vsp expression (121), i.e. VspA is turned off. In addition, contact between *M. bovis* and bovine macrophages also triggers changes in Vsp expression (141). It is important to note that, to date, only a few members of the Vsp family demonstrate expression (140). The majority of Vsp's, for example VspL,
currently exist as tentative gene products based on sequences characteristic of open reading frames.

In conjunction with the passive immune evasion function, VspS are known to play an active role in cellular adhesion (140, 142-144). Specific Vsp members appear to have stronger affinities for cellular adhesion than others, based on their repetitive epitopes (140). While the affinity of individual VspS for cellular adhesion may differ, it appears that their role in adherence is largely ubiquitous among Vsp family members (140, 142-144). This redundancy in adhesion ability is important due to the variability in Vsp expression; if one Vsp is down regulated or turned off, adhesion is not affected. Despite intense study, no additional function for any Vsp family member has been demonstrated to date.

2.3 Immune Response to *Mycoplasma bovis*

Immune responses to *M. bovis* infection are of particular interest as it is generally accepted that tissue damage (lung lesions) characteristic of respiratory mycoplasmal infection are, in part, due to the immune response (discussed earlier). Early evidence supporting immunopathogenesis in *M. bovis* infections includes the accumulation of lymphocytes in peribronchiolar and perivascular space, as with other mycoplasmal respiratory infections (76, 81). However, in *M. bovis* infections, accumulations of lymphoid cells surrounding the lung lesions (macroscopic focal regions of coagulative necrosis) are the primary inducers of increased lung pathology, and not the peribronchiolar and perivascular lymphocyte accumulations (76, 80, 81). This is in contrast to other mycoplasma respiratory infections (82, 145). More recently, the hypothesis of immune response involvement in lung damage has been upheld by a phenomenon known as vaccine induced susceptibility (VIS) or ‘priming’ (146, 147). VIS is defined as an increased amount of lung damage in vaccinated animals following challenge when compared to challenged/non-vaccinated control animals. While a recently developed *M. bovis* vaccine is purported not to lead to VIS (148), other vaccine strategies have routinely led to VIS (Dr. Ricardo Rosenbusch, Iowa State University - lab observation). The VIS phenomenon is consistent with adaptive immune response involvement in lung damage. Further evidence of immune mediated lung damage in mycoplasmal disease has been demonstrated in the mouse MRD model of *M. pulmonis*
infection (discussed earlier). Regardless of the roles T cells play in tissue damage, it is known that both adaptive and innate immune responses are important in protection against mycoplasmal infections (16, 21, 22).

Mycoplasma spp. have routinely been shown to activate cells of the innate immune system (21, 149-151). In particular, *M. fermentans* has been shown to activate monocytes via interaction with Toll-like receptor 2 (152, 153). Incubation of *M. bovis* with alveolar macrophages induces the production of NO and TNF-alpha, two potent mediators of immune activity, by the alveolar macrophages (149). Antigens derived from *M. bovis* have also been shown to activate bovine \( \gamma \delta \)-T cells in vitro, in a manner consistent with innate immunity (67). While T cells are traditionally thought of as adaptive immune cells, peripheral \( \gamma \delta \)-T cells in cattle are known to become activated in a manner consistent with innate immunity (e.g. as with mycobacterial antigens (154, 155)) and are able to present antigen to Th cells (156). The in vivo role of bovine \( \gamma \delta \)-T cells in innate immunity has yet to be elucidated.

While the activation of innate immune cells by *M. bovis* has been documented, it is currently unknown if toll-like receptors (TLRs) are involved in this activation. In contrast to macrophages, neutrophils appear to be inhibited by *M. bovis* (this will be discussed in a later section) (157). Following an initial response of innate cells during a *M. bovis* infection, adaptive immune cells are educated in order to mount a response toward *M. bovis*.

Adaptive immune responses to *M. bovis* are measurable by blastogenesis and/or serological assays; however, the level of proliferative response (blastogenesis) to *M. bovis* antigen is not very pronounced (67, 158) and in some studies has been lacking completely (159). Because of this, and the ease of serological study, serology has generally been used to measure adaptive immune responses to *M. bovis* (158, 160).

Studying both peripheral lymphocytes and serum during a *M. bovis* infection it was determined that the immune response to *M. bovis* lung infection is skewed towards a type 2 immune response (classically considered a humoral response) (67). Consistent with serological studies, immunohistochemical studies indicate a large number of IgG1 producing cells (IgG1 is an indication of a Th-2 response (49)) in the lungs of infected cattle (76). Despite the activity of both innate and adaptive immune cells, *M. bovis* tends to persist as a chronic infection, with organisms being recovered from post clinical animals up to several
months (97). It has been hypothesized that one contributing factor to the chronicity of a *M. bovis* infection is that the serological response may not be of the correct phenotype as IgG2 and IgM are superior opsonins compared to IgG1 (60). In addition, IgG1 is an inferior activator of complement compared to other IgG isoforms (61).

3. Lymphocyte Function and Suppression

3.1 T lymphocyte activation

This section is designed to give an overview of T cell activation by TCR engagement and mitogenic stimulation, followed by the description of T cell suppression by multiple compounds and their generally accepted mechanisms of lympho-suppression. These compounds have been divided into groups based largely on origin and molecular makeup. These groups include, endogenous lymphocyte inhibitors, immunosuppressive drugs, immunosuppressive peptides, bacterial products that inhibit lymphocyte activity, and mycoplasma induced immunosuppression. Following this portion is a brief description of apoptosis and its importance to lymphocyte homeostasis and pathogen induced lymphocyte suppression.

Lymphocytes, a group of cells making up the adaptive portion of an immune system, largely consist of B and T cells. Activation of these cells occurs through engagement of respective activating receptors (T cell receptor – TCR, and B cell receptor BCR) with their respective ligands (161-163). In the case of T cells, the TCR is a heterodimer consisting of either α and β subunits (αβ TCR) or γ and δ subunits (γδ TCR) (162, 164). The ligand for the TCR is an MHC:peptide complex presented on either target cells (any nucleated cell – MHC class I) or antigen presenting cells (macrophages, B-cells, dendritic cells – cells of the innate immune system, MHC class II) (161). On the T cell’s surface, the TCR complex is further associated with the hetero-hexamer CD3 (165). For naïve mature T cells the CD3 multimer is responsible for signal transduction perpetuated by TCR:ligand engagement (165, 166). Following TCR engagement, phosphorylation cascades are induced that ultimately lead to T cell activation (162, 167). Interruption of key signal transduction events will effectively inhibit T cell activation (proliferation, differentiation, and/or cytokine production) and therefore retard an effective immune response (168, 169). T cell activation can be
experimentally mimicked in vitro by engaging the CD3 with antagonizing antibodies or by the addition of lectin mitogens, such as concavalin A (ConA), phytohemagglutinin (PHA), or pokeweed mitogen (PWM) in the presence of APCs (antigen presenting cells). Although there is a difference in cytokine production between signals generated by TCR engagement and those induced by mitogen contact, the majority of intracellular signaling events (PLC-γ activation, Ca2+ influx, calcineurin activation, MAP kinase cascades, etc...) (170-172) are similar. The extent to which mitogen-stimulated T cells undergo CD3 hyperphosphorylation is unknown, however, the phosphorylation of the Src kinase Lck (involved in CD3 phosphorylation) is necessary for PHA induced maturation of T cells, presumably through an undefined interaction between Lck and MAP kinase pathways (173).

3.2 TCR signaling

Initially the interaction between TCR and MHC:peptide leads to a morphological rearrangement of lipid domains maintained in the T cell’s lipid bilayer known as rafts (174). During T cell activation (i.e. TCR and MHC:peptide engagement), TCRs and positive regulators of T cell signaling are found associated with rafts (174-176). Raft maintenance and formation, is dependent upon cholesterol (177); and, presumably, so too is cell signaling and T cell activation (174, 178). This may be important in understanding the effects of some mycoplasmas (cholesterol sequestering organisms (113-115, 179) on lymphocyte functionality (section 3.8).

If raft formation is uninterrupted upon TCR engagement, cytoplasmic domains of CD3 known as ITAMS (immunoreceptor tyrosine-based activation motifs) are phosphorylated by non-receptor tyrosine kinases (162, 165, 180). The ITAM motifs contain multiple phosphorylatable tyrosine residues. These phosphorylated tyrosines serve as ‘docking sites’ for a group of kinases containing phosphorylated tyrosine binding domains (180, 181). These domains are called SH2 (Src homology 2) domains after the protein kinase, Src (181). The kinases, aptly named Src family kinases, propagate and expand the signaling cascades initiated by TCR engagement (180, 182).

As with any cell, receptor induced responses are dependent on the coordination of intracellular signaling events. The phosphorylation cascades that are initiated by TCR
engagement are responsible for the T cell's differentiation into a mature, activated T effector cell, but not before these cascades have branched out to effect other necessary components of T cell activation, such as calcium influx (183). Phosphorylation cascades in the activation of T cells are linked to calcium flux via the activation of LAT (linker of activation in T cells) (165, 184, 185). LAT is a membrane bound tyrosine kinase activated downstream from CD3 ITAM phosphorylation following TCR engagement. The activation of LAT subsequently leads to the activation of the phospholipase-C gamma 1 (PLCy1) Ca\(^{2+}\) pathway (184). The activation of PLC\(\gamma\) leads to the cleavage of phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-triphosphate (IP3) (183). IP3 binds to its receptor on the endoplasmic reticulum (ER), ultimately leading to the release of intracellular Ca\(^{2+}\) stores from the ER (183). This release increases the level of cytoplasmic Ca\(^{2+}\), and induces the opening of membrane Ca\(^{2+}\) channels, known as CRAC channels (calcium release activated calcium channels), which allow the influx of external Ca\(^{2+}\) (183, 186). A sustained Ca\(^{2+}\) current is necessary for T cell activation (183), and is achieved by K\(^+\) channels/pumps used to maintain membrane hyperpolarization (187) (this is important in understanding the effects of some K\(^+\) pump inhibitors on T cell activation – discussed later). The dependency of T cell activation on Ca\(^{2+}\) influx is due to its indirect role in gene transcription (187).

The transcription of genes in T cells, as in all cells, is dependent upon the activation of transcription factors and regulators. An important transcription factor in T cells is known as nuclear factor of activated T cells or NFAT (of which there are multiple family members) (188). In its inactive state NFAT is phosphorylated and held within the cytoplasm (188, 189). Upon dephosphorylation, NFAT is translocated to the nucleus and augments transcriptional regulation of target genes such as IL-2 (190), an important cytokine with positive feedback effects on T cell activation/proliferation (191, 192). The activation/dephosphorylation of NFAT is catalyzed by the Ca\(^{2+}\) dependent phosphatase calcineurin (193), therefore linking Ca\(^{2+}\) influx and phosphorylation events to gene transcription and T cell activation. Calcineurin is thought to be the link between Ca\(^{2+}\) flux and regulation of gene transcription in multiple pathways, but is extremely important in T cell activation as blocking its activity abrogates TCR mediated T cell activation (194-196).
3.3 Immunosuppressive Drugs

Immunosuppressive compounds are commonly divided into 5 groups based on suppressive mechanisms (Table 1). Glucocorticoids are the model drug for Group 1, regulators of gene expression (Table 1). The use of glucocorticosteroids in treating autoimmune disease and their mechanisms in lymphocyte suppression will be covered briefly in a later section dedicated to endogenous lymphocyte suppressors (pages 21-23).

Groups 2, 3, and 4, from Table 1, specifically target nucleic acids in their mechanisms of immune suppression (168). Cyclophosphamide (Cy) is an alkylation model drug (group 2), that chemically modifies genomic DNA, subsequently suppressing cell division as its primary mechanism of therapeutic action (168, 197). Cyclophosphamide major effect is seen in suppression of antibody production (197), while the reasons for Cy’s preferential suppression of B cell responses vs. T cell responses is unknown. Cyclophosphamide is therefore used in cases of antibody dependent autoimmune diseases such as systemic lupus erythematosus (168). However, the use of Cy increases the risk of cancer due to its modification of DNA (168).

Table 1. Mechanisms of immunosuppressive drug action

<table>
<thead>
<tr>
<th>General Mechanism of action</th>
<th>Drug</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Regulation of gene expression</td>
<td>Glucocorticoids</td>
</tr>
<tr>
<td>2. Alkylation</td>
<td>Cyclophosphamide</td>
</tr>
<tr>
<td>3. De novo pyrimidine synthesis inhibition</td>
<td>Methotrexate</td>
</tr>
<tr>
<td></td>
<td>Leflunomide</td>
</tr>
<tr>
<td>4. De novo purine synthesis inhibition</td>
<td>Azathioprine</td>
</tr>
<tr>
<td></td>
<td>Methotrexate</td>
</tr>
<tr>
<td>5. Kinase/Phosphatase inhibition</td>
<td>Cyclosporin A</td>
</tr>
<tr>
<td></td>
<td>Tacrolimus (FK506)</td>
</tr>
<tr>
<td></td>
<td>Rapamycin</td>
</tr>
<tr>
<td></td>
<td>p38 kinase inhibitors</td>
</tr>
</tbody>
</table>

Adapted from: Anthony C. Allison, 2000, Immunosuppressive drugs: the first 50 years and a glance forward, Immunopharm, 47, page 64.

Groups 3 and 4 are inhibitors of pyrimidine and purine de novo synthesis respectively. These compounds halt effective production of nucleic acids needed for cellular division and general cell functions (168, 198). Because of their mode of action, these drugs
have the most effect on nucleotide dependent cells, such as blastogenic lymphocytes (rapidly dividing cells). The first immunosuppressive drug targeting purine synthesis was Azathioprine (AZA) (199), a compound that is metabolically converted to the purine homolog, 6-thioguaninenucleotide (200). This metabolite of AZA is an inhibitor of multiple enzymes involved in purine synthesis including PRPP-aminotransferase and adenylosuccinate synthase (201). Other metabolite derivatives of AZA also aid in lymphocyte inhibition, depleting lymphocytes of adenosine and guanine nucleotides (198).

Methotrexate (MTX), a drug originally designed to treat malignancies (168, 202), is a folate antagonist routinely used in treating rheumatoid arthritis. The mechanism of MTX therapeutic effects is attributed to its blockage of purine and pyrimidine de novo synthesis. The mechanism involved in blocking de novo pyrimidine synthesis is the inhibition of dihydrofolate reductase. This enzyme is responsible for the conversion of dihydrofolate to tetrahydrofolate. Tetrahydrofolate is in turn used by thymidylate synthase to convert dUMP to dTMP. Subsequently the use of MTX results in a shortage of dTTP used in DNA synthesis (168, 203). The inhibition of purine synthesis is believed to be through the inhibition of other folate-dependent enzymes such as AICAR (5-amino-imidizole carboxamide ribonucleotide) transformylase (203). This enzyme is responsible for the completion of purine de novo synthesis. Additional mechanisms may be utilized by MTX as administration of folic acid, shown to overcome the depletion of tetrahydrofolate, abrogates the side effects associated with MTX, but does not alter the therapeutic effects (204, 205). One addition to MTX suppressive capabilities is the induction of apoptosis in activated lymphocytes (206).

While no mycoplasmal products that are able to modify host DNA or inhibit purine/pyrimidine synthesis have been identified, multiple mycoplasmas have been shown to produce unique DNases able to digest host cell DNA (207-211). Presumably these DNases could induce apoptosis of host cells, including lymphocytes (209, 211); however, the identification of mycoplasmal DNases in apoptotic cells has only been realized when in vitro cell cultures contaminated with mycoplasmas were experimentally induced to undergo apoptosis (209, 210). Therefore a cause and effect relationship between mycoplasmal DNases and host cell apoptosis is lacking.
The final group of immunosuppressive drugs, Group 5, is the inhibitors of kinase and phosphatase activity. Due to the complex signaling cascades generated during the activation of T cells, kinase and phosphatase inhibitors are effective immunosuppressants. Many compounds have been identified as potential drugs based on their ability to suppress specific phosphorylation dependent pathways, such as the MAP kinase pathway (169). Two of the most often used immunosuppressive drugs in this group (kinase/phosphatase inhibitors) are Cyclosporin A (CsA) and Tacrolimus (FK506). Unlike the inhibitors of MAP kinase pathway, these drugs inhibit signaling cascades at a critical point of phosphatase and calcium dependent activities (187).

Cyclosporin A and FK506 both form complexes with heterogeneous binding proteins (immunophillins) known as cyclophilins and FK506 binding proteins, respectively (187, 212). Once engaged with cyclophilin (CsA/CP) and FKBP (FK506/FKBP) these complexes are each able to bind to and inhibit calcineurin’s phosphatase activity (212), therefore inhibiting nuclear translocation of NFAT transcription factors (187, 212). Due to their effects on calcineurin, these compounds are grouped in kinase/phosphatase inhibitors but are classified specifically as calcineurin inhibitors (168). It has recently been hypothesized that the binding of these complexes to calcineurin may invoke their NFAT suppressive activities via additional means. The complexes may block the co-transnuclear location of CN allowing the re-phosphorylation of NFAT by nuclear kinases subsequently leading to NFAT being shuttled out of the nucleus (213). In addition to the inhibition of NFAT translocation, CsA has been shown to block additional phosphorylation pathways such as janus kinase (JNK) and p38, two important signaling pathways in T cells (214).

Additional mechanisms, outside the 5 described groups of immunosuppressive drugs, have been described, including the induction of T cell apoptosis (168), and modulation of T cell peripheral mobility (215). Interestingly, multiple drugs posses more than one functional activity. For example, CsA is described as an inhibitor of calcineurin, but also induces the production of TGF-β by lymphocytes (168, 216). The production of this immune regulatory cytokine (section 3.4) is blamed for multiple side effects of CsA administration, but may also contribute to its lympho-suppressive attributes, as TGF-β is a known T cell regulator (217-219). The induction of a regulatory cytokine to control immune responses is an effective
strategy in immunotherapy as demonstrated by KM2210. KM2210 is a immunosuppressant whose mechanism of action is the induction of immuno-regulatory cytokines IL-10 and TGF-β (220).

3.4 Endogenous T lymphocyte suppressors

The following is a brief description of compounds that are able to suppress lymphocyte functions. This is not intended to be an exhaustive discussion of known endogenous T lymphocyte suppressors.

Glucocorticosteroids, such as cortisone, were the first group of endogenous compounds shown to be immune modulatory through their beneficial medicinal effects on diseases such as arthritis and asthma (221, 222). Further studies illustrated the usefulness of these compounds in preventing allograft rejection and treating immune disorders (168). The primary mechanism of lymphocyte inhibition by glucocorticosteroids is the negative regulation of immune regulatory genes such as IL-2 (168, 223). In addition to gene regulation, glucocorticosteroids have also been implicated in posttranslational mechanisms of immune modulation, for instance, decreasing mRNA stability (224) and inducing apoptosis of cells by disrupting mitochondrial membrane potentials (225). Other steroids and opioids, such as beta-endorphin and met-enkephalin, have modulatory effects on lymphocyte function as well (226, 227). Gonadal steroids have received most of the attention due to the gender differences in immune system functionality (226, 228-230). In general, it is said that estrogens enhance immune function while androgens suppress immune function (226). However, these effects have proven to be dose dependent as homeostatic levels of estrogens are shown to be immunosuppressive. As revealed by Simecka et al., these differences in gender steroids may play a direct role in mycoplasmal respiratory infections as male mice are more susceptibility to *M. pulmonis* MRD than female mice (231).

While the production of specific steroids or hormones may have a purposeful effect on lymphocytes, their production is generally associated with other non-immune biological functions. In some instances immune modulating biomolecules are produced specifically to control or ‘tamp down’ immune responses. During pregnancy, for example, EPFs (early pregnancy factors) are produced by the pregnant female (232). These serum proteins are able
to inhibit T cell function in vivo by down regulating adhesion molecules (VCAM-1, ICAM-1, and integrins LFA-1, VLA4, and MAC1) necessary for T cell tissue migration (232, 233). The up-regulation of EPFs therefore inhibits T-cell functionality in vivo by blocking extravasation, and potentially limits immune recognition of the fetus.

Other mechanisms of endogenous suppression are dependent upon immune tissues or cells themselves. The majority of these suppressive interactions are based on secreted factors such as cytokines. The microenvironment of Peyer's patches, gut associated lymphoid tissue located in the ileum of the small intestine, naturally suppresses T cell proliferation. The suppression was due to secreted factors. It was further suggested that the suppressive environment of the Peyer's patches was due to its Th2 cytokine phenotype, as the researchers failed to identify a single factor responsible for the suppressive effects (234).

It is also possible for ROS (reactive oxygen species) to inhibit T cell function. The source of which may be APC, innate immune cells, or the T cells themselves (235). It is believed that ROS may suppress T cell function by two mechanisms. The first is through the induction of apoptosis as peroxide induces the expression of the proapoptotic ligand, FasL (236-239) and decreases the expression of BcL-2 anti-apoptotic family members (240, 241). The second is through the misregulation of redox potentials and their effects on transcription factors and gene regulation (242, 243). The difference between inhibition and apoptosis induction by ROS is thought to be dose dependent (243).

One of the most recognized endogenous T cell suppressors is TGF-β (transforming growth factor beta). TGF-β is a cytokine associated with adaptive immune system regulatory functions (244, 245). TGF-β can be produced by a number of cells including, macrophages, neutrophils, dendritic cells, and T lymphocytes under various conditions (246, 247). While normally considered a negative regulator of lymphocyte function, TGF-β has been shown to act as a positive regulator of T cell function at low concentrations (0.1-1pg/ml) (219). In terms of negative regulation, TGF-β has been implicated in the down-regulation of IL-2 production, IL-2R alpha production, inhibition of tyrosine phosphorylation of specific Jak/Stat family members and a decrease in antigen induced T cell proliferation (219, 248, 249). TGF-β has also been shown to aid in induction of T cell apoptotic milieu (246, 249). The importance of TGF-β in lympho-proliferation and as a T cell regulator has been
demonstrated in multiple studies. For example, the loss of TGF-β receptor expression described in T cell malignancies (250), the correlation between TGF-β serum levels and posttraumatic immunosuppression (251), and TGF-β expression by CD4+CD25+ regulatory T cells (T_{reg}) as a membrane bound cytokine (252). In fact, the T_{reg} inhibitory effects on T cell proliferation are significantly modulated by the addition of anti-TGF-β antibodies (253). Interestingly, the production of TGF-β is also reported to be a mechanism utilized by some pathogens to dampen immune responses (discussed below) (254, 255).

3.5 Lymphocyte apoptosis

While inhibiting T cell differentiation will suppress an adaptive response, depletion of T cells will also suppress adaptive immunity. As a safeguard against uncontrolled proliferation, cells are equipped with means of suicide or programmed cell death (apoptosis). While apoptotic mechanisms are often discussed in terms of removing pre-cancerous cells, these mechanisms are also used in homeostatic maintenance of lymphocytes (256). Upon activation, T cells undergo clonal expansion in order to increase the numbers of antigen specific cells to fight infection (239). After the peak of initial T cell expansion, T cell numbers begin to decline. This phenomenon is referred to as contracting and often occurs prior to pathogen clearance (257). Contraction is a result of mass apoptosis via two mechanisms, AICD (activation induced cell death) and ACAD (activated T cell autonomous death) (235). AICD is controlled by exogenous signals delivered to the T cell, generally via interaction of membrane bound death receptors Fas (CD95) and/or TNF-alphaR with the respective ligands, FasL and TNF-α (235). In contrast ACAD, is controlled through intrinsic T cell signals, mediated by differential regulation/activation of Bcl-2 (pro and anti-apoptotic family of proteins) related proteins (235). Reactive oxygen species, NO, and TGF-β have been implicated in both forms of apoptosis (235, 239, 258). Again, some pathogens have been shown to induce the apoptosis of T lymphocytes as a possible mechanism of immune evasion. This is discussed in section 3.7, 'Bacterial products modulating lymphocyte responses'.
3.6 Immunosuppressive Peptides

A number of peptide toxins isolated from marine invertebrates and arachnids have been shown to inhibit T lymphocytes in vitro (259-265). These peptides share structural features while having little amino acid sequence homology with one another. The structural feature shared by these peptides is the conservation of 6 cystine residues that are involved in forming 3 di-sulfide bridges (266-269). As these peptides are usually no longer than 40 amino acids, these 3 bridges impose a great deal of stability and rigidity to these peptide structures.

This structural motif is also found in β-defensins, anti-microbial peptides secreted by a number of mammalian cells including mucosal epithelia (270-272). Unlike the β-defensins, however, these toxin peptides bind to and inhibit K⁺ selective proton pumps expressed on T cells (273). This binding renders the pump non-functional, subsequently affecting Ca²⁺ influx patterns (discussed earlier). These peptides therefore block Ca²⁺ dependent signaling events and inhibit the activation of the prospective T cell. The use of these peptides as immunosuppressant drugs is currently being tested and showing promising results in EAE (experimental autoimmune encephalitis), a mouse model of multiple sclerosis (260, 263).

Not all immunosuppressive peptides are K⁺ ion pump inhibitors. For example, the drug cyclosporin A (section 3.3) is a cyclic undeca-peptide inhibitor of calcineurin (274). An antimicrobial cecropin-like peptide produced by Helicobacter pylori has homology with members of the cecropin family (anti-microbial peptides produced by insects (275)) and is also able to suppress lymphocytes. The H. pylori cecropin-like peptide is an alpha-helical peptide, and does not contain any cystine residues, in contrast to the peptide toxins mentioned above. This peptide suppresses lymphocytes through an indirect mechanism, involving the activation of monocytes (discussed below).

3.7 Bacterial products modulating lymphocyte responses

Suppression of lymphocyte responses is a mechanism of immune evasion utilized by several microbial pathogens (276, 277). Suppressive mechanisms utilized by bacterial pathogens can largely be divided into 2 categories, 1) direct suppression of lymphocytes, and
2) lympho-suppression mediated by microbe-induced innate immune cells (macrophages, monocytes, etc.). The majority of direct, bacterial-mediated T cell suppression involves the induction of lymphocyte apoptosis via bacterial toxins (276, 277). The induction of apoptosis by these toxins is often dependent on the T cell's intrinsic apoptotic machinery; and is induced due to cellular stress caused by increased cell permeability, inhibition of protein production, or direct alteration of signal transduction (276-278). Based on these criteria, bacterial toxin induced T cell apoptosis is considered ACAD-like death.

The indirect lymphosuppression mediated through the activation of innate immune cells can occur via lymphocyte apoptosis or suppression without loss of cell viability. The induction of apoptosis by indirect means is generally considered a mechanism used by intracellular bacteria (276). Bacterial pathogens such as *Chlamydia* and *Mycobacteria* spp. direct their host cells (usually cells of innate immune system—such as macrophages) to induce ACID by orchestrating the delivery of apoptotic signals, such as TNF-α and FasL, to T cells (276). It is important to note that other mechanisms of apoptosis have been linked to the production of reactive oxygen species, nitric oxide, and TGF-β (235, 239, 249). While the majority of studies focused on these effects have been in vitro, resistance of T cells to apoptosis during in vitro assays correlates with resistance to chronic infection (279).

In contrast to the indirect lympho-suppression by apoptosis, multiple species of bacteria and/or bacterial products are able to suppress lymphocyte activation through induction of innate immune cell products such as TGF-β, ROS, and NO (254, 255, 280). The induction of these products can be mediated by whole bacteria, bacterial cell wall extracts, and/or LPS (254, 281-284). Suppression vs. apoptosis may be a question of TGF-β, ROS, and/or NO dose, as these products have also been linked to decrease in lymphocyte viability (mentioned above).

A special case of indirect induction of lymphocyte suppression through activation of innate cells, is the production of a cecropin-like peptide (cecrops are anti-microbial peptides related to β-defensins) by *H. pylori* (285). This 19 amino acid peptide fragment of Hsp70 gene product is chemotactic for monocytes and induces the production of ROS able to induce apoptosis of naïve lymphocytes and NK cells (280, 286). Interestingly, this peptide (known as Hp2-20) is also a bacteriocidin as it displays anti-microbial activity (286).
3.8 Mycoplasmas and immunosuppression

Multiple mycoplasma species have been reported to be immunosuppressive (Table 1). To date, the factors involved in mycoplasmal suppression of lymphocyte function have largely gone undiscovered. However, some groups have isolated inhibitory or cytotoxic mycoplasmal products. The superantigen MAM (M. arthritidis mitogen), produced by M. arthritidis, is able to suppress lymphocyte proliferation when lymphocytes are isolated from MAM immunized mice (287). This phenomenon is not fully understood, but may be due to activation induced cell death (AICD) or T cell anergy (288-291). Lipoproteins from M. fermentans and M. salivarium have shown cytotoxic effects on transformed lymphocytes and monocytes by an unknown mechanism (292, 293). An inflammatory toxin isolated from M. bovis was shown to be toxic to cells. The toxin is a 73 kDa polysaccharide containing no lipid or protein components (294). Its size and lack of other biomolecules (lipids or proteins) suggest that it is an envelope carbohydrate. However, methods used to determine the existence of polysaccharide coats produced by mycoplasmas (295) have not detected this structure in association with any tested strains of M. bovis (Dr. Ricardo Rosenbusch, Iowa State University -- personal communication).

Mycoplasma bovis is included in the group of immunosuppressive mycoplasma by modulating both neutrophils and lymphocytes (157, 296). Early research into the study of immune responses to M. bovis indicated that peripheral blood mononuclear cells (PBMCs) from M. bovis-infected animals have an impaired proliferative response to lectin mitogens in vitro, when compared to responses from non-infected animals (297). It was also shown that animals infected with M. bovis had a reduction in immune reactivity (298, 299). The effects of M. bovis infection on general animal health was subsequently described in a field case study conducted in UK. The study reported that calves born to a herd infected with M. bovis had a higher rate of mortality due to common pathogens than those born in the herd prior to M. bovis introduction (300). While this study could suggest an immunosuppressive ability of M. bovis, this hypothesis was never directly tested.
In vitro studies using purified bovine neutrophils indicated that *M. bovis* is able to bind to neutrophils and inhibit experimentally induced oxidative burst (157). Additional studies were conducted to determine the effects of *M. bovis* on bovine lymphocytes. C.J. Howard and C. Thomas independently showed that *M. bovis* is able to suppress bovine PBMC proliferative responses to lectin mitogens in vitro, and that the mycoplasmal factor(s) is heat (30min 56C) but not formalin labile (296, 309). These discoveries coupled with the identification of the immune evasion proteins, VspS, suggest that *M. bovis* is equipped with multiple mechanisms to elude immune clearance subsequently aiding in persistence and pathogenicity.
Since the discovery of mycoplasmal immunosuppression, multiple species have been described as modulating immune function. The mechanisms of immune modulation range from lymphocyte suppression through induction of apoptosis to down-regulation of immune modulatory receptors such as MHC class II (Table 2). Despite years of investigations into immunosuppressive phenomena, the molecules responsible for these effects are largely unknown. To date no mycoplasmal factor able to inhibit naive lymphocyte function has been isolated and/or characterized.

**Literature cited**


291. Wang, J. K., B. Zhu, S. T. Ju, J. Tschopp, and A. Marshak-Rothstein. 1997. CD4+ T cells reactivated with superantigen are both more sensitive to FasL-mediated killing and express a higher level of FasL. Cell Immunol 179:153-64.


CHAPTER 3. MYCOPLASMA BOVIS INDUCES APOPTOSIS OF BOVINE LYMPHOCYTES

A paper published in FEMS Immunology and Medical Microbiology†
Tony J. Vanden Bush† and Ricardo F. Rosenbusch*

Abstract

We report Mycoplasma bovis induces apoptotic death of bovine lymphocytes. Using flow-cytometry analyzed propidium iodide inclusion we observed a loss in viable lymphocytes upon incubation of freshly isolated bovine PBMCs with M. bovis. The use of Annexin V staining as well as TUNEL assays corroborated these findings. In addition, these assays indicated that the M. bovis-induced lymphocyte death is apoptotic in nature. Subsequent experiments demonstrated that the prokaryotic protein production inhibitor chloramphenicol inhibited lymphocyte death induced by M. bovis, showing that M. bovis protein production is necessary for the induction of lymphocyte death, and that the death is not dependent upon the addition of apoptotic inducers as shown with other mycoplasma. We also show that M. bovis is different from other bovine mycoplasmas (both pathogenic and non-pathogenic) with regards to this characteristic.

1. Introduction

Mycoplasma bovis is a wall-less prokaryotic (class Mollicutes) pathogen of cattle. Recently, M. bovis is gaining attention due to its negative impact on US beef and dairy industries, accounting for substantial economic losses annually. While this pathogen is most often associated with bovine pneumonia, it has the capability to become systemic causing arthritis and mastitis.

Pathogenic factors have been described for multiple species of mycoplasmas [1]. Mycoplasma bovis itself has been shown to induce bovine macrophages to produce TNF-

---

‡ Primary Researcher and Author
§ Corresponding Author. E-mail: rfrosenb@iastate.edu
alpha and nitric oxide, two powerful initiators of immune activity [2]. Along with the ability to induce immune cells to produce cytokines, previous publications have reported that *M. bovis* can modulate immune cell functions *in vitro* and *in vivo* [3-6]. Specifically, *M. bovis* is able to inhibit both mitogen mediated lympho-proliferative responses and neutrophil degranulation *in vitro* [4-6]. Using trypan blue to determine cell viability, it was reported that the observed lympho-proliferative suppression of *M. bovis* was not due to a loss of lymphocyte viability [4,5]. Because of the systemic capabilities of *Mycoplasma bovis*, we view its inhibition of lympho-proliferative responses as a potential pathogenic mechanism. We therefore studied this suppressive phenomenon in an effort to characterize the interaction between bovine lymphocytes and *M. bovis*.

2. Materials and Methods

2.1. *Mycoplasma*

*Mycoplasma* species were obtained either through ATCC (*M. bovis* Jasper 25025) or from Dr. R. Rosenbusch (*M. bovis* strain M23 [7], *M. bovirhinis* strain 352i, *M. bovocoli* strain 233, and *M. bovocoli* strain C52). All Mycoplasma were grown in modified Friis broth at 37°C, 2.5% CO₂ as previously described [8]. Mycoplasma were harvested by centrifugation (18,000 g for 20 minutes at 4°C), washed 3 times in PBS pH 7.4, and suspended at a concentration of 1 mg/ml (BioRad DC protein Assay) in RPMI 1640 complete media (RPMI supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM Gluta-Max (GibcoBRL), 5 X 10⁻³ mM β-mercaptoethanol, 25 mM Hepes, 1 mM sodium pyruvate, and 10% fetal bovine serum (FBS)). Washed mycoplasma were stored at -70°C until needed.

2.2. Bovine PBMCs harvest

Bovine PBMCs were harvested from healthy, Mycoplasma free (by nasal swab), 8 month old steers (sero-negative for *M. bovis*) in accordance with animal care regulations. Blood drawn from cattle was diluted 1:1 with 37°C PBS and layered on Histopaque 1077 (Sigma). Upon centrifugation (500 X g for 40 minutes at room temperature), the resulting band was collected, washed 2 times in PBS (centrifuged at 250 X g for 10 minutes for each wash), and counted. The cells were resuspended in RPMI 1640 complete at the desired
concentration depending on the experimental parameters. To ensure that the effect was due to *M. bovis*, cell suspensions were periodically tested for mycoplasmal contamination by PCR, and all solutions used were dedicated to individual experiments [9]. Each experiment used a single donor calf with each treatment performed in triplicate. Comparing treatment samples to an internal control from the same donor generated data for individual experiments. Mycoplasmas were added to cell cultures at amounts determined to give optimum results.

2.3. Blastogenesis

In a 96 well plate format, 4 X 10^5 PBMCs were incubated with 4 μg/ml ConA in the presence or absence of various bovine mycoplasmas (200 ng/well). After 44 hours, a trace amount of ^3H-thymidine was added to each well followed by incubation for an additional 20 hours. Cells were harvested onto a glass filter and counts per minute (cpm) read by a scintillation counter. The proliferative response was measured by stimulation index (treatment cpm/media blank cpm).

2.4. Flow cytometry

All FACS analyses were performed on a flow cytometer FACScan (Becton Dickenson), acquiring 10,000 events and analyzed using CellQuest FACS software. Percentages are based on the population of cells defined as lymphocytes. The population was designated as being lymphocytic in nature by previous experiments staining the isolated PBMCs with T cell subset markers CD4 and CD8.

2.4.1. Propidium iodide viability assay

PBMCs (9 X 10^5 cells/well) were incubated in a 24 well plate format for 40 hours in the presence or absence of M23 or Jasper (200 ng/well) strains of *M. bovis*. The cells were washed 2 times in PBS 1%BSA (FACS buffer) by centrifugation at 200 X g for 7 minutes per wash. Cells were then resuspended in 1 ml of FACS buffer containing 1.25 μg/ml propidium iodide (PI). PBMCs were subjected to flow analysis and the lymphocyte population gated. Chloramphenicol was added to cell cultures (at the same point as the mycoplasma, time = 0) to a final concentration of 50 μg/ml.
2.4.2. Apoptosis assays

PBMCs (9 X 10^5 cells/well) were incubated in a 24 well plate format for 18 hours in the presence or absence of M23 (200 ng/well). Cells from each well were then prepared according to manufacturers’ specifications (Annexin V apoptosis kit, Sigma: TUNEL apoptosis kit, R&D systems). In brief, cells were washed 2 times in FACS buffer before being subjected to either of the assays. For Annexin binding determination, the cells were resuspended in Annexin binding buffer and allowed to incubate at RT with FITC labeled Annexin V and PI, all of which were provided with the kit. With regards to the TUNEL assay, the washed cells were fixed in 3.7% paraformaldehyde in PBS. Cells were then permeabilized and incubated with a reaction mixture containing terminal dinucleotide transferase enzyme and biotinylated dNTPs. A positive control was generated by adding a nuclease (provided) to a control sample of PBMCs (negative for M. bovis) and incubating at 37°C for 15 minutes prior to incubation with the reaction mixture. The negative control did not receive the terminal dinucleotide transferase. The cells were then reacted with FITC conjugated streptavidin and washed. After their respective treatments, the cells were subjected to flow cytometry and the lymphocyte population gated and analyzed for Annexin V and PI staining or an increase in fluorescence intensity (TUNEL).

To ascertain whether the DNA damage was nuclear or cytoplasmic, cells from the TUNEL assay, described above, were placed onto glass slides by centrifugation and analyzed by confocal fluorescence microscopy. PBMCs treated with M. bovis, as described, were washed, fixed in 3.7% buffered formaldehyde, washed in PBS, and spun onto a glass slide. Cell morphology was visualized by creating a digital image through a 100X objective (oil immersion) and the digital image increased in size by a zoom factor of 1.25. Images of individual cells showing classic apoptotic bodies were cropped and sharpened using Photoshop. No images were digitally enhanced.

DNA laddering was analyzed by the extraction and purification of DNA (Quiagen blood kit) from cells incubated with and without M. bovis strain M23 for 24 hours. The DNA was then quantified, loaded in a 1.5% agarose gel at equal amounts determined by 260nm absorbance, and subjected to electrophoresis. Gels were then stained with ethidium bromide, illuminated with UV light, and photographed.
2.6. Statistics

All determinations were done in triplicate wells. Mean values were generated and compared by individual t-tests. Values are reported in Table 1 as means ± 2 X standard error of the mean (SEM).

3. Results & Discussion

3.1. Inhibition of blastogenesis responses is due to Mycoplasma bovis induced lymphocyte death. Previous publications have stated that the Jasper strain of Mycoplasma bovis, a highly passaged lab strain, is able to inhibit bovine PBMC's lympho-proliferative responses to mitogens [4,5]. To determine if the lympho-proliferative inhibitory trait was species specific, 2 other bovine mycoplasma species were tested for the inhibitory effect, utilizing 3H-thymidine incorporation based blastogenesis. Mycoplasma bovis strains M23 and Jasper were able to inhibit lympho-proliferative responses to ConA. Neither the non-pathogenic Mycoplasma bovirhinis nor the ocular pathogen Mycoplasma bovocoli have an inhibitory affect on the ConA induced lymphocyte proliferation (Fig. 1). Interestingly, M. bovirhinis was able to increase 3H-thymidine uptake by bovine PBMCs in the absence of ConA, indicating that this non-pathogenic mycoplasma has mitogenic capablities (data not shown). Even at a 10 fold increase (protein amounts), M. bovocoli and M. bovirhinis did not inhibit ConA stimulated proliferation (data not shown). It was therefore concluded that the inhibitory effect of M. bovis is not common to all bovine mycoplasmas, supporting the view that the effect may be a specific pathogenic characteristic.

The blastogenesis results obtained from the M23 strain treated wells were consistent with the previously reported results for Jasper strain. Using trypan blue exclusion after 48 hours of incubation with Jasper strain, previous publications ruled out that the blastogenesis results were due to cell death. This is in contrast to more recent data showing that infection with other species of mycoplasma increased death of T cells incubated with ConA [10]. We hypothesized that cells susceptible to M. bovis-induced death would be destroyed by 48 hour incubation, and not detected by trypan blue exclusion. We therefore used flow analyzed propidium iodide (PI) exclusion, a population based technique, to determine cell viability.
Fig. 1. *Mycoplasma bovis* inhibits proliferative responses of PBMC’s stimulated with ConA. PBMC’s were incubated in the presence of ConA, with or without mycoplasmas (1 μg/ml protein) and subjected to a blastogenesis assay. Columns are average SI ± standard deviation.

Data is represented as stimulation index (SI) and is representative of 3 individual experiments.

SI equals the treatment sample CPM divided by the CPM of the nonstimulated PBMC’s

* Statistical significance (p< 0.05)
In propidium iodide viability assays, bovine PBMC preps incubated with either M. bovis strain led to a statistically significant increase in PI staining of lymphocytes as compared to the negative control (Fig. 2a). Not only was a higher percentage of cells PI positive within the lymphocyte population, but the overall percent of cells within the lymphocyte gate was decreased (Table 1). Based on the cell scatter information the incubation of PBMC's with *M. bovis* induced a shift in cell size and granularity, creating a new cell population (Fig. 2b, c). These cells were PI positive and correlated well with the loss of cells within the lymphocyte gate (Table 1 vs. Fig. 2a and b). Even though there was variability in control values between experiments, such as seen in Table 1, the effect on lymphocyte viability upon incubation with *M. bovis* was the same. We note that incubation of PBMCs with *M. bovirhinis* or *M. bovoculi* did not reduce the number of cells within the lymphocyte gate (Table 1) or induce an increase in PI positive cells (data not shown). In fact, incubation of PBMCs with *M. bovirhinis* led to an increase in cells within the lymphocyte population as compared to the control. This is consistent with our blastogenesis assay observations that *M. bovirhinis* is mitogenic to bovine PBMCs.

### Table 1. Effects of bovine mycoplasmas on cell numbers within lymphocyte population

<table>
<thead>
<tr>
<th>Treatment of PBMCs</th>
<th>% total population within lymphocyte gate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (RPMI)</td>
<td>82.51% ± 2.26</td>
</tr>
<tr>
<td>330 ng/ml Jasper strain <em>M. bovis</em></td>
<td>65.26% ± 5.60*</td>
</tr>
<tr>
<td>330 ng/ml M23 strain <em>M. bovis</em></td>
<td>54.85% ± 1.42*</td>
</tr>
<tr>
<td>Control (RPMI) b</td>
<td>60.91% ± 1.96</td>
</tr>
<tr>
<td>330 ng/ml strain C52 <em>M. bovoculi</em> b</td>
<td>59.52% ± 1.83</td>
</tr>
<tr>
<td>330 ng/ml strain 352i <em>M. bovirhinis</em> b</td>
<td>71.46% ± 6.93*</td>
</tr>
<tr>
<td>330 ng/ml strain M23 <em>M. bovis</em> b</td>
<td>31.77% ± 4.65*</td>
</tr>
</tbody>
</table>

*Statistically different from corresponding control value (t-test P < 0.05)

* PBMCs harvested separately from those in the previous experiment.

Values are % population ± standard deviation
Percent within M and treatment (shaded) 2.73% PBMCs alone
- - - - - - 41.14% Jasper
- - - - - - 37.97% M23

Propidium Iodide

A

B

C
Viability assay results with *M. bovis* are consistent with a decrease in cell viability and demonstrate that *M. bovis* has lymphotoxic capabilities. We therefore consider that the previous work proposing *M. bovis* as an inhibitor of lympho-proliferation should be interpreted as an observation consistent with a decline in lymphocyte viability. While the inhibition of lymphocyte proliferation is most likely due to a loss in viability and not a mechanistic suppression, the phenomenon is still a potential pathogenic mechanism.

3.2. Lymphocyte death due to *Mycoplasma bovis* is apoptotic in nature.

Based on the decrease in lymphocyte size and an increase in granularity, it was proposed that the cells were undergoing an apoptotic-like death. To test this hypothesis we dual labeled PBMC samples with FITC conjugated Annexin V and PI (Sigma) after incubation with or without *M. bovis* (strain M23). Annexin V binds phosphatidylserine, the translocation of which is an early indicator of apoptosis [11]. Since Annexin V enables the visualization of early apoptotic events, the incubation time was shortened to 18 hours. Incubation with M23 increased the Annexin V positive population as well as the double labeled (Annexin V/PI) cell population as compared to the negative control, indicating an increase in apoptotic cells (Fig. 3). While the data shows an increase in Annexin+/PI- cells, indicative of early apoptosis, the majority of the shift is seen in the double positive (Annexin V+/PI+) quadrant. This result could be interpreted as either late apoptosis or necrosis, as annexin binding is not limited to apoptotic cells [12]. However, the decrease in cell size and increase in granularity shown in Figure 2 combined with the annexin V data from Figure 3 indicates that the event is apoptosis and not necrosis, as necrotic or oncotic cells increase in size and decrease in density [12]. In addition, multiple cells within the *M. bovis* treated group of PBMCs showed the characteristic apoptotic bodies on their surfaces (Fig. 3C). These cells were also positive for *in situ* TUNEL staining (data not shown).

To ensure that these cells were not activated or mitotic B cells, or necrotic cells binding Annexin V, the experiment was repeated using a TUNEL assay (R&D) [12,13]. The flow analyzed TUNEL assay showed that incubation with *M. bovis* induced a PBMC shift in fluorescence intensity (Fig. 4). This shift is directly correlated with the amount of DNA fragmentation within each cell. DNA fragmentation is a phenomenon closely linked to apoptosis. Also, we note that the cell count is lower in the *M. bovis* treated group. This
Fig. 3. *M. bovis* induces apoptotic death of lymphocytes. PBMCs were stained with Annexin V vs. PI after 18 hours incubation with: a) RPMI, or b) *M. bovis* strain M23. Lymphocyte population was gated and analyzed for Annexin (FL1-H) binding and PI incorporation (FL2-H). Numbers within each quadrant represent the percentage of lymphocytes within that quadrant. Each plot is representative of 3 individual experiments.
Figure 3C. Apoptotic cells from PBMCs cultured with *M. bovis*. Confocal bright field image of an apoptotic cell (see materials and methods). The arrows indicate apoptotic bodies, structures indicative of apoptosis. These cells represent multiple cells within the field. Figure is representative of 3 fields from each of 3 individual experiments.
Fig. 4. *Mycoplasma bovis* increases the amount of DNA fragmentation in PBMCs. Bovine PMBCs were analyzed using a TUNEL based flow assay for DNA damage after 18 hours of incubation with: a) RPMI (solide thin line), or b) *M. bovis* strain M23 (thick gray line). The dashed grey line represents the positive control (see materials and methods). Histogram is representative of two individual experiments. M represents cells positive for DNA fragmentation.
decrease in cell count is the result of cells leaving the lymphocyte gate due to morphological changes similar to those seen in figure 2c. These results were also confirmed by observing a characteristic DNA ladder, a phenomenon associated with apoptosis, from those PBMCs treated with *M. bovis* (data not shown).

Based on the translocation of phosphatidylserine, incorporation of PI, morphological changes, and an increase in nuclear DNA fragmentation (TUNEL and ladder assays) seen in PBMCs infected with *M. bovis*, we conclude that the incubation of *M. bovis* with bovine PBMCs leads to an induction of lymphocyte apoptosis.

3.3. Lymphotoxicity is dependent upon *M. bovis* protein synthesis.

To determine if the lymphotoxic effect witnessed was dependent upon prokaryotic protein production, viability assays, as described previously (PI exclusion), were performed on cells incubated in the presence of chloramphenicol (an inhibitor of prokaryotic protein production). The addition of this compound at the chosen dose had no effect on lymphocyte viability (data not shown). Chloramphenicol was able to inhibit the lymphotoxicity otherwise seen in non-chloramphenicol treated wells (Fig. 5). This result shows that the lymphotoxic effect is dependent upon mycoplasmal protein production.

While the addition of *M. bovis* to PBMCs induces lymphocyte apoptosis, it is not clear if this effect is a direct or indirect effect of *M. bovis*. In response to antigen, cells within the culture may have been producing pro-apoptotic peptides or been induced to release pre-formed pro-apoptotic peptides [14-16]. We note, however, that if the effect is indirect it is still dependent upon mycoplasmal protein production specific to *M. bovis*, as other mycoplasma species did not have the same effect.

Recent advances in flow cytometry have discredited Annexin V/PI assays as being specific for apoptosis [12]. Oncotic cells are also able to bind annexin and are permeable to PI, causing difficulty in interpreting results. However, morphological changes can be used to distinguish the two types of death. Apoptotic cells are characterized by a decrease in cell size and a condensed cytoplasm while oncotic cells are characterized by cellular swelling [12]. The cell population resulting from *M. bovis* infection (Fig 2B and C) is smaller in size and shows an increase in granularity, consistent with the morphological changes
Fig. 5. Suppression of lymphotoxic effect by chloramphenicol. Propidium iodide exclusion in lymphocytes was measured after 40 hours of PBMC incubation with, Medium (filled), Medium + chloramphenicol + *M. bovis* (broken line), or Medium + *M. bovis* (solid line). Histogram is representative of 3 individual experiments. Cells within M1 are PI positive.
characterizing apoptosis. In addition, eukaryotic cells undergoing apoptosis exhibit DNA fragmentation and laddering due to the cleavage of histone bound DNA, and this effect was also observed in \textit{M. bovis} treated PBMCs.

Multiple laboratories have described an increase in cell death due to mycoplasmal contamination \cite{10,17,18}. However, cell death in these studies was experimentally induced by the addition of pro-apoptotic chemicals or peptides. We did not add, nor were any pro-apoptotic agents present within the cell suspensions as indicated by the low percentage of non-viable cells in the control groups from each experiment.

In conjunction with previous reports of an increase in mycoplasmal-induced cell death upon induction of apoptosis by experimental means, endonucleases of mycoplasmal origin have been found in association with cultured cells’ DNA. However, these nucleases were absent from intracellular fractions if the cultured cells were not induced to undergo apoptosis \cite{10,17,18}. In short, these researchers failed to see a mycoplasma-based exacerbation of cell death in the absence of apoptotic inducers. This observation has been used to suggest that mycoplasmal endonucleases are not able to cause apoptosis per se, but are simply able to enhance the apoptotic effect once the host cell is permeablized to the endonucleases \cite{1}. In our research, \textit{M. bovoccoli}, a bovine pathogen known to produce an endonuclease similar in size and activity to that of \textit{M. bovis}, has no significant effect on the viability of lymphocytes (Figure 1 and Table 1)\cite{19}. This, along with the data presented here -- showing \textit{M. bovis} as capable of inducing apoptosis without the aid of apoptotic inducers -- indicates that lymphotoxicity is a pathogenic determinant associated with \textit{M. bovis}.

The ability of mycoplasmas to induce biologically significant processes in cell culture is of great interest as it has been estimated that from 10 to 80\% of all tissue cultures are contaminated by mycoplasmas \cite{1}. Whereas the importance of these findings is evident to researchers working in cell culture, the importance of these activities as mechanisms of pathogenesis is still undetermined. This research suggests that lymphocytes may be specifically targeted by toxic factor(s), potentially arming \textit{Mycoplasma bovis} with a unique mechanism of pathogenesis.

The induction of bovine lymphocyte apoptosis by \textit{M. bovis}, a pathogen causing respiratory disease as well as diseases such as arthritis and mastitis, seems counterintuitive;
arthritis and other forms of inflammatory diseases are often associated with mass lymphocyte infiltration and nonspecific activation, not lymphocyte death [20]. However, *M. bovis* may not be inducing the apoptosis of all lymphocytes in vivo, and the apoptotic effect may not inhibit lymphocyte infiltration or the production of pro-inflammatory cytokines by additional cell types. Furthermore, the arthritis and mastitis caused by *M. bovis* are acute and less dependent on lymphocyte infiltration and non-specific activation than chronic inflammatory disease states.

We are herein the first to report that: 1) *Mycoplasma bovis* induces lymphocyte death, 2) that *M. bovis* protein production is necessary for the induction of lymphocyte death, 3) that the death is apoptotic in nature, and 4) that the death is not dependent upon the addition of apoptotic inducers as shown with other mycoplasma. We also show that *M. bovis* is different from other bovine mycoplasmas (both pathogenic and non-pathogenic) with regards to this characteristic.

**Acknowledgements**

This work was supported in part by funding from the Iowa Livestock Health Advisory Council.

We thank Donghui Cheng for flow-cytometry technical assistance and the following persons for assistance in animal handling: Amanda Ramer, Tony Peterson, Brad Jordison, and Jessica Katzman.

**References**


CHAPTER 4. CHARACTERIZATION OF A LYMPHO-INHIBITORY PEPTIDE PRODUCED BY MYCOPLASMA BOVIS

A paper submitted to Biochemical and Biophysical Research Communications

November, 2003

Tony J. Vanden Bush and Ricardo F. Rosenbusch

Abstract

Mycoplasma bovis is able to inhibit the mitogen-induced proliferation of bovine lymphocytes. Herein is described the isolation of an immuno-inhibitory peptide from M. bovis. Using size exclusion chromatography, three lympho-suppressive fractions were isolated from M. bovis free supernatant. MALDI-TOF analysis revealed a common peak throughout the suppressive fractions. The purest of these fractions was subjected to N-terminal sequencing, revealing an 84% homologous match with the C-terminus of the M. bovis surface protein VspL (variable surface protein – L). A recombinant of the 26 amino acid peptide was also able to suppress ConA induced proliferation of bovine lymphocytes. This describes a unique immunosuppressive peptide produced by the bovine respiratory pathogen, M. bovis.

Introduction

Mycoplasmas are wall-less prokaryotes that represent the smallest self-replicating organisms known. In comparison to other prokaryotes, mycoplasmas have a relatively small genome, limited metabolic activities (may be related to their small genomes), and lack a cell wall [1]. Pathogenic species of mycoplasma are of great significance to both human and animal health, being linked to diseases such as mastitis, urethritis, arthritis, and pneumonia in animals and humans [1-3]. Despite their small genome, these obligatory parasites have evolved complex methods of immune evasion including antigenic modulation [4-6], and immune cell modification [7-9].

Antigenic modulation is accomplished through variations in surface protein expression. In the case of M. bovis, a family of highly antigenic proteins known as variable surface proteins (VspS) undergo high rates of recombination [10-12]. Through these
recombinatory events, individual VspS can be ‘turned off’, or their primary structure altered, therefore changing the bacteria’s antigenic phenotype [5, 13]. In contrast, immune cell modulation involves the suppression or alteration of immune cell function. For example, *M. pulmonis* (murine pathogen) is able to suppress DHT responses, *M. hyorhinis* (porcine pathogen) is able to suppress mitogen induced proliferation of PBMCs, and *M. pneumoniae* (human pathogen) is able to suppress cellular immune functions [14-16]. Despite these observations, characterization of a mycoplasmal product(s) capable of suppressing naive lymphocyte activation has yet to be accomplished.

*Mycoplasma bovis* is a pathogen of cattle associated with pneumonia, arthritis, and mastitis [17]. This mycoplasma’s genome contains multiple *vsp* genes. In addition to their role in immune evasion through antigenic modulation, the VspS of *M. bovis* have been characterized as adhesion factors, orchestrating the attachment of these obligatory parasites to host cells [5, 18-20]. Along with the ability to alter its antigenic phenotype, *M. bovis* is presumed to modulate immune function through induction of lymphocyte apoptosis and suppression of neutrophil function [7, 8, 21]. To date, no independent function has been assigned to an individual *vsp* gene product. In this study, the isolation of a *M. bovis* produced immunosuppressive peptide homologous to the C-terminus of Vsp-L (*Mb*-LIP – *Mycoplasma bovis* inhibitory peptide) is described.

**Material and methods**

*Bacterial Culture.* Mycoplasma bovis strain M23 was grown and maintained in Friis+ broth as previously described [22].

*Peripheral Blood Mononuclear Cell Isolation.* Bovine peripheral blood mononuclear cells (PBMCs) were isolated as previously described [22]. In brief, blood was collected from donor cattle by veinupuncture and mixed 1:10 with 2X ACD (anticoagulant). Whole blood was mixed with an equal volume of PBS and layered on lymphocyte separation media (CellGrow), and centrifuged at 400 X g for 40 minutes. The resulting band of PBMCs was collected. PBMCs were then washed in PBS three times and resuspended in supplemented
RPMI 1640 medium (4 X 10^6 cells/ml). One hundred micro-liters of cell suspension were dispensed into the wells of a 96 well, U-bottom culture plate.

**Mb-LIP Isolation.** *M. bovis* from a log phase broth culture were pelleted by centrifugation as previously described. The pellet was resuspended in PBS to wash the bacteria, followed by centrifugation. After 3 washes in PBS, the pellet was resuspended at 35X log phase concentration in PBS:HBSS w/Ca2+ (50/50 v/v) and placed at 37°C for 1.5 hours. *M. bovis* was pelleted by centrifugation, and the supernatant collected. This supernatant was subsequently passed through a 50kDa exclusion filter (Millipore). The filtrate was lyophilized. The lyophilized sample was resuspended at 5X concentration (3ml) and loaded onto a size exclusion column packed with BioGel-4, BioRad (2cm X 70cm). Double distilled water was used as the running solution. Five ml fractions were collected and lyophilized. Lyophilized samples were resuspended at 10X (0.5ml) in PBS and tested for free amine groups (ninhydrin test) and conductivity (to ensure isotonicity for the biological assay). Samples displaying both a positive ninhydrin test and conductivity similar to PBS control solution were tested for lympho-suppressive capabilities.

**Ninhydrin and conductivity tests.** Five µl of test sample was spotted onto a silicon coated glass plate (TLC plate). The spots were allowed to dry and then subjected to an aerosol of ninhyrdin reagent (0.2% ninhydrin in butanol saturated H2O). The plate was then baked at 180°C for 10 minutes. Visible, violet spots were recorded as a positive. Conductivity of 10 mls of distilled H2O was calibrated (using a conductivity meter, Fisher Scientific Co. serial # 019982) before and after the addition of 10µl of resuspended sample (above). Sample conductivity was background corrected and compared to the background corrected conductivity of PBS (10µl into 10ml).

**Lymphocyte Proliferation Assay.** Lymphocyte proliferation assays were done as previously described [22]. Mitogen stimulation of PBMCs was done by treating plated cells (as described above) with ConA (1µg/ml final concentration - Sigma). Negative controls received equal amounts of PBS instead of ConA. All treatments were performed in triplicate
including PBS blanks (negative control). Test samples were 50μl of either chromatographic fraction in PBS, recombinant Mb-LIP in PBS (concentrations as designated), or GST-control in PBS. Growth media (supplemented RPMI 1640) was then added to individual wells to a final volume of 200μl. Treated cells were incubated for 36 hr in a humidified, 37°C incubator. At 36 hrs 0.5μCi of ³H-thymidine was added to each well and the samples incubated for an additional 12 hrs. Cellular DNA was then harvested onto glass filter paper using a cell harvester, and the counts per minute (CPM) determined by a scintillation counter. Results are recorded as stimulation indexes (SI). SI is equal to CPM of the treatment divided by the CPM of the corresponding negative control (CPM treatment/CPM negative control).

**MALDI-TOF mass spectrometry.** Matrix assisted desorption ionization-time of flight (MALDI-TOF) mass spectra were recorded using a spectrometer (Thermo BioAnalysis DYNAMO). Test sample was diluted 1:5 in distilled H₂O. Sample was mixed with matrix (ACH -- α-cyano-4-hydrocinnamic acid – at 20mg/ml in 50% acetonitrile solution containing 0.1% TFA) in equal volumes. One μl was then loaded onto the sample spot, dried, and loaded into the instrument.

**N-terminal Sequencing.** N-terminal sequencing was done by using an automated Edman degradation system (Procise Protein Sequencer, Applied Biosystems) according to manufacturer's recommended protocols.

**Recombinant Construction.** Primers were constructed to amplify the DNA encoding the 26 amino acid peptide (NCBI, DNA acquisition # AF162146). Primers were designed with BamH1 and EcoRI adapters, respectively (capitalized letters are restriction sites) GGATCCtctaaaggtgaaaactac and GAATTCtttacttatttgcttttgt. PCR was performed using DNA from *M. bovis* strain M23 (isolated using the Quiagen blood kit) as template DNA. Thermocycler program: 90°C, 2min, 1X: 90C, 1min; 44C, 1.5min; 72C, 2min, 35X; 72C, 4min. The product was first cloned into the vector pCR®2.1-TOPO® (Invitrogen) and then transferred into the pGEX-2T (Amersham Biosciences) vector at the BamH1/EcoR1 site. *E. coli* were transformed with the pGEX-2T construct, and the colonies grown on selective
media (ampicillin). Colonies were selected, grown in broth culture, and plasmids isolated. The inserts were sequenced (Iowa State University - DNA facility) to ensure proper insert and frame. Colonies were grown in LB broth (to an OD of 0.600) and expression induced by IPTG (adding to a final concentration of 0.5 mM). Induced *E. coli* were isolated by centrifugation and lysed (BugBuster, Novagene) according to manufacturer's instruction. Lysed mixture was clarified by centrifugation (13,000 X g for 10 minutes) and the supernatant collected. The recombinant GST-fusion protein was isolated using Glutathione columns (Amersham Biosciences) by manufacturer's instruction. The recombinant peptide was released from bound GST by incubation with 80 Units of thrombin (Amersham Biosciences) for 4 hrs at RT. The thrombin and free recombinant peptide were then subjected to centrifugation through a 10kDa microcentricon (amicon). This procedure in its entirety was also performed on an unrelated GST-construct as a control (denoted GST-con). The GST-fusion protein was compared to GST alone and thrombin cleaved GST-fusion by SDS-PAGE to ensure production of the recombinant. The filtrate containing the recombinant (after thrombin cleavage and 10kDa filtration) was assayed for presence and purity of the recombinant Mb-LIP by MALDI (as described previously).

*Western blotting.* GST and GST-Mb-LIP fusion proteins were isolated using Glutathione columns (Amersham Biosciences) according to manufacturer's instruction and run through a 4-15% gradient SDS-PAGE gel (BioRad). Upon separation, proteins were blotted to PVDF and the membrane blocked with 5% Nonfat dry milk in PBS containing 0.1% Tween. Serum was collected from experimentally infected animals as described [23]. The serum was diluted in the blocking solution at 1:200 and incubated with the membrane for 1 hour at room temperature. The membrane was then washed in PBS-0.1% Tween 3 times. Peroxidase labeled rabbit anti-bovine was used as the secondary antibody (Jackson ImmunoResearch labs) at 1:500 dilution (in PBS-T) and incubated with membrane for 1 hour at room temperature.

An anti-GST antibody (Pierce) was used to verify the presence of both the GST and GST-fusion on the PVDF membrane. Using the same blotting and washing procedures as above with the following exceptions: primary antibody 1:10,000 and secondary antibody
(goat anti-mouse) at 1:1000. Visualization of all bands was done by incubating blots in substrate mixture (4mg/ml 4-chloro-1-naphthol in methanol and diluted into PBS at 1:6).

Results

*Lymphocyte inhibitory factor is secreted from M. bovis*

Live and formalin, but not heat, inactivated *M. bovis* are able to suppress lectin-mitogen induced proliferation of bovine lymphocytes, through a mechanism that may be associated with loss of lymphocyte viability [8, 21, 23]. To determine if the lympho-inhibitory factor(s) was easily separated from *M. bovis*, live *M. bovis* were incubated in PBS/HBSS for 1.5 hours (Materials and Methods). Supernatants were then passed through 50kDa and 10kDa filters and tested for suppressive function by $^3$H-thymidine incorporation-based blastogenesis. Both of the *M. bovis* free filtrates suppressed the mitogen directed proliferation of bovine lymphocytes, indicating that the factor was either secreted or easily separated from the bacteria (Fig.1). Retention of biological function (i.e. lympho-proliferative suppression) after 10kDa filtration suggested that the factor(s) was of a low molecular weight.

*Isolation of suppressive fraction*

The suppressive filtrate (50kDa exclusion filter) was subjected to size exclusion chromatography in an effort to further isolate the suppressive factor(s). After separation, each of the fractions was concentrated and tested for the ability to suppress lympho-proliferative responses of bovine PBMCs to ConA. To ensure isotonicity within the biological assay, only those fractions that maintained a low (equal or below that of PBS) conductivity level were tested for suppression of PBMCs (fractions 1-9 – data not shown). Fractions 7-9 tested positive for free amine groups by ninhydrin reaction (Table I), and all were able to suppress the lympho-proliferation of bovine PBMCs induced by ConA (Fig. 2). While all of these fractions were able to significantly decrease the level of lymphocyte proliferation, fraction 7 was not as potent an inhibitor as fractions 8 and 9.

Suppressive fractions were then analyzed by MALDI, to estimate the size and concentration of products within the fractions. MALDI analysis of fraction 9 revealed two
peaks, one minor and one major (Fig. 3A). The major peak (MW 2891) was believed to be the suppressive factor because it was found in all of the suppressive fractions (Table I); while the other, minor peak (1606 Da) contained within fraction 9, was not found in all suppressive fractions (Table I).

Table I. Suppressive fractions contain free amine groups and a common component based on molecular mass.

<table>
<thead>
<tr>
<th>Fraction Number</th>
<th>Ninhydrin test</th>
<th>Conductivity(^a)</th>
<th>Suppression</th>
<th>MALDI peaks(^{cd})</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>negative</td>
<td>isotonic</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>7</td>
<td>positive</td>
<td>isotonic</td>
<td>slight positive(^b)</td>
<td>1252, 1616, 1817, 2505, 2525, 2778, 2891</td>
</tr>
<tr>
<td>8</td>
<td>positive</td>
<td>isotonic</td>
<td>positive</td>
<td>2503, 2525, 2689, 2777, 2890</td>
</tr>
<tr>
<td>9</td>
<td>positive</td>
<td>isotonic</td>
<td>positive</td>
<td>1616, 2891</td>
</tr>
<tr>
<td>10</td>
<td>positive</td>
<td>hypertonic</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

\(^a\) Definition of iso, and hypertonic are based on comparison with PBS.
\(^b\) Slightly suppressive fraction decreased the amount of lymphocyte proliferation from control wells, but not to the extent of other suppressive fractions (See figure 2).
\(^c\) Bold text signifies the dominant peak by MALDI analysis.
\(^d\) Underlined text indicates peaks (MALDI determination) contained in all three suppressive fractions.

ND - Not Determined

**Suppressive peptide is homologous to variable surface protein L (VspL)**

N-terminal sequencing of fraction 9 was used to elucidate the primary amino acid sequence of the predominant peptide. N-terminal sequencing of the amino acids 2-20 revealed a sequence 84% homologous to the C-terminus of a theoretical gene product of *M. bovis* (NCBI protein acquisition # AAD53531) (Fig. 3B). Calculation of the C-terminal 26 amino acid, theoretical gene product’s molecular weight matched the MALDI analysis
Figure 1. *Mycoplasma bovis* free supernatant is able to suppress lympho-proliferation.

PBMC cultures were incubated with 25% v/v HBSS with or without *M. bovis* free supernatant filtrates passed through either 50 or 10kDa exclusion filters (Material and Methods). Cultures were then stimulated with ConA to induce lymphocyte proliferation. Filtrates from *M. bovis* suspension supernatants inhibited ConA lympho-proliferation as determined by $^3$H-thymidine uptake. *Significantly different than the positive control (HBSS + ConA) P < 0.05, T-test.*
Figure 2. Chromatography fractions able to suppress lympho-proliferation. Bovine PBMC cultures were incubated with 25% v/v PBS with or without fraction components (Material and Methods). Cultures were then stimulated with ConA to induce lymphocyte proliferation. Fractions 7-9 suppressed lympho-proliferative responses when compared to PBS control. Graphed values are average S.I. ± standard deviation. *Statistically different than the positive control (PBS + ConA) P < 0.05, T-test. **Statistically different from both PBS control and fraction #7, P < 0.05, T-test.
Figure 3A. MALDI analysis of fraction 9 reveals two components. Suppressive fraction #9 (figure 2) was subjected to MALDI to determine the number of components and to estimate the molecular weight of components. The analysis revealed 2 peaks (1616 Da, and 2891 Da).

3B

Identities (Query 2-20) = 16/19 (84%), Positives = 17/19 (89%)

Query: 1  XSKGPNYLPTGFKXGEEAK 20
   SKG NYLPI+GFK GEEAK
Sbjct: 252 NSKGENYLPSGFKGEEAK.....ETKANK 277

Figure 3B. Fraction 9 dominant peak (2891) is homologous to the C-terminus of VspL surface protein of *M. bovis*. N-terminal sequencing of the suppressive fraction #9 produced a 19 amino acid sequence. This sequence was used as a query to search for homologous sequences in the NCBI database. The 19 amino acid sequence was 84% homologous to C-terminus of VspL.
(2.87 kDa), confirming its identity as the dominant peak found in the MALDI analysis of fraction 9.

Based on the theoretical sequence information primers were designed that encompassed the 26 amino acid peptide. The fragment was amplified (PCR) and used to create a GST-fusion recombinant construct. Purification of the peptide was facilitated by thrombin cleavage. The recombinant (28 amino acids – GSSN SKGE NYLP ISGF KFG EAKE TKAN K) peptide was able to inhibit bovine PBMC proliferation in response to ConA while the GST-Con sample did not (Fig 4).

Production of Mb-LIP during infection

Vsp family members have been identified based on their (i) inclusion in a gene loci, (ii) open reading frame characteristics, and (iii) lipoprotein targeting sequence and containment of repeat regions [24]. Despite years of research, the expression of some VspS, including VspL, is currently undetermined. To ascertain if Mb-LIP is expressed by M. bovis during a respiratory infection, serum from experimentally infected animals was tested for recognition of the GST-Mb-LIP construct. Serum collected post infection reacted with the GST-Mb-LIP fusion protein and not with GST alone (Fig. 5). These data indicate that the Mb-LIP peptide is produced during an infection.

Discussion

A number of short (< 50 amino acids), linear, lympho-suppressive peptides have been isolated from the toxins of scorpions and mollusks [25-32]. These peptides share a high number of cystines (6) within their short (33-37amino acid) sequences. These 6 cystines are used to form 3 disulfide bridges within these small peptides, linking these peptides by secondary structure [27, 33, 34]. The Mb-LIP peptide however, does not contain cystines, therefore excluding it from this group of peptides. One other lympho-suppressive bacterial peptide lacking cystines is the cecropin like peptide Hp2-20 produced by Helicobacter pylori [35].

The immunosuppressive peptide produced by M. bovis described herein marks the first mycoplasmal suppressor of naive lymphocytes to be isolated and fully sequenced. The
Figure 4. **Recombinant peptide able to suppress lympho-proliferation.** Bovine PBMC cultures were incubated in the presence of ConA with or without 50μl of recombinant peptide Mb-LIP in PBS or 50μl of respective GST-Con thrombin cleavage product. *Statistically different from PBS control T-test, p value < 0.05. **Statistically different from both PBS control and other Mb-LIP concentrations, T-test, p value < 0.05.
Figure 5. Serum from *M. bovis* infected calf contains antibodies against Mb-LIP. GST-Mb-LIP fusion and GST were subjected to SDS-PAGE and transferred to PVDF membranes. Monoclonal antibody against GST was used to probe blot A. Serum from *M. bovis*-infected calf was used to probe blot B. Blot C was probed with anti-GST monoclonal antibody. Arrow indicates GST-Mb-LIP fusion band.
M. arthitidis superantigen MAM (M. arthitidis mitogen) is able to inhibit lymphocyte proliferation, but only if the lymphocytes are recovered from a mouse previously immunized against MAM, and therefore not naive [36]. This observation is similar to that of an anergic response of the cultured lymphocytes, or activation induced cell death [37-39]. When cultured with naïve lymphocytes in the presence of accessory cells, MAM induces lympho-proliferation [36]. Lipoproteins from M. salivarium and M. fermentans are capable of inducing the death of transformed monocytes and lymphocytes, but the effect has not been documented for naïve lymphocytes [40, 41]. The elucidation of Mb-LIP is therefore an important step in understanding the interactions between mycoplasmas and their hosts’ immune systems.

The inhibition of lymphocyte proliferation may be due to mechanistic suppression or the induction of lymphocyte apoptosis by the peptide Mb-LIP. Either of these possibilities can be a consequence of direct or indirect mechanisms. Indirect mechanisms would entail the employment of eukaryotic devices to suppress or destroy lymphocyte populations, through the production of suppressive cytokines (TGF-β) or pro-apoptotic compounds (ROS, FasL, etc.) [42-44]. Direct mechanisms would require direct activity of Mb-LIP on lymphocytes such as the toxin produced by Staphylococcus aureus, able to directly kill lymphocytes [45].

Due to the increase in transplantation surgery and diagnosis of autoimmune diseases, the importance of immunosuppressive molecules in research and modern medicine is evident. A recent and attractive application of immunosuppressant peptides is in the treatment of multiple sclerosis (MS). Lympho-suppressive peptides isolated from the toxins of invertebrates have been successful in treating experimental autoimmune encephalopathy (EAE), an experimental mouse model of MS [26, 46]. The potential of Mb-LIP to be used as an immunosuppressant is dependent upon multiple factors; even still, the elucidation of Mb-LIP’s activity may open the door to new immunosuppressive drug targets and strategies.

In addition to the discovery of a novel immunosuppressive peptide, we are herein the first to 1) isolate and characterize a mycoplasma product able to inhibit naïve lymphocyte activation, and 2) define a specific pathogenic function to a Vsp family member.
REFERENCES


[38] J. K. Wang, B. Zhu, S. T. Ju, J. Tschopp, and A. Marshak-Rothstein, CD4+ T cells reactivated with superantigen are both more sensitive to FasL-mediated killing and express a higher level of FasL, Cell Immunol 179 (1997) 153-164.


CHAPTER 5. GENERAL CONCLUSIONS

Summary

Evasion of host immune clearance is paramount to the survival of any parasitic pathogen. The bovine respiratory pathogen Mycoplasma bovis is able to modulate bovine lymphocyte function as a possible mechanism of immune evasion. Immune modulation by M. bovis through the induction of lymphocyte apoptosis is dependent upon prokaryotic protein production, indicating that the mechanism may be related to a M. bovis produced lympho-toxin, and not an indirect activation induced cell death (AICD) mechanism.

Consistent with the hypothesis of a proteinacious product able to directly inhibit bovine lymphocyte function, a lympho-suppressive peptide produced by M. bovis was identified. This 26 amino acid peptide, referred to as Mb-LIP, is able to inhibit the lympho-proliferative responses of bovine lymphocytes to the mitogen ConA. This potential lymphotoxin is easily separated from M. bovis suggesting that it is a secreted product. Owing to its homology with the C-terminal end of an externally exposed surface protein (VspL), Mb-LIP may be a cleavage product from VspL. Release of such protein fragments by protease activity is a recognized mechanism of secretion utilized by several mycoplasma species.

Recommendations for future research

The discovery of a novel immunosuppressive peptide can open many areas of continuing research. With regards to Mb-LIP, research can be divided into 2 general directions based on discipline. The first direction is immunological in nature, and focuses on the immunosuppression induced by M. bovis, with particular interests on the mechanism(s) of suppression. Introductory studies could determine the effects of Mb-LIP, or M. bovis on lymphocytes from other species (human or mouse). This would not only compare the species specificity of M. bovis effects, but also allow researchers to utilize a larger pool of reagents designed for model species. Using purified Mb-LIP, determine the effectiveness of it as an in vivo immunosuppressant, and determine if the Mb-LIP mediated lympho-suppression is dependent on eukaryotic activation via TGF-β, FasL, or ROS production. Whether the effects of Mb-LIP are direct or indirect, elucidation of its molecular interaction with
eukaryotic cells would be of great interest, including determining the receptor for Mb-LIP. Elucidating Mb-LIP's mechanisms of lymphocyte inhibition could prove valuable to medicine as a prototype for immunosuppressive drugs or the discovery of novel drug targets for immunosuppression.

The second direction is microbiological in nature, and focuses on the regulation of Mb-LIP and VspL by *M. bovis*. It should be determined if Mb-LIP is the only immunosuppressive factor produced by *M. bovis*. The expression of VspL by immunosuppressive strains of *M. bovis* should be tested, i.e. do all *M. bovis* strains produce VspL or Mb-LIP? Are all *M. bovis* strains immunosuppressive? It should be determined if the source of Mb-LIP is the cleavage of surface VspL, cytoplasmic VspL, or if Mb-LIP is not a cleavage product of VspL at all.

Having discovered an immunosuppressive mechanism utilized by *M. bovis*, defining the first mycoplasmal factor able to inhibit naïve lymphocytes, and characterizing a novel immunosuppressive peptide, the stage is now set for continued research into the immunosuppressive mechanisms used by mycoplasmas as well as other bacterial species. Studies into these areas will open new avenues of research culminating in a better understanding of mycoplasma/lymphocyte interactions.
ACKNOWLEDGEMENTS

I would here like to thank the people whose influence, support, and understanding were paramount in the completion of this degree. To my mother for her support, strength, and ability to lead by example (I’m proud of you too mom); to my mother and father for indulging my inquisitive, sometimes rebellious, nature (sorry for all the trouble); Larry Javens for his interest (or at least the appearance of being interested) in my work; my friends, whose words of encouragement helped me through times of doubt (Todd, Brad, Dan, Dave, John, Jason, and Paul); the members of Rosenbusch’s lab who’ve kept me company through the years; and, of course, my wife, Jane. The last few years would have been near impossible without her love, support, and understanding.

Thanks to the members of my committee for their guidance and interest in my progress. A special thanks to Dr. Mike Wannemuehler for making me feel like a scientist and not a student; our ‘parking-lot’ talks made this experience not only endurable but also memorable. In closing I would like to give my gratitude and thanks to Dr. Ricardo Rosenbusch for his guidance and attitude toward his role as a major professor. The lessons learned under your tutelage will not be lost, thank you for treating me as a peer.
APPENDIX A: CHARACTERIZATION OF THE IMMUNE RESPONSE TO MYCOPLASMA BOVIS LUNG INFECTION

Article published in the journal, Veterinary Immunology and Immunopathology

Tony J. Vanden Bush§ and Ricardo F. Rosenbusch*

Abstract

To better understand the interaction between Mycoplasma bovis and its bovine host, we have characterized the immune response generated during an experimental lung infection with M. bovis. Proliferation (3H-thymidine blastogenesis) and Th1/Th2 cytokine production were used to monitor peripheral cellular immune responses. Flow cytometry analysis was used to determine T cell subset activity by CD25 expression. Humoral immune response was monitored by the identification of antigen-specific IgG1 and IgG2 isotypes over time. Herein we show that M. bovis antigen stimulates activation of CD4+ and CD8+ cells in vitro in a manner consistent with memory, and that γδ-T cells are activated by antigen in a manner consistent with innate immunity. In addition, the percent of cells producing IFN-γ during recall response is equal to that of IL-4 producing cells. Serological analysis shows M. bovis stimulates increased production of antigen-specific IgG1 while very little IgG2 is produced. We therefore submit that experimental lung infection of cattle with M. bovis results in a Th2 skewed immune response.

Introduction

Mycoplasmas are wall-less prokaryotes (class Mollicutes) that encompass a diverse group of organisms considered the smallest self-replicating organisms known. While some of these (obligatory) parasites are harmless commensals within their hosts, others are capable of causing severe disease (Razin, et al., 1998). Mycoplasma bovis is a pathogen of considerable importance to beef and dairy industries. While M. bovis is primarily considered a respiratory or mammary pathogen (causing pneumonia and/or mastitis), it has the

---

§ Primary Researcher and Author
* Corresponding Author. E-mail: rfrosemb@iastate.edu
capabilities to become systemic causing several other diseases including arthritis (Pfutzner, et al., 1996).

As with many mycoplasma species, M. bovis has been reported to be both immune reactive and immunosuppressive (Razin, et al., 1998). Upon incubation with M. bovis, alveolar macrophages are activated and produce TNF-alpha and nitric oxide (Jungi, et al., 1996). In contrast, M. bovis also displays immunosuppressive characteristics inhibiting neutrophil degranulation and oxidative bursts and proliferation of lymphocytes directed by mitogens (Finch, 1990; Thomas, et al., 1990; Thomas, et al., 1991). Recently it was determined that M. bovis induces bovine lymphocyte apoptosis in vitro (Vanden Bush, et al., 2002). Despite its deleterious effects on lymphocytes, infected cattle are able to generate measurable humoral and cellular immune responses against M. bovis (Howard, et al., 1986). The importance of understanding the immune response generated against M. bovis is underscored by reports that the immune system is partly responsible for the lung damage in mycoplasma-induced pneumonia, as reported in the mouse M. pulmonis infection model (Cartner, et al., 1998). Characterization of the immune response to M. bovis is therefore critical in understanding the interaction between M. bovis and the bovine immune system, and it's potential relation to lung pathology.

Materials and Methods

Animals

Eight 12 week old calves, negative for respiratory mycoplasmas (nasal swab culture) and sero-negative for M. bovis (ELISA), were infected by intratracheal inoculation with 25ml PBS containing 7 X 10⁹ CFU/ml fresh culture M. bovis strain M23. An additional 10ml of inoculum was delivered intranasally (5ml/nostril). Calves were housed at an animal research facility located on site. All procedures were done with consent from Iowa State University's Animal Use Committee.

Sample Collection and Preparation

One day prior to infection, and at indicated times thereafter, PBMCs and/or serum was collected from each animal. Blood was collected via venipuncture. Aliquots allowed to
clot were then centrifuged and the serum collected and stored at -20°C until use. Other aliquots were collected over sodium citrate anticoagulant, and PBMCs isolated (Vanden Bush, et al., 2002). In brief, noncoagulated blood sample was diluted 1:2 with 37°C PBS and layered on Histopaque 1077 (Sigma). Upon centrifugation (500 X g for 40 minutes at room temperature), the resulting band was collected, washed 2 times in PBS (centrifuged at 250 X g for 10 minutes for each wash), and cells counted. The cells were resuspended in complete media (RPPI 1640 containing 10% FBS – tested BVDV free, 100 units ml$^{-1}$ penicillin, 100 μg ml$^{-1}$ streptomycin, and 2mM Gluta-Max (Gibco BRL, Grand Island, NY)) at the desired concentration depending on the experimental parameters.

**Blastogenesis**

In a 96 well plate format, $4 \times 10^5$ PBMCs were incubated with either $2\mu$g/ml ConA, 1.5μg/ml heat killed *M. bovis* strain M23 (56°C for 30 min.), or complete media. After 72 hours, 0.5μCi of $^3$H-thymidine was added to each well followed by incubation for an additional 20 hours. Cells were harvested onto a glass filter and counts per minute (cpm) read by a scintillation counter. The proliferative response was measured by background corrected stimulation index (treatment cpm/complete media cpm).

**IFN-$\gamma$ secretion**

In a 96 well plate format, $4 \times 10^5$ PBMCs were incubated with either $2\mu$g/ml ConA, 1.5μg/ml heat killed *M. bovis* strain M23, or equal volume PBS (each treatment in triplicate). After 72 hours supernatants from individual wells were collected and frozen at -70°C. Supernatants were tested for IFN-$\gamma$ by Bovi-gam ELISA (BioCore, Omaha, NE) according to manufacturer's instructions. Comparisons were made between the three treatments. Results are representative of 2 trials using cells collected from the same group of 4 animals.

**Serum ELISA**

Serum was subjected to antigen-specific, direct or isotype specific ELISA. In brief, Tween-20 extracted *M. bovis* proteins were used to coat an 96 well Immulon I ELISA plate at 5μg protein/well. After antigen attachment, plates were washed 4 times (isotonic saline
containing 0.1% Tween, Sal-T), then blocked (1% FCS in Sal-T) for 2 hours at RT. After blocking, the plates were washed 3 times with Sal-T and diluted serum samples added to the wells in triplicate -- serum dilutions for the direct ELISA (using Protein G) were 1:100 (50mM Tris containing BSA 0.1%, 0.8% saline, and 0.05% Tween 20, TSB-T). The plates were then incubated for 30 minutes at 37°C. After incubation, plates were washed 3 times with TSB-T, and horse-radish peroxidase conjugated Protein-G (diluted in TSB-T 1:1000 from 1mg/ml stock) added to the wells. The plates were then incubated for an additional 30 minutes at 37°C, subsequently washed (TSB-T for 3 times), and peroxidase reaction mixture (ABTS containing H$_2$O$_2$ -- Kirkegaard and Perry, Gaithersburg, MD) added to each well. Isotype-specific ELISAs were performed using serum at 1:20 dilution (in TSB-T). DAS-2 (mouse anti-bovine IgG2) and DAS-16 (mouse anti-bovine IgG1) monoclonal antibodies were gifts from Celia O'Brien (Beltsville, MD) and used as secondary antibodies (1:500 dilution of ascites fluid) followed by goat anti-mouse HRPO conjugate (Jackson Immunologicals, West Grove, PA) at 0.8μg/ml. All incubations were 30 minutes at 37°C and were followed by 3 washes with TSB-T (as described above). Peroxidase reaction mixture was added to wells and incubated for 10 minutes before reading absorbance at 405nm.

To determine comparability between IgG1 and IgG2 amounts using DAS-2 and DAS-16 secondary antibodies, isotype ELISA validations were run and the absorbances compared. In brief, one 96 well Immulon I plate was divided in half, and each half coated with serial dilutions (in quadruplicate) of either bovine IgG1 or IgG2 (Jackson Immunologicals, West Grove, PA) normalized to decreasing protein amounts per well. DAS monoclonals and goat anti-mouse HRPO conjugated antibody were used in a direct ELISA (as described above). Absorbance readings based on IgG1 and IgG2 amounts showed no statistical difference (P>0.05), thus indicating that relative amounts of serum IgG isotypes could be compared using the DAS-2 and DAS-16 monoclonal antibodies.

Flow Cytometry

PBMCs were purified and used to seed 96 well plates at 4 X 10$^5$ cells per well with or without 5μg of heat inactivated *M. bovis* (Vanden Bush, et al., 2002). After 5 days of
incubation the cells were stained with appropriate antibodies, and subjected to flow-cytometric analysis. For dual labeling of CD25 and T cell subset markers, the PBMCs were washed 3 times in PBS containing 0.1% NaN₃. The cells were then incubated for 30 minutes at 4°C with mouse anti-CD25 and either mouse anti-CD4, mouse anti-CD8, or mouse anti-γδ TCR (Table 1). Cells were then washed 3 additional times before incubating with appropriate fluorochrome-conjugated, isotype-specific secondary antibodies (Table 1). Cells were incubated with secondary antibodies for 30 minutes at 4°C according to manufacturer’s instructions. The cells were then washed 3 times and fixed in PBS 1% formalin, 0.1% NaN₃.

Intracellular cytokine staining was performed in 96 well format. Upon incubation with and without *M. bovis* antigen for 5 days, the culture media was replaced with media containing 5μg/ml Brefeldin A, 25ng/ml PMA, and 1μg/ml ionomycin and allowed to incubate an additional 5 hours. Cells were then washed 2 times in PBS, fixed and permeabilized using Cytofix/Cytoperm (Becton Dickinson, San Jose, Ca) according to manufacturer’s instructions. Cells were then washed 2 times in PBS, fixed and permeabilized using Cytofix/Cytoperm (Becton Dickinson, San Jose, Ca) according to manufacturer’s instructions. Cells were again washed (using Cytowash – Becton Dickinson) and incubated for 30 minutes with mouse anti-bovine IFN-γ (biotin-conjugated) or mouse anti-bovine IL-4 (Serotech, Raleigh, NC). Cells were again washed and incubated respectively with Avidin-PE (Sigma) -- according to manufacturer’s instructions -- or goat anti-mouse IgG2b – PE conjugate (Southern Biotechnology Associates, Birmingham, AL) at 0.5μg/ml. Cells were washed and subjected to flow cytometry analysis. Gates for all flow cytometry analysis were determined using appropriate isotype controls (Table 1).

**Statistics**

Flow cytometry analysis was done on a FACScan flow cytometer (Becton Dickinson), acquiring 5000 events and data analyzed with CellQuest FACS software. Each experiment was repeated 2 times using 2 different groups of four cattle (unless otherwise stated). Background corrected percent CD25 positive cells were values from treated cells minus values from non-treated cells. In blastogenesis and IFN-γ ELISA assays, stimulation indexes were calculated as corrected value from treated cells divided by value from non-treated cells. P-values less than 0.05 were considered statistically significant, analyzed by t-tests.
<table>
<thead>
<tr>
<th>$1^\circ$ Antibody/specificity</th>
<th>Isotype</th>
<th>Clone/Vendor</th>
<th>$2^\circ$ Antibody/specificity</th>
<th>Conjugate</th>
</tr>
</thead>
<tbody>
<tr>
<td>mouse anti-bo CD25</td>
<td>IgG2a</td>
<td>CACT108A / VMRD Pullman, WA</td>
<td>goat anti-mouse IgG2a</td>
<td>PE</td>
</tr>
<tr>
<td>mouse anti-bo CD4</td>
<td>IgG1</td>
<td>CACT138A / VMRD Pullman, WA</td>
<td>goat anti-mouse IgG1</td>
<td>FITC</td>
</tr>
<tr>
<td>mouse anti-bo CD8</td>
<td>IgG1</td>
<td>CACT80C / VMRD Pullman, WA</td>
<td>goat anti-mouse IgG1</td>
<td>FITC</td>
</tr>
<tr>
<td>mouse anti-bo γδ-TCR</td>
<td>IgG2b</td>
<td>TcR1-N24 / VMRD Pullman, WA</td>
<td>goat anti-mouse IgG2b</td>
<td>FITC</td>
</tr>
<tr>
<td>mouse anti-bo IFN-γ (biotin)</td>
<td>IgG1</td>
<td>7B6 / SeroTec Oxford UK.</td>
<td>Avidin</td>
<td>PE</td>
</tr>
<tr>
<td>mouse anti-bo IL-4</td>
<td>IgG2b</td>
<td>IOH8 / SeroTec Oxford UK.</td>
<td>goat anti-mouse IgG2b</td>
<td>PE</td>
</tr>
<tr>
<td>mouse IgG2a isotype control</td>
<td></td>
<td></td>
<td>goat anti-mouse IgG2a</td>
<td>PE</td>
</tr>
<tr>
<td>mouse IgG1 isotype control</td>
<td></td>
<td>CACT80C / VMRD Pullman, WA</td>
<td>goat anti-mouse IgG1 or Avidin</td>
<td>PE</td>
</tr>
<tr>
<td>mouse IgG2b isotype control</td>
<td></td>
<td>TcR1-N24 / VMRD Pullman, WA</td>
<td>goat anti-mouse IgG2b</td>
<td>FITC</td>
</tr>
</tbody>
</table>

Table 1. List of the antibodies used for flow cytometric analysis of T cell subset activation and cytokine production. All secondary antibodies were purchased from Southern Biotech (Birmingham AL). PE-conjugated avidin was purchased from Sigma.
Results

Subset Activation

The recall responses of peripheral lymphocytes can be used to characterize immune cell function to an infection (Rhodes, et al., 2001; Sandbulte, et al., 2002). Lymphocyte activation upon incubation with heat inactivated *M. bovis* was monitored using both blastogenesis and fluorescence based detection of CD25 expression. The cells from infected animals proliferated in response to antigenic stimulation, and in contrast, those from non-infected animals did not (FIG. 1). Using dual color analysis of PBMCs from infected animals (labeling both the subset marker and CD25), the subset of cells responding to the *in vitro* stimulation was determined. All three T cell subsets CD4, CD8, and γδ-T cell groups had an increase in the percentage of CD25 positive cells when incubated with heat inactivated *M. bovis* as compared to no antigen addition (FIG. 2). The increase in the percentage of CD8/CD25 double positive cells however, was proportionally smaller than either the CD4 or γδ-T cell subset increases.

To ensure that the increase in CD25 positive cells was a recall response to antigen and not simply an *in vitro* effect of antigen presentation, PBMCs harvested from infected animals were compared to those harvested from non-infected animals (FIG. 2). Expression of CD25 on naive animals' CD4 and CD8 groups was statistically lower than those from the infected cattle, indicating that the infected animals' cells were responding to *M. bovis* antigen. This confirmed that the CD25 positive PBMCs from infected animals were responding to *M. bovis* antigen in a manner consistent with a memory response. However, the γδ-T cells of both groups, infected and non-infected, became CD25 positive upon stimulation with *M. bovis*. This is consistent with the view that bovine circulating γδ-T cells may become activated in an innate immune fashion (Smyth, et al., 2001; Kennedy, et al., 2002).

Cytokine production

To help characterize the type of immune response to *M. bovis*, IFN-γ secretion during recall responses was monitored (FIG. 3). PBMCs from infected animals produced IFN-γ in response to inactivated *M. bovis*. The IFN-γ production was considered to be a result of
Figure 1. PBMCs from *Mycoplasma bovis* infected animals proliferate in response to *in vitro* antigen stimulation. PBMCs from infected (I#1-I#4, 21 dpi) and non-infected (U#1, and U#2) animals were purified and incubated with or without heat inactivated *M. bovis*. $^3$H-thymidine uptake was used as an indication of proliferation. Recorded values are averages of Stimulation Indexes (SI = CPM treatment/CPM untreated) ± standard deviation.

*Statistically different from 1 (zero stimulation) P< 0.05. ** Statistically different from 1 (zero stimulation) P< 0.01
Figure 2. All T cell subsets are activated in response to *M. bovis* antigen. PBMCs were harvested from infected (21 dpi, n=4) and non-infected cattle (n=2), incubated with or without heat inactivated *M. bovis* and subsequently labeled for the expression of both CD25 and T cell specific subset markers (CD4, CD8 and γδ-TCR). Values are background corrected mean percentages of activated T cell subsets ± standard deviation. *Statistically different from infected group results (P< 0.05). †Statistically different from background (values differ from zero).
Figure 3. PBMCs from infected animals secrete IFN-γ in response to antigen. Supernatants from in vitro recall assays (isolated 35 dpi) were subjected to BOVIGAM™ ELISA and the relative absorbance (Abs) levels converted to a stimulation index (SI = Abs treated/Abs untreated control). * Statistically different from untreated (P< 0.001). **Statistically different from untreated (P< 0.05).
antigen specific recall response since PBMCs from naive animals did not produce significant levels of IFN-γ when incubated with *M. bovis* antigen.

PBMCs from infected and naive animals were subjected to intracellular cytokine staining for IFN-γ and IL-4 after incubation with or without *M. bovis* antigen (FIG. 4A). There is a significant difference between infected and non-infected animals in both IFN-γ and IL-4 production. Percentage (background corrected) of lymphocytes, from infected animals, positive for IFN-γ production was then compared to the percent of IL-4 producing lymphocytes (FIG. 4B). In three of the four infected animals, IL-4 and IFN-γ producing cells were equal in number i.e. showed no statistical difference. PBMCs from calf I#3 however had a statistically greater number of IL-4 than IFN-γ producing cells. While the results are not conclusive, these data would suggest that the immune response is either mixed, or skewed to a Th2 response. To help ascertain if the response is skewed or mixed, antigen-specific antibody responses were studied.

**Serum Responses**

Sera collected every 7 days over a 63 day period were tested for the presence of antigen specific IgG antibodies. All four cattle showed an increase in antigen-specific antibody titers over the 63 day period (FIG. 5). These findings are consistent with a functional humoral response.

To better characterize the humoral response, ELISA tests were performed using isotype specific (IgG1 and IgG2) secondary antibodies to determine if the response had a skewed IgG isotype phenotype with regards to IgG1 and IgG2. The levels of antigen-specific IgG1 continually increased during the testing period, while IgG2 levels barely rose above background levels (FIG. 5B and C). The response was dominated by an IgG1 isotype production, consistent with a Th2 response corroborating the earlier cytokine data.

**Discussion**

Due to both the immunosuppressive nature of *M. bovis* and reports that the immune response is partly responsible for lung pathology, we have characterized the type of adaptive immune response to a *M. bovis* lung infection. While *M. bovis* infection does induce a
Percent PBMCs positive for cytokine production

A

B

#1

#2

#3

#4

IFN-γ

IL-4

Infected

Non-Infected
Figure 5A. *Mycoplasma bovis* infection results in antigen-specific antibody production consistent with a Th2 immune response. Serum collected over 9 weeks was subjected to antigen-specific ELISA. Total IgG was detected using peroxidase conjugated Protein-G as a signaling molecule. Individual points represent the mean of triplicate wells. The graph is representative of 4 individual ELISA trials (2 trials for each group of 4 animals).
measurable cellular response (Bennett, et al., 1977) (blastogenesis) (Howard, et al., 1986) (humoral), neither the subsets of T lymphocytes activated during a recall assay nor the phenotype of the response had been identified. CD25 (IL2R-alpha subunit) can be used as an indicator of lymphocyte activation as this marker's expression is increased upon activation of lymphocytes (Dutton, et al., 1998; Quade, et al., 1999; Endsley, et al., 2002; Sandbulte, et al., 2002). Using CD25 as an indication of activation we report that the major subsets of T cells (CD4, γδ-T cells, and, to a lesser extent, CD8) are all activated during incubation with antigen.

IFN-γ production has been used as a measure of cellular immune responsiveness and is tightly associated with a Th1 immune response. The production of IFN-γ was not limited to infected animals, as one of the control animal’s PBMCs responded to antigen with limited production of IFN-γ (Fig. 3). The production of this IFN-γ could have come from γδ-T cells activated in a manner consistent with innate immunity. Previous studies have shown that bovine peripheral γδ-T cells are able to produce IFN-γ (Sopp, et al., 2001). However, the level of IFN-γ produced by the PBMCs from the naive animal is significantly lower than that of the infected animals.

As IFN-γ production is indicative of a Th1 response, IL-4 is an indicator of Th2 responses. In a murine polarized response (Th1 or Th2) there are large increases in characteristic cytokines (either IL-4 or IFN-γ) and cytokine producing T cells, while the opposing cytokine is produced in low amounts by relatively few T cells (Morel, et al., 1998). In the bovine, however, clear divisions in cytokine production, i.e. immune response phenotype, are not as evident (Brown, et al., 1998). Currently, ratios of IL-4 and IFN-γ amounts have been used to determine Th1 vs. Th2 responsiveness in out-bred animals (Koh, et al., 2001). Given that a reliable ELISA for bovine IL-4 has yet to be developed, we presented our data as direct comparisons of IFN-γ vs. IL-4 producing cells (similar to data gathered using an ELISPOT assay).

Because the driving forces of Th1 and Th2 responses include the specific antigen and the genetics of the host, a “polarized response” depends on the individual host as well as the pathogen. Due to the genetic variables in immune response phenotype, out-bred animals often give less polarized responses than in-bred animal models. In the bovine model, high
levels of IFN-γ, low levels of IL-4 and an increase in IgG2 antigen-specific antibody would represent a Th1 response (Estes, et al., 1994). A Th2 response would be represented by high levels of IL-4, low to moderate levels of IFN-γ, and an increase in IgG1 antigen-specific antibodies (Estes, et al., 1995). Differences in antigen-specific immunoglobulin isotype production are largely due to the differing effects of the Th1 and Th2 characteristic cytokines on B cell function and immunoglobulin gene rearrangement (Estes, et al., 1994; Estes, et al., 1995). For this reason, ratios of cytokine amounts and antigen-specific, antibody isotype determination are used as indicators of response phenotyping. In the case of *M. bovis* experimental infection, the number of PBMCs producing IL-4 was as great or greater than the number of PBMCs producing IFN-γ. While the number of cells producing IL-4 and IFN-γ are statistically similar, the immunoglobulin phenotype (high levels of Ag-specific IgG1, low levels of Ag-specific IgG2) was consistent with a Th2 response. We therefore conclude that the immune response mounted against a *M. bovis* lung infection, while not polarized, is skewed toward a Th2 phenotype. This skewed response is consistent with other mycoplasma respiratory infections, including *Mycoplasma pneumoniae* infections of humans and *M. pulmonis* infections of mice (Koh, et al., 2001; Romero-Rojas, et al., 2001). The high level of IgG1 vs. IgG2 not only aids in our response characterization, but may have serious implications with regards to *M. bovis* caused diseases as it is thought that IgG2 is the superior opsonin compared to IgG1. The low IgG2 response may therefore contribute to the chronicity of a *M. bovis* infection [Howard, 1984].

The activation of CD8 cells by *M. bovis* was unexpected as *M. bovis* is an extracellular pathogen and CD8 cells are normally activated by intracellular pathogens (Harty et. al., 2000). However, antigen presenting cells can “cross-prime” in their antigen presenting capabilities, activating CD8 cells with antigen derived from extracellular pathogens (Gromme et. al., 2002; Maecker et. al., 2001). Although the upregulation of CD25 on the CD8 cells could be attributed to IL-2 production by activated CD4 cells, we consider the activation of CD8 cells as antigen driven due to reports of CD8 cells being involved in other mycoplasma infections (Gaunson et. al., 2000; Hayakawa et. al., 2002; Jones et. al. 2002; Rodriguez et. al., 2000).
The impact of cytokines on lung cells during mycoplasmal respiratory disease is not currently known. Cytokine production during an immune response to *M. bovis* may have direct effects on lung pathology. The Th1 cytokine IFN-γ sensitizes human alveolar type II cells to FasL mediated apoptosis (Wen, et al., 1997). FasL is expressed on the surface of neutrophils and activated lymphocytes (two cell types that migrate to lung during *M. bovis* infection) and has been implicated in the immuno-pathology of mycobacterial and listerial infections (Jensen, et al., 1998; Mustafa, et al., 2001). It has been hypothesized that the effect of IFN-γ on lung cells is a mechanism of natural cell turnover in an organ that is continuously exposed to foreign particles (Ahdieh, et al., 2001). The mis-regulation of an apoptosis facilitating cytokine, such as IFN-γ could potentially have serious consequences. In contrast, cytokines of a Th2 response weaken lung cell junctions and slow healing of lung tissue (Ahdieh, et al., 2001). Again, the mis-regulation of Th2 cytokines may lead to significant lung pathology (Henderson, et al., 2000; Temann, et al., 2002). A mixed or less robust immune response to *M. bovis* may therefore have benefits over a polarized response with regards to infection and immune lung damage.

We here provide the first report that: 1) All lymphocyte subsets are activated in response to a *Mycoplasma bovis* lung infection, 2) Bovine γδ-T cells exhibit “innate immune reactivity” to *Mycoplasma bovis*, and 3) The response to *M. bovis* infection is characterized by a Th2-skewed immune response.

**Acknowledgements**

This work was supported in part by funding from the Iowa Livestock Health Advisory Council. We thank Dr. R. Waters for technical assistance and the following persons for assistance with animal experiments: Tony Peterson, Brad Jordison, Jessica Katzman, and Leticia Calvente.
References


APPENDIX B: ACTIVATION OF WC1+ γδT CELLS BY MYCOPLASMA BOVIS IS CONSISTENT WITH INNATE IMMUNITY

A poster (#152) presented at the 14th International Congress of the International Organization of Mycoplasmology (IOM), 2002, July 7-12, Vienna, Austria
Tony J. Vanden Bush*, and Ricardo F. Rosenbusch

Abstract

While it has been reported that WC1+ γδT cell activation is consistent with an adaptive immune response, the role of these cells once activated is still an area of debate. Due to the high numbers and unknown importance of γδT cells in the bovine immune system, the activation of this and other T cell subsets was monitored for responsiveness to Mycoplasma bovis in infected and non-infected cattle. Mycoplasma bovis-driven activation of bovine T cell subsets during recall assays was monitored using flow cytometry analysis of CD25 expression. Lymphocytes from both infected and non-infected animals were monitored over the course of 3 months. During the early stages of the infection (1-49 dpi), activation of all T cell subsets was evident. The activity of γδT cells, however, were not dependent upon memory, as the activity of cells from non-infected animals was similar to the activity from infected animals. Using flow cytometry it was determined that these active γδT cells were WC1+ in phenotype, a distinct subset of γδT cells. We conclude that M. bovis activates WC1+/ γδT cells in a manner consistent with innate and not adaptive immunity.

Introduction

Innate immune cells are recruited to an area of infection, activated, and phagocytize antigen. These cells then activate members of the adaptive arm of the immune system, T cells. The primary difference between adaptive and innate immunity is specificity. Innate immune cells recognize general bacterial biomolecules, while adaptive immune cells recognize specific antigens.

In mammals, the T cell group consists of three primary subsets, CD4+, CD8+, and the γδT cells. The distinction between subsets is based on surface markers and T cell receptor phenotype. While the role of CD4+ and CD8+ cells in infection and immunity is well...
defined, little is known about the roles γδT cells play in immunity.

Previous studies dealing with the roles of bovine γδT cells indicate that these cells may play roles in both innate and adaptive immunity. These cells can act in a memory cell capacity, while also able to present antigen to CD4 cells; therefore linking them to innate immunity (1,2). Due to their large numbers and prevalence in the lung, we studied γδT cell activation with regards to *M. bovis* infection (3). Our findings indicate that while there may be gamma delta cells that act in an adaptive nature, gamma delta cells of the WC1+ population are activated during *in vitro* culture with *M. bovis* in a manner consistent with innate immunity.

**Results and Discussion**

The IL-2 receptor subunit alpha is expressed upon activation of T lymphocytes, and can therefore be used as an indication of T lymphocyte activation. Using flow cytometry, it was discovered that γδT cells isolated from naïve cattle (non-infected and non-vaccinated) were activated in response to *M. bovis* antigen *in vitro*. This activation does not appear to be driven by CD4 or CD8 activation, as these subsets of T cells were not activated within the naïve PBMC samples (Fig. 1 & 2).

Peripheral bovine γδT cells can be divided into 2 subsets based on the expression of either CD8 or WC1+. Non-CD8 γδT cells, or WC1+ cells, constitute from 68 to 90% of the peripheral γδT cells in cattle. Using three-color flow cytometry is was determined that the γδT cells that were activated expressed WC1 (Fig.3).

Despite the innate like activation of these T lymphocytes, the cells do not express Toll-Like Receptor 4, this however does not rule out the expression of other TLRs (Fig. 4). WC1+ cells may play an important role in *M. bovis* immunity as they constitute a large percent of the T cells in cattle and respond in an innate immune function. A recent report shows that WC1+ cells have little effect on Mycobacterial infections in cattle (4). This may or may not reflect the importance of this cell to Mycoplasmal infection, as other infection models have shown γδT cells to either be important for survival or not very important to immunity (5,6).
Figure 1. γδ T-cells from naïve cattle become activated upon incubation with *M. bovis*. PBMCs from naïve and infected animals were subjected to an *in vitro* recall assay using heat inactivated *M. bovis*.  

A) γδ T-cells from naïve animals (n=2) responded to the antigen while CD4 and CD8 T cells did not (Top).  

B) All of the T cell subsets from infected animals (n=4) became activated upon incubation with *M. bovis* antigen (Bottom).
A

B

C

Neg Calf-1 gd Stim.005

Percent GD T cells

Calf #15 Calf #18 Calf #14

Percent PBMCs CD25/gd Double Positive

Infected Non-infect.
Figure 3. Activated γδ T-cells are WC1+ in phenotype. Tri-color staining of PBMCs from naïve animals incubated with *M. bovis* shows that the CD25+/γδ TCR double positive cells (upper right quadrant) express WC1+.
Figure 4. WC1+ /γδ T cells does not express Toll-like Receptors 4. RNA was extracted from FACs sorted WC1+ T cells and neutrophils. The purified RNA was subjected to RT-PCR (reverse transcription) using primers specific for TLR-4. L-1, WC1+ RT-PCR w/o reverse transcriptase; L-2, WC1+ RNA; L-3, Neutrophil RT-PCR w/o reverse transcriptase; L-4, Neutrophil RNA.
References