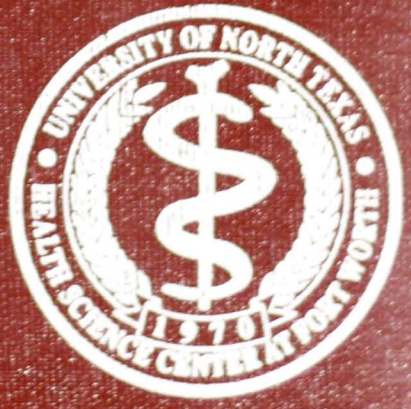
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The purpose of these studies was to investigate the Toll-like receptors (TLR) responsible for the recognition of invading mycoplasmas. They were also meant to evaluate the role of Toll-like receptors in the generation of immune responses and disease progression in mycoplasma respiratory disease. To determine the role of TLRs in recognizing viable *Mycoplasma pulmonis,* we utilized human embryonic kidney (HEK) cell lines that are known to have low basal expression of TLRs. The HEK cell lines used were stably transfected to express various combinations of TLRs including TLR1, 2 and 6. The current paradigm of TLR recognition of mycoplasma is that TLR2 dimerizes with either TLR1 or TLR6 to recognize different subclasses of mycoplasma lipoproteins. However, the recognition of viable *M. pulmonis* organisms remains unclear. When stimulated with viable *M. pulmonis,* it was discovered that TLR2 was pivotal in mediating the host's pro-inflammatory cytokine production and that the co-expression of TLR1 or TLR6 enhanced the response.

To study their role in mycoplasma recognition and disease progression, we utilized TLR2 knockout (KO) mice. Bone-marrow derived dendritic cells (BMDC) from TLR2 KO mice showed an impaired ability to produce pro-inflammatory cytokines such as IL-12p40 in response to viable *M. pulmonis.* In addition, the host's ability to clear the infection was also impaired in TLR2 KO

animals. There were higher numbers of cfu in the lower respiratory tract where alveolar macrophages are known to mediate the host's intrapulmonary clearance of organism. In the upper respiratory tract, where alveolar macrophages (AM) are absent, the production of anti-microbial peptides (e.g. β-defensin) in response to TLR2 agonists has been demonstrated. Thus, TLR2 does mediate the host's immune response to mycoplasma infection, by interfering with the host's ability to clear the infection and by interfering with the host's ability to mount an effective inflammatory response. These results also suggest that the TLR2 mediated anti-mycoplasma effects vary and are compartmentalized along the respiratory tract. These studies demonstrated diverse and novel roles of TLRs in respiratory infections and will serve as a platform for future studies investigating mycoplasma respiratory infections.

Toll-like Receptor 2 Mediates the Host's Respones in Murine Respiratory Mycoplasmosis

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THE ROLE OF TOLL-LIKE RECEPTOR 2 IN MEDIATING THE HOST'S DEFENSES TOWARDS MYCOPLASMA INFECTION IN THE UPPER AND LOWER RESPIRATORY TRACTS.

DISSERTATION

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Table

Abbreviation	Definition
NK	Natural Killer
AM	Alveolar Macrophage
HEK	Human Embryonic Kidney
BMDC	Bone-marrow Derived Dendritic Cell
DC	Dendritic Cell
BAL	Bronchoalveolar Lavage
TLR	Toll-like Receptor
MRM	Murine Respiratory Mycoplasmosis
PAMP	Pathogen Associated Molecular Patterns
ROS	Reactive Oxygen Species
SP-D	e.g. Surfactant Protein D
Sag	Superantigen
LPS	Lipopolysaccharide
MOI	Multiplicity of Infection
IRAK	IL-1R Associated Kinase
DMEM	Dulbecco's Modified Eagle's Medium
PRR	Pattern Recognition Receptor
IL-8	Interleukin 8

CHAPTER I

INTRODUCTION TO THE STUDY

Mycoplasma infection is a leading cause of pneumonia worldwide. In the United States, *Mycoplasma pneumoniae* accounts for as many as 30% of all cases of pneumonia [1-3]. Mycoplasmas cause an atypical pneumonia, and in humans, *M. pneumoniae* infection can exacerbate pre-existing respiratory diseases such as asthma [4-7]. Mycoplasma infection is also a noted problem in livestock where it has a major economic impact worldwide [8-12]. *M. pulmonis* is a naturally occurring pathogen of rats and mice and is the etiological agent responsible for murine respiratory mycoplasmosis (MRM) [13]. MRM is an atypical pneumonia with both acute and chronic stages to the disease. MRM caused by *M. pulmonis* is an excellent animal model used to study human respiratory mycoplasmosis [14]. As with other mycoplasma diseases, both have an immunopathologic element to disease progression and share similar pathologies and symptoms, such as rhinitis, otitis media, laryngotracheitis and bronchopneumonia [13]. In terms of histology, MRM is characterized by an influx of inflammatory cells along the respiratory airways. This influx of pro-inflammatory cells damages the host's tissues through the propagation of an exaggerated inflammatory response [13]. This infiltration suggests that recruitment and activation of these inflammatory cells are key events in both the

acute and chronic disease. In fact, elements of both the innate [15-17] and adaptive [18-22], arms of the immune system play a role in the progression and intrapulmonary clearance of the disease. Thus, it is clear that the mechanisms governing the recruitment of inflammatory cells and control of mycoplasma infection will ultimately determine the severity of mycoplasma respiratory disease. Our hypothesis is that *M. pulmonis* is recognized by Toll-like receptors on epithelial and/or antigen-presenting cells, which leads to cytokine production that determines the subsequent immune response

The initial molecular interactions between the invading mycoplasma and the hosts which occur during an infection are unknown. Currently, it is believed that attachment to the respiratory epithelium is the first step in colonization of the host, and attachment to the alveolar macrophages (AM) is critical in determining the levels of infection [1]. However, the molecular intermediates which mediate this recognition remain obscure. Recent reports indicate that mycoplasma lipoproteins are agonistic for Toll-like receptors (TLR) [23-25]. TLRs are a highly conserved family of type I transmembrane receptors that recognize specific pathogen-associated molecular patterns (PAMPs). It is clear that TLRs play a role in the recognition of mycoplasmas. The association of mycoplasma lipoproteins to TLRs is well documented [23-25]. Specifically, TLR1, TLR2 and TLR6 are implicated in the recognition of mycoplasma lipoproteins stemming from several mycoplasma strains [24, 25]. The dimerization of TLR1 and TLR6 with TLR2 enhances the recognition of the lipoproteins and augments the cellular cytokine response. Reports in the literature show that the dimerization of TLR1 or

TLR6 with TLR2 causes discrimination in the recognition of distinct classes of lipoproteins [26]. The stimulation of macrophages with TLR agonists can initiate the production of pro-inflammatory [27-29], chemotactic [30, 31], and antimicrobial immune responses [27, 32]. This suggests that the host's responses to mycoplasma infection might be a TLR coordinated immune response.

The intrapulmonary clearance of *M. pulmonis* is dependent on innate immune mechanisms. There is a growing body of evidence which links AMs to the intrapulmonary clearance of the organism. AMs are able to bind, ingest and kill *M. pulmonis* [15, 33, 34]. To demonstrate the role of AM in the intrapulmonary clearance of the organism, the depletion of AM was shown to decrease resistance to infection in a mouse strain resistant to lung infection (e.g. C57BL/6) to levels consistent with a susceptible mouse strain (e.g. C3H) [15]. The receptors which mediate the recognition of AM with viable *M. pulmonis* were unknown. We hypothesize that AM recognize viable *M. pulmonis* through TLR2 dependent mechanisms, and that this recognition augments the host's cytokine response and their ability to resist infection. In this study, we are the first to investigate the TLR recognition of viable mycoplasmas and determine the effects this recognition has on disease pathogenesis. In our current study, we show the expression of TLR1, TLR2 and TLR6 in Bronchoalveolar lavage (BAL) cells of C57BL/6 animals. In addition, we demonstrated that TLR1, TLR2 and TLR6 play a role in the recognition of viable *M. pulmonis*. Furthermore, we show the effects that TLR2 has on the resistance to the organism. Together, our findings support

the hypothesis that AM interact with invading *M. pulmonis* through TLR2-dependent mechanisms and opens the possibility of other AM-independent mechanisms of resistance.

MYCOPLASMA

Genus *Mycoplasma* represents the largest genus of the class Mollicutes with 107 species and subspecies being described to date. Mycoplasmas are regarded as highly evolved microorganisms that find residency in a number of different hosts which range from plants, animals and insects. There are currently 16 mycoplasma species known to infect humans, with most of them being commensal extracellular microorganisms that colonize the mucosal surfaces of the respiratory and urogenital tract [1]. However, a growing number of mycoplasma species have been identified as being pathogenic in recent years, which has prompted increased efforts into understanding the molecular biology and pathogenesis of these highly evolved organisms. *M. pneumoniae* is a naturally-occurring pathogen of humans where it is an etiological agent of atypical pneumoniae. *M. pneumoniae* infections of the upper and lower respiratory tract have the tendency to cause an acute localized disease which is followed with a chronic infection [1]. The clinical signs of *M. pneumoniae* range from mild respiratory symptoms, tracheobronchitis, interstitial pneumonia, with the rare case of extrapulmonary complications such as arthritis which is seen in most animal models [13]. *M. pneumoniae* outbreaks occur every 3-5 years and are often prevalent during community interactions found within households, military barracks and schools [1]. These series of investigations utilize *M. pulmonis,* which is indigenous to mice and rats and is the etiological agent responsible for MRM, where it causes a respiratory pathology similar to that found with *M. pneumoniae* in humans.

The Immune System

The Immune system is a collection of organs and cells which act in concert, offering multi-layered resistance to infection with increasing specificity. The immune system is capable of responding to a multitude of agents stemming from several backgrounds including bacterial, fungal, and viral [35]. In order to describe the immune system's complexity, its responses are designated by two major branches; the adaptive immune system and the innate immune system. The innate immune system responds quickly to infection, in a non-specific manner, and is found in all organisms. Its first line of defense can be found in physical barriers, e.g. the skin and respiratory epithelium. These anatomical barriers prevent infectious agents from having access to the internal milieu of the organism [35].

The second layer of defense, which interacts with the invading organisms should they breach one of the anatomical barriers, are the phagocytic/cellular responses (e.g. macrophages, neutrophils and NK cells) of the innate system. Innate cell recognition of foreign agents mediates their phagocytosis, generates an inflammatory response, and initiates an adaptive immune response [35]. The inflammatory response is one of the first signs of an infection and results in the influx of effector immune cells needed to fight the infection.

The final layer of protection offered by the immune system is the adaptive immune response. Adaptive immunity is found in vertebrates, is specific, and confers immunological memory towards the host. Its primary role is to increase the specificity of the host's response to the infectious agents while maintaining

self/non-self discrimination [35]. This increased specificity is retained as immunological memory which allows the host to mount a swifter and more pronounced response once the antigen is encountered again [35].

Mycoplasma respiratory infections are usually characterized by an acute inflammatory response followed by a chronic and often persistent infection. The infections produce mycoplasma specific and non-specific reactions and the host's immune response have been shown to play a pivotal role in exacerbating disease progression. The purpose of these studies was to investigate the role that Toll-like receptors have in contributing to the host's immune responses in a mycoplasma respiratory infection.

Inflammatory response

The purpose of the inflammatory response is the removal and/or localization of any pathogens, as well as to promote the healing of damaged tissues. The inflammatory response is one of the first signs of an infection and is characterized by redness, pain, swelling, and heat [35]. The redness and swelling is caused by increased blood flow to the tissues. This increase in blood flow is a direct result of the cytokines, released by the innate cells in response to the infection, that effect tissue vasculature [35]. These cytokines result in the dilation of blood vessels and the influx of effector cell populations needed to fight the infection. The heat and swelling associated with an inflammatory response are simply bi-products of the physiological changes.

Cytokines

Cytokines are signaling proteins or glycoproteins that are used extensively for cellular communications within the immune system. Cytokines interact with receptors located within the plasma membrane of the target cells. They are produced by a variety of cell types, and their methods of actions are grouped as paracrine, autocrine or endocrine. Autocrine pathways are those in which the cytokine reacts with the cell that secretes it. Paracrine mechanisms occur when cytokines interact with cells other than the one that secretes it, and endocrine mechanisms occur when the cytokine diffuses to different parts of the body to interact with tissues at distant locations. Engagement of cytokines with their target receptors leads to internal signaling cascades within the target cells and leads to an alteration of cell function.

Adaptive Immunity

The adaptive arm of the immune system is the most evolved branch of the immune system. Specificity, immunological memory, self/non-self discrimination and diversity are the main characteristics of the adaptive immune system. The effector cells of the adaptive immune system are the B and T lymphocytes. Both B and T lymphocytes have surface receptors that allow for antigen specificity. B lymphocytes express immunoglobulin (Ig) molecules on their surface and once they encounter their specific antigen they become plasma cells whose main function is the production of antigen-specific antibodies. Once exposed to antigen, certain B lymphocytes are capable of becoming memory B lymphocytes which confers immunological memory to the host. The T lymphocytes receptor (TCR) is similar to the immunoglobulin molecules of B cells, but can only

recognize antigen which is complexed to the major histocompatibility complex (MHC) molecules found on antigen presenting cells. These receptors are the products of extensive gene rearrangement which allows for the recognition of an extremely diverse population of antigens, and enhanced antigen specificity. In addition, the antigen presentation process allows B and T lymphocytes to distinguish between self and non-self antigens. Together, these characteristics allow for the adaptive immune system to adapt to a wide array of infectious agents, hence the name 'adaptive immunity'.

The adaptive immune system is believed to participate in preventing the dissemination of the disease from the pulmonary cavity and contribute to the development of the inflammatory lesions through the propagation of an exaggerated inflammatory response [22]. Many of the studies investigating the adaptive response to mycoplasma infection involve the use of immunodeficient animals challenged with *M. pulmonis* or immunocompromised patients suffering from *M. pneumoniae* [22]. Through the use of these *in vivo* models much has been learned concerning the activities of lymphocytes in mycoplasma lung disease.

Components of Innate Immunity

Innate immune mechanisms act immediately, and are followed by early induced responses, which help keep the infection under control while providing time for the adaptive immune response to develop [35]. The innate immune system consists of cell mediated responses, humoral factors, and physical

barriers. These three layers of defense are aimed at preventing infection and colonization of the host's tissues.

Cells of Innate Immunity

Natural Killer cells

Natural killer cells (NK) are large granular lymphocytes of the innate immune system. Originally, NK cells were discovered due to their ability to directly lyse tumor cells without prior stimulation [36]. However, since that time, their participation in other immunomodulatory processes was determined [37]. Unlike the B and T lymphocytes, they do not rearrange their genes to gain specificity to antigens or express TCR or immunoglobulins on their surface. Instead, NK cells express a series of germline-encoded receptors which mediate their activity and allow them to discriminate between self and non-self [37]. NK cells contribute to innate immunity in the defense from viruses and other intracellular pathogens, as well as contributing to antibody-dependent cellular cytotoxicity (ADCC). NK cell activation results in cytokine production and the secretion of lytic granules which can lyse microbes and induce apoptosis in the target cells.

Originally, in mycoplasma respiratory infections, NK cells were believed to assist in the innate immune response by secreting IFN-γ. This NK cell-derived IFN-γ would then activate other immune cell populations, enhancing their phagocytosis of mycoplasma, ultimately protecting the host from infection [38]. However, IFN-γ KO mice have a 2 log higher number of mycoplasmas in lungs, but the depletion of NK cells from IFN-γ KO animals resulted in the number of

organisms consistent with wild-type animals. In fact, there are increases in intracellular, IFN-γ-expressing NK cells within the lungs at 72 hours post-infection [38]. BAL cells also show increased IFN-γ mRNA expression at 72 hours post infection [38]. These data suggest that NK cells are the early source of IFN-γ after mycoplasma infection and that IFN-γ could play a role in the early response to mycoplasma infection.

Neutrophils

Neutrophils are the major class of white blood cells in human peripheral blood. They are characterized as having multi-lobed nuclei with cytoplasmic granules. Neutrophils are phagocytes and play an important role in engulfing extracellular parasites [35]. Extracellular pathogens or particles engulfed by neutrophils are lysed within the phagosome where they are exposed to reactive oxygen species (ROS), in a process often referred to as the 'respiratory burst'. Usually found in the bloodstream, during times of infection they relocate to the site of infection via chemotaxis.

There is no current evidence that supports the phagocytosis of mycoplasma by neutrophils in the absence of antibody [13, 39]. However, there is evidence *in vitro* that indicates mycoplasmas are phagocytized by neutrophils once they become opsonized with specific antibody [39]. To further corroborate the neutrophilic contribution to the innate response, recent reports indicate that neutrophil derived histamine contributes to pulmonary inflammation [40]. Histamine is a vasoactive amine most-often associated with allergic and infectious inflammation. Histamine contributes to the inflammatory response by

dilating blood vessels and promoting smooth muscle contractions [35]. It is routinely released from basophils or mast cells upon pathogen interaction with IgE molecules attached to their surface. However, recent findings indicate that histamine levels increased in the BAL fluid and serum of mast-cell KO animals at 7 and 14 days post infection [40]. This increase was also independent of increased basophil levels. In addition, it was demonstrated that mycoplasmas promote the release of histamine from neutrophils *in vivo* and *in vitro [40].* Together, these findings suggest that neutrophils contribute to the host's immune response after a mycoplasma infection.

Alveolar Macrophages

The literature supports the notion that alveolar macrophages (AM) play a large role in the intrapulmonary clearance of organisms from the lung during a mycoplasma infection [15, 33, 34] The primary phagocytic population, residing in the airways, are the AMs which have been determined by bronchoalveolar lavage samples to constitute ~95% [13]. *In vitro* studies have shown that AMs are incapable of ingesting mycoplasmas in the absence of specific antibodies [41, 42]. However, these studies contradict those conducted *in vivo* that seem to indicate AM as the primary agents responsible for the intrapulmonary clearance of the organisms [15, 17, 33, 34, 40]. These findings stem from studies investigating the functionality of AM in the lungs, that discovered impairment of AM functionality by NO_2 exposure abrogated the intrapulmonary clearance of the organisms [15, 43-45]. Furthermore, the recovery of the *in vivo* intrapulmonary clearance paralleled the recovery from damage of alveolar macrophages

following NO_2 exposure [13]. Further studies indicated the exacerbation of respiratory mycoplasmosis disease upon the depletion of alveolar macrophages. These findings corroborate the hypothesis that innate host defenses play a large role in the protection and clearance of the organisms from the lungs. In addition, they highlight the possible mechanisms by which the agents of the innate immune systems are interacting with the mycoplasma.

Opsonins

Opsonins are any molecules that bind pathogen and aid in the process of phagocytosis [35]. The process of coating pathogens is referred to as opsonization. Antibody, complement and surfactants all serve as opsonins within the pulmonary cavity.

Surfactant

Surfactants are a group of lipoproteins secreted by type II alveolar cells found in the alveoli, which aid in regulating the surface tension of the alveolae during gas exchange. There are four surfactant proteins, (SP-A, SP-B, SP-C and SP-D). Two of the proteins, SP-D and SP-A, have been implicated in augmenting the innate immune responses of the host during mycoplasma respiratory infections [46, 47]. SP-A acts as opsonin by coating invading microorganisms and enhancing their phagocytosis by AM [17, 46]. Recent reports in the literature indicate that some mycoplasma species have ligands on their surface which bind to the surfactant proteins with high affinity as a means to initiate colonization of the lungs [47, 48]. Furthermore, the binding of surfactants to TLR has been shown to enhance the inflammatory response in a microbial specific manner [46].

Human SP-D has recently been shown to aid in antigen recognition by binding to *M. pneumoniae* through high affinity interactions with surface lipids [49]. SP-A has been indicated as mediating the mycoplasmacidal activities of activated AM *in vitro* in a dose and time dependent manner [17]. These findings corroborate the hypothesis that non-specific host responses contribute to the intrapulmonary clearance of organisms.

Complement

Complement is a heat-labile component of plasma that augments the opsonization of bacteria by antibodies and allows some antibodies to kill bacteria [35]. This activity was said to 'complement' the antibacterial activity of antibody, hence the name. It is composed of a large number of plasma proteins and can perform its effector functions through either a classical or alternative pathways [35]. The classical pathway of complement is dependent upon specific antibody bound to the invading pathogen, which then trigger a cascade of effector functions which results in the killing of pathogens, the opsonization of pathogens, or the recruitment of other inflammatory cells. The alternative pathway of complement acts as part of the innate immune response. It can be triggered in the absence of antibody-bound antigens, with its cascade resulting in the same antimicrobial effects as the classical pathway [35]. The alternative pathway is regarded as the humoral component of innate immunity.

Complement is another nonspecific host defense known to contribute to the intrapulmonary clearance of the organism. Complement killing of mycoplasmas appears to be mediated by both the classical and alternative

mechanisms [50]. *M. pneumoniae* and *M. pulmonis* have both been identified as being susceptible to complement killing. One study, conducted *in vitro*, has shown the ability of *M. pneumoniae* to activate both pathways and implicated macrophages as major contributors to the classical pathway [16]. The role of the classical pathway was further highlighted in a murine *in vivo* model of experimental *M. pulmonis* infection. These studies showed the ability of *M. pulmonis* specific immunoglobulins to protect the host via complement mediated mechanisms [42]. The current body of evidence supports the *in vivo* and *in vitro* anti-microbial effects of complement against *M. pulmonis* [51-53].

Respiratory Epithelium

The lungs are constantly inundated with contaminants from the environment. The dogma pertaining to the respiratory epithelium is that it serves as a physical barrier protecting the internal milieu from the external environment. However, an increasing body of evidence is portraying the respiratory epithelium as a metabolically active modulator of respiratory function [54]. There are currently eight recognized morphologically different cell types that comprise the respiratory epithelium and these can be divided into three basic categories; Basal, secretory, and ciliated [54]. The dominant ciliated cell type, found throughout the lungs and comprising greater than 50% of the total population, are columnar ciliated epithelial cells. The dominating secretory cell type is the mucin-producing mucous (goblet) cells. During chronic inflammatory diseases, mucous cell hyperplasia is common and is believed to contribute to the productive cough that accompanies many of these conditions. The basal cells see a decrease in

their numbers as airway size decreases. Their main function is to serve as an attachment site for the more superficial cell types. They are the only cells of the respiratory epithelium that are firmly attached to the basement membrane and there is a direct correlation between epithelium thickness and basal cell number [54].

Perhaps the most recognized function of the respiratory epithelium is its mechanical defense of the lungs through the mucociliary clearance mechanism. The acidic mucin granules produced by the goblet cells are composed mainly of glycoproteins which serve as an effective "sticky" medium meant to trap foreign particles. The mucociliary movement on the luminal surface of epithelial cells provides a directional transport of these trapped particles from the lung to the throat [54]. During a mycoplasma respiratory infection, invading mycoplasmas are capable of bypassing this mechanism upon their attachment to the ciliated epithelium where they cause ciliostasis, inevitably ceasing ciliary action. In addition, recent reports indicate the respiratory epithelium's ability to contribute to the defense of the lungs through the production of cytokines [55-59]. Furthermore, the TLR-mediated production of pro-inflammatory cytokines in response to mycoplasma antigens by the respiratory epithelium has also been cited [60-63]. The studies described here seek to add to the current body of knowledge by investigating the initial interactions which occur between invading mycoplasmas and the respiratory epithelium and to furthermore understand what impacts these interactions have on the development of the immune responses.

Host's Genetic Background

As the concepts involved with the host's response to mycoplasma infection become clearer, it is becoming increasingly evident that diseasprogression is dependent on the genetic background of the host. The innate immune system appears to be responsible for controlling the proliferation of the organisms *in vivo*. In a study in which a number of mouse strains were screened for their susceptibility to *M. pulmonis* infection, it was discovered that resistance to mycoplasma lung disease is a complex genetic trait that depends on the genetic background of the host [64]. This study found that some strains of mice (C57BL, C57BR) were resistant to disease while the remaining strains tested (C3H and others) had varying susceptibilities. One study investigated the clearance of organisms in C57BL (resistant) and C3H (susceptible) mice and found that within 72 hr post-infection the numbers of microorganisms in the lungs of the C57BL mice decreased by 83% whereas those in the lungs of C3H increased by 18,000% [44]. Most likely, these genetic differences influence immune function which would explain the noted differences which occur between the different strains of mice. The finding of mouse strains with varied predispositions to developing mycoplasma lung disease, helped advance the understanding of the host's immune response to the microorganisms and the eventual discovery that the innate immune system played a large role in the intrapulmonary clearance of the organisms.

Toll-Like Receptors

At the forefront of understanding the complexity involved in host-parasite interactions is the recognition of the invading microbe by the host. Our current

understanding of the innate mechanisms governing this process centers on members of the toll-like receptor family. The toll-like receptors (TLRs) are a family of transmembrane glycoprotein receptors characterized by extracellular motifs containing multiple copies of leucine-rich repeats and a highly conserved cytoplasmic Toll/IL-1R (TIR) homology domain. There are currently 11 toll-like receptors that have been reported in mammalian species, and their primary function is the recognition of pathogen-associated molecular patterns (PAMPs). TLR1 dimerizes with TLR2 to recognize triacylated lipoproteins [26]. TLR2 recognizes a number of components of gram positive bacteria including lipopeptides, peptidoglycan, and lipoteichoic acid. TLR3 recognizes double-stranded RNA. TLR4 recognizes lipopolysaccharide and a number of host ligands including heat-shock proteins 60/70 (hsp60 and hsp70), and fibrinogen. TLR5 recognizes bacterial derived flagellin. TLR6 recognizes lipoteichoic acid and dimerizes with TLR2 in the recognition of diacylated bacterial lipopeptides. TLR7 and TLR8 recognize a variety of synthetic compounds and single stranded RNA from viruses. TLR9 recognizes CpG-containing DNA derived from viruses and bacteria and TLR10's ligand has yet to be identified. TLR11 also has a yet unidentified ligand, but due to its localization to the kidneys it has been found to have a role in the defense from uropathogenic organisms [65-67]. These distinct PAMPs are those evolutionary conserved molecular sequences specific to distinct classes of microbes including viral, fungal and bacterial.

TLR expression is cell-type-specific and found on most cells of the body. TLRs are found on a number of innate immune cells including

macrophages/monocytes [68-72], neutrophils [72-74] and natural killer cells [75]. They are found on the cells of the adaptive immune system such as T cells, Treg cells, B cells and Dendritic cells [76, 77]. In addition to these immune cells, epithelial cells lining the mucosal surfaces of the body have also been shown to express TLRs [56, 78-81]. Often, cell types which routinely encounter similar microbes will express similar regimens of TLRs. For example, the host's antiviral defense relies on those TLRs which recognize viral PAMPs, e.g. TLR 3, 7 and 9. These TLRs are found on bronchial epithelial cells, DC and NK cells which all have a role in the defense from viruses which attack the pulmonary cavity [82]. In summary, TLR activation is utilized to mediate a wide array of cellular functions including cell maturation [83, 84], inflammation [85-87], chemotaxis/migration [31, 57, 73, 74, 79], Immunosurveillance [85, 88], the production of anti-microbial peptides [89-91] and antigen presentation [28]. Their wide cellular expression and contributions to host defense make them undeniable links between the innate and adaptive arms of the immune system.

TLRs signal through a conserved IL-1R signaling pathway. All TLRs and IL-1R family members contain a cytoplasmic TIR domain, which is a region of ~200 conserved amino acids [67]. Upon binding with ligand, the TLR TIR domain undergoes conformational changes which lead to the recruitment of TIR domain-containing adaptor molecules like myeloid differentiation primary-response protein 88 (MyD88) [67]. After MyD88 interacts with the receptor complex it recruits members of the IL-1R associated kinase (IRAK) family, IRAK1 and IRAK4. After phosphorylation, the IRAK family members dissociate from the

receptor complex and recruit TRAF6 (tumor-necrosis-factor-receptor-associated factor 6). Activated TRAF6 then complexes with members of the mitogen-activated protein (MAP) kinase kinase kinase family (e.g. TAK1) which lead to the transcription factor NF-κβ through kinase cascades involving the IκB complex and MAP kinases (ERK, JNK, p38) [66, 67]. NF-κβ controls inflammatory responses through the induction of inflammatory cytokines [65]. There are two types of NF-κβ activation in TLR signaling: the MyD88 dependent pathway, which mediates early phase activation of NF-κβ, and the TRIF (TIR-domain-containing adaptor molecule1)-dependent pathway, which mediates the late phase activation of NF-κβ [67]. The MyD88 dependent signaling cascade results in the acute induction of inflammatory cytokines, chemokines, etc [65, 67]. The TRIF dependent signaling cascade results in the delayed induction of co-stimulatory molecules, IFN-β, and other IFN-inducible gene products [65, 67]. All TLRs with the exception of TLR3 are believed to signal through MyD88 dependent signaling cascades [66]. TLR3 and TLR4 are the only TLRs known to utilize the MyD88 independent signaling pathway [65].

TLR signaling serves as a bridge between the innate and adaptive immune responses. When the innate immune system fails to clear an infection the adaptive immune system responds. The adaptive immune response is initiated through the presentation of pathogen-derived peptides by antigen-presenting cells (e.g. DC) to T cells [35]. The stimulation of immature DC with TLR agonists leads to the production of cytokines which influence the differentiation of naïve T cells towards a Th1 or Th2 lineage [92]. It also

contributes to the DC maturation process by inducing the expression of co-stimulatory molecules utilized during the antigen-presenting process [92, 93]. Thus, TLR activation acts as a bridge between the adaptive and innate immune responses by linking microbial recognition with the antigen-presentation process.

Mycoplasma membrane lipoproteins, expressed on the surface, are agonistic for TLRs [23-25]. Recent reports indicate that recognition of these lipoproteins is mediated through TLR1, 2, and 6 [23, 24]. Recently, a dipalmitoylated lipoprotein from *M. pneumoniae* was found to activate NF-κβ through TLR1, TLR2 and TLR6 [24]. The current literature demonstrates that TLR2 dimerizes with TLR1 in the recognition of triacylated lipoproteins [26] and with TLR6 in the recognition of diacylated lipoproteins [94]. These heterotypic associations are preformed on the surface of the cell and serve to increase the ligand spectrum but fail to alter the cellular response [95, 96]. In addition to dimerizing with TLR1 or TLR6, it has been demonstrated that TLR2 is capable of interacting with TLR4 in the recognition of a mycoplasma superantigen (Sag) MAM isolated from *M. arthritidis* [97]. Also, it's been demonstrated that the activation of TLRs with mycoplasma membrane lipoproteins has the potential to affect pre-existing conditions. Membrane lipoproteins purified from *M. penetrans* and *M. fermentans* have been demonstrated to activate the long-terminal repeats (LTR) of the human immunodeficiency virus (HIV) [25]. LTRs are found in retroviral DNA or RNA flanking functional genes. During the viral infection process they mediate the integration of the viral DNA into the host's chromosome which is the basic mechanism utilized by HIV [25]. The activation of TLRs by

mycoplasma membrane proteins contributes to the immune response through the induction of inflammatory cytokines or anti-microbial peptides [85, 89, 91].

Rationale

Recent advances in TLR research have identified the TLR members involved in the recognition of purified mycoplasma membrane lipoproteins. Currently, there are gaps in the literature pertaining to the recognition of viable mycoplasma by TLRs. There are also gaps concerning a comparative analysis between mycoplasma species in regards to their membrane lipopeptide expression profiles. These studies sought to determine the TLRs involved in the recognition of viable mycoplasmas and what contributions this recognition makes to the developing immune response.

CHAPTER II

MYCOPLASMA PULMONIS CAN DIRECTLY STIMULATE A RESPIRATORY EPITHELIAL CELL LINE TO PRODUCE A CHEMOKINE, AND TLR2 LIKELY MEDIATES CELL STIMULATION

Mycoplasmas cause chronic respiratory diseases in animals and humans. It is clear that the mechanisms governing the recruitment of inflammatory cells ultimately determine severity of mycoplasma respiratory disease. Chemokine production is implicated in cell recruitment from studies using a murine model of mycoplasma pneumonia due to *Mycoplasma pulmonis*. The interaction of mycoplasma with respiratory epithelium is one of the early events after infection, but it is currently unknown whether respiratory epithelial cells are capable of responding to infection with the murine pathogen, and the molecular basis of this recognition. In this study, we demonstrated that *M. pulmonis* infection stimulates a pulmonary epithelial cell line to produce a chemokine. Furthermore, TLR2 was shown using stably transfected cells to be important in optimal responses to mycoplasma infection. Thus, the TLR2-mediated recognition of mycoplasma by respiratory epithelium likely plays a role in the recruitment of inflammatory cells involved in disease pathogenesis.

Introduction

Mycoplasma infection is a leading cause of pneumonia worldwide. In the United States, *Mycoplasma pneumoniae* accounts for as many as 30% of all

cases of pneumonia [1-3]. Mycoplasma causes an atypical pneumonia, and *M. pneumoniae* infection can exacerbate pre-existing respiratory diseases such as asthma. *Mycoplasma pulmonis* is a naturally occurring pathogen of rats and mice and is the etiological agent responsible for murine respiratory mycoplasmosis (MRM) [98]. MRM caused by *M. pulmonis* is an excellent animal model used to study human respiratory mycoplasmosis. Both diseases have an immunopathologic element to disease progression and share similar pathologies and symptoms, such as rhinitis, otitis media, laryngotracheitis and bronchopneumonia. As with *M. pneumonia* disease, MRM is characterized by an exaggerated inflammatory response, with an influx of inflammatory cells that in turn, damage the host's tissues. Thus, it is clear that the mechanisms governing the recruitment of inflammatory cells will ultimately determine the severity of mycoplasma respiratory disease.

Immune responses against pathogenic mycoplasma species contribute to lesion formation as well as resistance to infection [20, 22]. However, the initial mycoplasma-host interactions responsible for the cascade of events leading to lesion development or resistance to infection are unknown. One likely possibility is that the interaction of mycoplasma with respiratory epithelium and/or macrophages triggers secretion of chemotactic cytokines, resulting in the recruitment and activation of inflammatory cells. In fact, experimental infection of mice with *M. pulmonis* does result in the rapid production of inflammatory cytokine responses, such as TNF-α, IL-6 and IL-1, in the lungs of susceptible and resistant strains of mice [99], supporting the idea that early cytokine responses

can contribute to both resistance to infection as well as lesion pathogenesis. Thus, the early interactions between mycoplasma and host cells are likely critical in determining the outcome of the infection; these interactions influence the balance between whether the host response controls infection or promotes the development of inflammatory lesions.

The Toll-like receptor (TLR) family plays a role in the recognition of microbial ligands and potentially plays a role in the interactions that mediate the initial responses to mycoplasma infection. The activation of TLRs by microbial ligands is a key step in the initiation of immune and other host responses to many other infectious agents [85]. Importantly, lipoproteins derived from various mycoplasma species can interact with cells via TLR2 [23, 24]. Studies however have focused on the TLR response to purified mycoplasma lipoproteins, but the roles of TLR in responses to whole organisms are largely unknown. For example, *M. pneumoniae* produces a dipalmitoylated lipoprotein that stimulates cells through TLR1, TLR2, and TLR6 [24], but although whole (viable) *M. pneumoniae* stimulates cells [16, 60, 100], it is not shown that these responses are limited to TLR2 mediated mechanisms. *M. pulmonis* can stimulate the activation of macrophages [17, 101], but it is not clear whether *M. pulmonis* can stimulate respiratory epithelial cells to produce cytokines, similar to that described for *M. pneumoniae [61, 63, 102, 103].* Furthermore, it is unknown whether whole (viable) *M. pulmonis* can stimulate cells through TLR. In the present study we demonstrate that viable *M. pulmonis* can directly stimulate a respiratory epithelial

cell line to produce a chemokine, and TLR2 likely mediates this cellular stimulation.

Materials and methods

Mycoplasma

The UAB CT strain of *M. pulmonis* was used in all experiments. Stock cultures were grown, as previously described [104], 1-ml aliquots frozen at -80°C were thawed and grown in 9-ml Hayflick's medium for 3 hours at 200 rpm. The resulting growth was centrifuged at 10,000 rpm for 20 minutes, washed in serum-free Dulbecco's Modified Eagle Medium (DMEM) high glucose (Hyclone, Logan, UT), and reconstituted in a total volume of 5 ml of serum-free DMEM.

Cell lines

The human type II alveolar epithelial cell line, A549, was obtained from American Type Culture Collection (ATCC, Manassas, VA). Human embryonic kidney 293 (HEK) cell lines were obtained from (Invivogen, San Diego, CA), and included HEK (control) and HEK cells stably transfected to express TLR2 or TLR4/hMD2/CD14. All cells were cultured in DMEM high glucose containing 10% FBS, and 2 mM L-glutamine, and the HEK cell lines were used prior to 10 passages.

Cell stimulation

A549 or HEK293 stable transfectant cells (2×10^5 cells/well) were placed into wells of flat-bottom 24-well plates (BD Biosciences, Baltimore, MA) and stimulated with *M. pulmonis* at a multiplicity of infection (MOI) of 0.7 (whole organisms/cell), *E. coli* Ultra pure LPS at 10 μg/ml (Invivogen), or the synthetic

diacylated lipoprotein FSL-1 at 1 μg/ml (Invivogen) derived from *Mycoplasma salivarium* in a total volume of 2 ml for 24 hours. Cell culture supernatants were collected and stored at -80°C until assayed for their IL-8 levels. Data is expressed as the fold change of IL-8 pg/ml production of each agonist normalized to unstimulated controls.

IL-8 assay

Human IL-8 levels were measured using OptEIA IL-8 ELISA set (BD Pharmingen, San Jose, CA). Briefly, Pro-Bind 96-well flat-bottom microtiter plates (BD Biosciences, Bedford, MA) were coated overnight at 4°C with 100 μl mAb specific for human IL-8 diluted in 0.1M Na_2HPO_4, pH 9.5. Plates were washed three times with PBS/0.05% TWEEN 20 and blocked with 200 μl PBS supplemented with 10% FBS overnight at 4°C. Following an additional three washes with PBS/0.05% TWEEN 20, 100 μl of sample supernatants were placed into the appropriate wells and incubated overnight at 4°C. Plates were then washed 5 times with PBS/0.05% TWEEN 20, and 100 μl of biotinylated rat anti-human IL-8 mAb and avidin-horseradish peroxidase (HRP) were added to each well and incubated for 15 min. To reveal the chromatic change, the substrate Tetramethylbenzidine (TMB) (Moss Inc., Pasadena, MD) was added for approximately 20 min, and the indicative color change determined using an MX80 plate reader (Dynatech, Chantilly, VA) at an absorbance of 630 nm. IL-8 levels (pg/ml) were determined to standard curves generated from recombinant human IL-8.

Statistical analyses

These analyses were done using GraphPad Prism 4.0 (GraphPad Software, El Camino Real, CA) computer program. A *p* value of ≤0.05 was considered significant. All experiments are n=6 and were performed at least twice.

Results

M. pulmonis can stimulate respiratory epithelial cells to produce IL-8

To examine whether *M. pulmonis* can stimulate pulmonary epithelial cells, A549 cells were exposed to viable *M. pulmonis* at a low MOI (0.7 cfu/cell) for 24 hours. As a control, cells were incubated in the presence of culture medium alone. IL-8 levels in culture supernatants were monitored to determine the level of stimulation. After exposure to viable *M. pulmonis*, there was a significant ($P \leq 0.05$) increase in the levels of IL-8 produced as compared with unstimulated control cells (Fig. 1). Thus, the data suggest that pulmonary epithelial cells are capable of responding to *M. pulmonis* with the production of chemokines.

The recognition of whole organism *M. pulmonis* by TLR2 mediates the production of IL-8

To investigate whether TLR2 mediates the recognition of viable *M. pulmonis*, HEK cells, stably transfected to express individual human TLRs and/or their adaptor proteins, were stimulated *in vitro*. The levels of IL-8 in culture supernatants were determined. Stimulation of the cell lines with viable *M. pulmonis* demonstrated a role for TLR2 in the recognition of *M. pulmonis*. *M. pulmonis* stimulated TLR2 expressing cells to produce IL-8, while TLR4 expressing cells were unresponsive (Fig. 2). To confirm specificity of TLR mediated responses, HEK cell lines were stimulated with either a TLR4 (LPS) or

TLR2 (FSL-1) agonist. LPS stimulation is optimal when TLR4, MD2 and CD14 are present [105], but ultrapure preparations of LPS should not mediate responses in cells expressing only TLR2. As expected, TLR4/MD2/CD14-expressing cells responded to LPS stimulation ($P \leq 0.05$), while TLR2-expressing cells did not. Similarly FSL-1 is a TLR2/6 agonist [106], and as expected, it elicited a larger response in the TLR2 expressing cells when compared to the control HEK293 or the HEK TLR4-expressing cell lines. These results confirmed the specificity of the responses. Thus, *M. pulmonis* stimulated these cells using TLR2, but not TLR4, to produce the chemokine IL-8.

Discussion

One of the most consistent characteristics of chronic mycoplasma respiratory diseases in humans and animals is the accumulation of large numbers of inflammatory cells along the airways of the respiratory tract infection [98]. Cell infiltration is critical in the development of lesions and/or control of infection. Importantly, respiratory mycoplasmas, including *M. pulmonis*, adhere to respiratory epithelium along the airways, and this intimate interaction is thought to be the initial step in the pathogenesis of disease [1, 98]. A number of studies demonstrate that bronchoepithelial cells can produce chemokines and cytokines in response to TLR agonists and other stimuli [55, 57, 107-110], and it is likely these cells and the chemotactic mediators they produce are important in the early and later stages of mycoplasma infection. Furthermore, TLR are thought to be involved in mycoplasma cells interactions, as purified mycoplasma lipoproteins are capable of cell stimulation through TLR2 [24, 62, 70, 111, 112].

However, it is not clear if TLR2 recognition of intact, viable pathogenic mycoplasma leads to cell stimulation and subsequent release of cytokines or chemokines. Despite cell recruitment being a key event in the pathogenesis of mycoplasma respiratory disease, the responsible molecular cascade of events following the initial interaction between the host and mycoplasma are unknown. The purpose of these studies was to determine if the murine respiratory pathogen, *M. pulmonis*, can stimulate a respiratory cell line to produce a chemokine and if TLR2 mediates cell stimulation after *in vitro* infection with intact, viable mycoplasma. Respiratory epithelial cells are likely to produce chemokines in response to infection with *M. pulmonis*. In the current study, *M. pulmonis* infection stimulated human lung alveolar type II epithelial cells (A549 cells) to increase their production of IL-8 at least 2-fold. This is consistent with a previous study with the human mycoplasma, *M. pneumoniae [63]*. Although alveolar type II epithelial cells likely differ from bronchoepithelium in their cytokine responses to mycoplasma, preliminary *in situ* hybridization studies from our laboratory indicate that TNF-α is produced by bronchial and tracheal epithelium in the early stages of murine *M. pulmonis* respiratory disease (Unpublished data). Furthermore, we demonstrated that either protein or mRNA levels of the murine homologues of human IL-8, e.g. KC, MIP-2, and LIX, are elevated in the lungs of mice infected with *M. pulmonis [38, 113]*. Thus, adherence of *M. pulmonis* to airway epithelial cells leads to production of chemokines and likely other cytokines, and this response has the potential subsequently to influence the

severity of inflammatory lesions, including recruitment of lymphocytes and other inflammatory cells.

After infection, *M. pulmonis* most likely stimulates chemokine production from cells through interactions with TLR2. As indicated above, previous studies demonstrate that lipoproteins from other mycoplasma can stimulate cells via TLR2 [24, 26, 57, 62, 70, 112], but there was a gap in the literature pertaining to the TLR recognition of viable organisms. Our results, however, demonstrated that when stimulated with viable *M. pulmonis*, the TLR2 expressing cell line had a greater than 80-fold increase in IL-8 production when compared to uninfected cells, whereas, there was little response from cells that did not express similar levels of TLR. The TLR4/MD2/CD14-expressing cell line also had a slightly higher production of IL-8 when compared to control. This could be due to its expression of CD14, which has been shown to play an integral part in the recognition of mycoplasma lipoproteins [114]. In fact, there is no difference in disease severity in *M. pulmonis*-infected mice that express (C3H/HeN) or lack TLR4 (C3H/HeJ) [64], supporting a minimal role for TLR4 in disease pathogenesis. The use of the HEK cell line is widespread due to the belief that this cell line expresses only basal levels of the TLRs. As *M. pulmonis* was able to stimulate HEK cells, it is possible that the basal level of TLR2 expression is enough for some activation; however, another possibility is that there are other TLR2 independent mechanisms involved in the recognition of viable *M. pulmonis.* However, our results do clearly demonstrate that TLR2 is important in optimal

responses to *M. pulmonis* infection, and thus, likely contributes to production of chemokines and cytokines involved in disease pathogenesis.

The present study reflects the responses of human TLRs to the murine pathogen *M. pulmonis*. There is a high sequence homology between all members of the TLR family, with TLR2 showing 65% identical residues in the extra cellular region and 84% identity in the intracellular residues [115, 116]. Even with such high homology, there is a species-specific discrimination detected in response to known TLR agonists [117]. However, comparative analysis of hTLR2 and mTLR2 stimulated with a variety of distinct microbes did not reveal distinct differences. Based on the data presented, studies from our lab demonstrate that *M. pulmonis* is also recognized by murine TLR2 (Love and Simecka, Manuscript in Preparation). This suggests that a group of TLR2 agonists, e.g. those with mycoplasma characteristics, do not have significant species-specific TLR responses. Thus, even though the present study utilized human TLRs stimulated with a murine pathogen, it has provided insights to potential mechanisms involved in the development of inflammatory lesions in mycoplasma infections.

In summary, the present study provides insight into the mechanisms involved in triggering host responses by viable mycoplasma. Adherence of *M. pulmonis* to airway epithelial cells likely leads to production of chemokines and likely other cytokines, and this response has the potential subsequently to influence the severity of inflammatory lesions, including recruitment of lymphocytes and other inflammatory cells. Our results do clearly demonstrate

that TLR2 is important in optimal responses to *M. pulmonis* infection. This is the first report that we are aware of describing the ability of viable mycoplasma to interact with TLR2 in stimulating a cytokine response, as previous studies focused on the ability of purified mycoplasma lipoproteins to stimulate cells through TLR2 [24, 26, 57, 62, 70, 112]. In addition, demonstration of *M. pulmonis* interacting with TLR2 is new and suggests that TLR2-mediated events contribute to the initial interactions between the host and organism leading to the recruitment of inflammatory cells responsible for the pathogenesis of disease. Because the recognition of *M. pulmonis* by TLR2 is capable of eliciting a chemokine response, future studies investigating its role in the recruitment of inflammatory cells and ultimately the intrapulmonary clearance of the organism will undoubtedly prove fruitful.

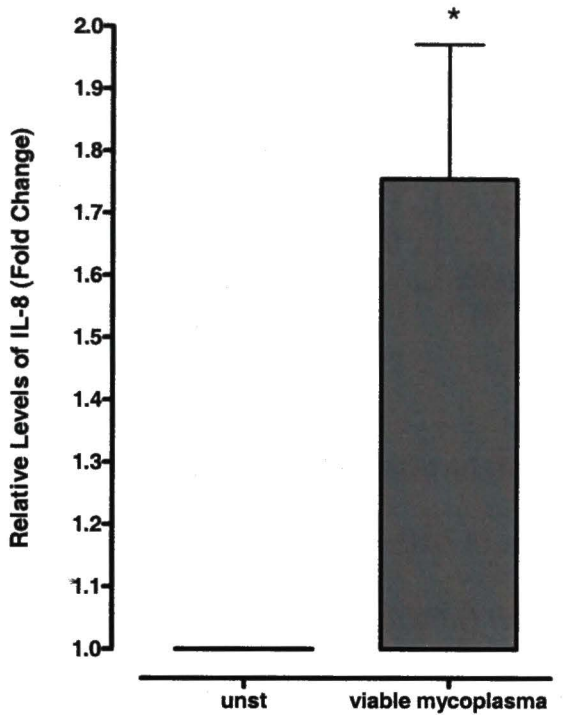

Fig. 1. *M. pulmonis* can stimulate respiratory epithelial cells to produce IL-8.

Data are expressed as the fold change of IL-8 pg/ml production normalized to unstimulated controls. A549 cells were incubated for 24 hours with an MOI (0.7 cfu/cell) after which the cell culture supernatants were assayed for their IL-8 content. An asterisk "*" denotes a p value ≤ 0.05.

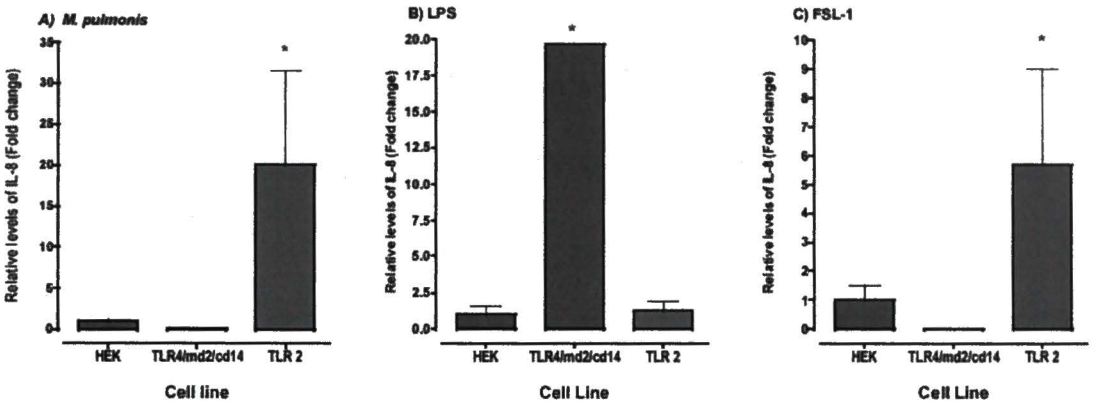

Fig. 2. Viable *M. pulmonis* stimulated IL-8 production using TLR2. HEK293 (HEK) or those stably transfected to express individual TLRs and their adaptor proteins (TLR2 or TLR4/md2/cd14) were stimulated for 24 hours with (A) viable *M. pulmonis* at 0.7 MOI, (B) an *E. coli* derived Ultrapure LPS preparation at 10 μg/ml, or (C) the TLR2/6 agonists FSL-1 at 1 μg/ml. The supernatants were then collected and assayed for their IL-8 content. Data are expressed as the fold change of IL-8 pg/ml production of each agonist normalized to unstimulated controls. An asterisk "*" denotes a p value ≤ 0.05.

CHAPTER III

TOLL-LIKE RECEPTOR 2 MEDIATES THE INTRAPULMONARY CLEARANCE OF ORGANISMS IN MURINE RESPIRATORY MYCOPLASMOSIS

Previous studies have indicated that mycoplasmas can be recognized by Toll-like receptors (TLR). However, studies have not shown that TLRs are capable of mediating the host's responses in disease progression. *Mycoplasma pulmonis* is a naturally occurring pathogen in mice which causes a respiratory disease. Currently, it is unknown which TLR recognizes this organism. TLRs are a family of pattern-recognition receptors (PRR) which contribute to the development of other inflammatory diseases such as asthma and atherosclerosis. The purpose of the current study was to determine the TLRs involved in the recognition of *M. pulmonis* and their activities during disease progression. To determine the physiological function of TLR2 *in vivo*, wild-type and TLR2 -/- (C57BL/6) mice were infected 72hours and colony forming units (cfu) were determined for the upper and lower respiratory tract. In addition, bone-marrow derived dendritic cells were generated from wild-type and TLR2 -/- animals and co-cultured *in vitro* with *M. pulmonis* whole organism. To determine stimulation, culture supernatants were collected and IL-12p40 levels were determined using a sandwich ELISA. Preliminary studies demonstrate that C57BL/6 TLR2 -/- animals have an increased cfu in the lower respiratory tract. Also, TLR2 expression mediates the recognition of the whole organism of *M. pulmonis* on BMDC stimulated *in vitro*. In

these studies, we determined that TLR2 mediates responses to *M. pulmonis* antigens *in vitro*. We also determined the TLR mRNA and protein expression changes in pulmonary DC and macrophages following infection.

Introduction:

Mycoplasma infection is a leading cause of pneumonia worldwide. In the United States, *Mycoplasma pneumoniae* accounts for as many as 30% of all cases of pneumonia [1-3]. Mycoplasmas cause an atypical pneumonia, and in humans, *M. pneumoniae* infection can exacerbate pre-existing respiratory diseases, such as asthma [4-7]. Mycoplasma infection is also a noted problem in livestock, where it has a major economic impact worldwide [8, 9, 11, 12]. *M. pulmonis* is a naturally occurring pathogen of rats and mice and is the etiological agent responsible for murine respiratory mycoplasmosis (MRM) [13]. MRM causes an atypical pneumonia with both acute and chronic stages to the disease. MRM caused by *M. pulmonis* is an excellent animal model used to gain insight into the pathologies caused by mycoplasma respiratory diseases [14]. As with other mycoplasma diseases, *M. pulmonis* disease has an immunopathologic element to disease progression. In terms of histology, MRM is characterized by an influx of inflammatory cells along the respiratory airways. This influx of pro-inflammatory cells damages the host's tissues through the propagation of an exaggerated inflammatory response. This infiltration suggests that recruitment and activation of these inflammatory cells are key events in both the acute and chronic disease. In fact, elements of both the innate [15-17] and adaptive [18-21]

arms of the immune system play a role in the progression and intrapulmonary clearance of the disease. Thus, it is clear that the mechanisms governing the recruitment of inflammatory cells and control of mycoplasma infection will ultimately determine the severity of mycoplasma respiratory disease.

The initial molecular interactions between the invading mycoplasma and the host during an infection are unknown. Currently, it is believed that attachment to the respiratory epithelium is the first step in colonization of the host and interactions with the alveolar macrophages (AM) are critical in determining the levels of infection [1]. However, the molecular intermediates which mediate this recognition remain obscure. TLRs are a highly conserved family of type I transmembrane receptors that recognize specific pathogen-associated molecular patterns (PAMPs), e.g. LPS, lipotechoic acid and other bacterial wall components. The recognition of purified mycoplasma lipoproteins to TLRs is also well documented [23-25]. Specifically, TLR1, TLR2 and TLR6 are implicated in the recognition of mycoplasma lipoproteins stemming from several mycoplasma strains [24, 25]. TLR2 dimerizes with either TLR1 or TLR6 to enhance the recognition of lipoproteins and augment the cellular cytokine response. The stimulation of macrophages with TLR agonists have been shown to initiate the production of pro-inflammatory [27-29], chemotactic [30, 31], and antimicrobial immune responses [27, 32]. Thus, the host's responses to mycoplasma infection might be initiated and/or influenced by TLR signaling. It is clear that TLRs play a role in the recognition of mycoplasmas lipoprotein, however, the role of TLRs in the early response to disease has yet to be described.

The intrapulmonary clearance of *M. pulmonis* is dependent on innate immune mechanisms. There is a growing body of evidence which links AMs to the intrapulmonary clearance of the organism. AM are able to bind, ingest and kill *M. pulmonis in vitro* and *in vivo* [15, 32, 33]. The intrapulmonary depletion of AMs decreases the resistance to infection of resistant mice strains (e.g. C57BL/6) to levels consistent with susceptible strains (e.g. C3H) [15]. Currently, the receptors which mediate the recognition of viable *M. pulmonis* by AM is unknown. We hypothesize that AM and other innate immune cells recognize viable *M. pulmonis* through TLR2 dependent mechanisms, and that this recognition augments the host's cytokine response and their ability to resist infection. In this study, we are the first to investigate the TLR recognition of viable mycoplasmas and determine the effects this recognition has on disease pathogenesis. We show the expression of TLR1, TLR2 and TLR6 in BAL cells of C57BL/6J animals. In addition, we show that TLR1, TLR2 and TLR6 are utilized in the recognition of viable *M. pulmonis in vitro*. Furthermore, TLR2 KO animals show an impaired resistance to infection in the upper and lower respiratory tract. Together, our findings show that TLR2 recognizes *M. pulmonis* and this interaction mediates resistance to mycoplasma infection throughout the respiratory tract.

Materials and Methods:

Mice

C57BL/6J and B6.129S1-*TLR2*^{tm1Dgen}/J (TLR2$^{-/-}$) mice, tested to be viral and mycoplasma free, were obtained from The Jackson Laboratory (Bar Harbor, MN). Mice were housed in sterile microisolator cages supplied with sterile bedding,

food and water given *ad libitum*. Mice used in the studies were between 9-15 weeks of age. Female mice were used in all studies. Before experimental infection, mice were anesthetized with an intramuscular injection of ketamine/xylazine.

Cell lines

Human embryonic kidney 293 (HEK) cell lines were obtained from Invivogen, (San Diego, CA), and included HEK (control) and HEK cells stably transfected to express murine TLR1, TLR2, TLR6, TLR2/6, TLR2/1 or TLR4/MD2/CD14. All cells were cultured in DMEM high glucose containing 10% FBS and 2 mM L-glutamine, and the HEK cell lines were used prior to 10 passages.

Mycoplasma

The UAB CT strain of *M. pulmonis* was used in all experiments. Stock cultures were grown as previously described [118]; for in vitro stimulation studies 1-ml aliquots frozen at -80°C were thawed and grown in 9-ml Hayflick's medium for 3 hours at 200 rpm. The resulting growth was centrifuged at 10,000 rpm for 20 minutes, washed in serum-free Dulbecco's Modified Eagle Medium (DMEM) high glucose (Hyclone, Logan, UT) and reconstituted in a total volume of 5 ml of serum-free DMEM. For infections, mice were anesthetized with an intraperitoneal injection of ketamine/xyalzine, then they received an intranasal inoculum of 20 μl containing 2 x 10^6 cfu of *M. pulmonis* strain CT [118].

BAL

The BAL was performed as previously described [111]. Briefly, two pathogen-free C57BL/6J mice were killed by lethal injection, and their tracheas were exposed

and ligated distal to the larynx. A sterile 22G ProtectIV Plus catheter (MEDEX, Carlsbad, CA) was inserted approximately 2 to 3mm into the lumen, and the lungs were then lavaged 3 times with 1 ml aliquots of phosphate-buffered saline solution (Hyclone). The lavages were pooled and cells pelleted using centrifugation at 200 x g for 10 min at 4°C. BAL cell pellets were resuspended in 500 μl of Trizol (Invitrogen Life Technologies, Carlsbad CA).

RNA extraction

RNA from the pelleted samples of BAL from C57BL/6J mice was isolated using Trizol RNA isolation reagent, as recommended by the manufacture (Invitrogen) [119]. Briefly, 500 μl of TRIZOL was added to the pellet and pipetted up and down repeatedly to ensure lysis of the sample. The homogenates were frozen at -80°C until further use. Chloroform was added to the thawed homogenates and centrifuged at 12,000 x *g* (4°C) for 30 min. The RNA was precipitated by adding isopropanol to the aqueous phase and centrifuging samples at 12,000 x *g* (4°C) for 10 min. The RNA pellet from each sample was washed twice with 75% ethanol by vortexing and subsequent centrifugation for 5 min at 7,500 x *g*, and then resuspended in diethylpyrocarbonate-treated water. The samples were stored at -80°C until further use.

Toll-Like receptor mRNA detection by real time-PCR

cDNA was synthesized from total RNA samples in a 100 μl reaction using the Taqman Gold RT-PCR kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Sybr-green real-time PCR was performed using RT^2 SYBR Green/ROX qPCR Master Mix and primers for TLR1, TLR2, TLR6 and the

housekeeping gene GAPDH (SuperArray Bioscience Corporation, Frederick, MD). The real-time PCR was performed in 25-μl SmartCycler tubes (Cepheid, Sunnyvale, CA), and the real-time PCR products were amplified using a SmartCycler system (Cepheid) at 95.0°C for 10 min, followed by 40 cycles of 95.0°C for 15 s and 60.0°C for 60 s. The threshold of the growth curve (C_T) was set at a value of 30 using the SmartCycler software. The expression of the housekeeping gene, GAPDH, was used to normalize the data. The formula for the normalization (ΔC_T) between the amplified TLR gene and the normalizer (GAPDH) is $\Delta C_T = C_T$ (GAPDH) – C_T (cytokine).

Generation of bone-marrow derived dendritic cells (BMDC)

Bone marrow derived dendritic cells were generated as previously described [111]. Femora and tibia were collected from mice, and the bones were suspended in wash medium containing RPMI 1640 (Hyclone), 10% FBS (Hyclone), antibiotic/antimycotic solution (Life Technologies, Grand Island, NY), and HEPES buffer (Fisher Scientific, Pittsburgh, PA). The bones were twice washed in medium and also soaked in 70% ethanol between washes. The epiphyses were cut, and the marrow flushed from the bones with wash medium. Epiphyses were finely minced and added with the flushed marrow. The suspension was passed through a 250-μm nylon mesh to remove any unwanted debris. The cell suspension was then spun down and subjected to an ACK lysis buffer. The cells were then spun down, re-suspended in wash medium and counted. Once counted, the cells were placed in cell culture flasks at a concentration of 1 x 10^7 cells ml^{-1}. In addition to wash medium, the cells were

incubated in the presence of IL-4 (20ng/ml, Invitrogen) and GM-CSF (20ng/ml, Invitrogen). Culture medium was changed every two days, and the cells were harvested on day 6.

In vitro cell stimulations

The HEK stable transfectants (Invivogen) were seeded at $2x10^5$ cells $well^{-1}$ in 24-well tissue culture treated plates. The cells were washed and seeded in serum-free DMEM high glucose cell culture medium (Hyclone). The agonists, FSL-1 (1μg ml^{-1}, Invivogen), Ultrapure LPS, *E. coli* 0111:B4 (10 μg ml^{-1}, Invivogen), and whole organism, *M. pulmonis* [multiplicity of infection (MOI) of 0.7 cfu $cell^{-1}$], were suspended in serum-free DMEM high glucose (Hyclone) and added to the wells for a total volume of 500 μl $well^{-1}$ and the supernatants were collected at 6 and 24 hour time points. BMDC were similarly stimulated with Ultrapure LPS and *M. pulmonis* and cell culture supernatants were collected at 24 hours after stimulation.

Cytokine ELISA

The level of cytokine in culture supernatants was determined by sandwich ELISA. Murine IL-12p40 and human IL-8 were measured using a sandwich ELISA OptEIA IL-12p40 or Human IL-8 ELISA set (BD pharmingen) as previously described [20].

Determination of mycoplasma numbers.

Following infection, lungs and nasal passages were quantitatively cultured as described elsewhere [118]. Briefly, lungs were minced, and both lungs and nasal passage washes were placed in mycoplasma broth medium. The samples were

sonicated for 45-60 seconds, and 1:10 serial dilutions were prepared. Twenty microliters of each dilution were plated onto mycoplasma agar medium. After 7 days of incubation at 37°C, cfu were counted.

Statistical Analyses

Data results were analyzed using two-way ANOVA for group comparisons and Students's t-test when appropriate. When necessary, the data was logarithmically transformed prior to analysis. The data was analyzed using the Instat and GraphPad Prism software programs (San Diego, CA).

Results:

TLR1, TLR2, and TLR6 mRNA are expressed in BAL from C57BL/6J mice

To determine if TLR1, TLR2, and TLR6 are expressed in BAL cells, the BAL fluid of two C57BL/6J mice was collected and pooled. The cells were pelleted, RNA isolated, and cDNA was generated. The primary transcript levels of TLR1, TLR2, and TLR6 were determined using sybr-green real-time PCR. This experiment was done a total of three times. TLR1, TLR2, and TLR6 mRNA are expressed in BAL cells (Figure 1). TLR1 was expressed at the lowest levels of the three TLRs assayed, with TLR6 having the highest expression.

Viable *M. pulmonis* recognition is mediated by TLR2 and enhanced when TLR1 or TLR6 are co-expressed

To determine which TLRs are involved in recognizing viable *M. pulmonis*, HEK cell lines, stably transfected to express murine TLRs, were stimulated with viable *M. pulmonis*. As a positive control, we stimulated the HEK cell lines with

the synthetic diacylated lipoprotein, FSL-1, a known TLR2 agonist. As a negative control we utilized the stock HEK cell line known to have null or low basal expression of all the TLRs. To further determine the specificity of our cell culture system, we also stimulated the cell lines with Ultrapure LPS, a known TLR4/MD2/CD14 agonist.

Stimulation of the cell lines with viable *M. pulmonis* demonstrated a role for TLR1, 2, and 6 in the recognition of *M. pulmonis*. *M. pulmonis* stimulated the TLR2 expressing cell lines to produce IL-8, while the TLR4/MD2/CD14 expressing cell line was unresponsive (Figure 3). To confirm specificity of TLR mediated responses, HEK cell lines were stimulated with either a TLR4 or TLR2 agonist. LPS stimulation is optimal when TLR4, MD2 and CD14 are present, but ultrapure preparations of LPS should not mediate responses in cells expressing TLR2. As expected, the TLR4/MD2/CD14 expressing cells responded to LPS stimulation ($P \leq 0.05$) while TLR2 expressing cells did not. Similarly, with FSL-1, which is a TLR2 agonist, we saw an increase in IL-8 production by the TLR2 expressing cell lines with an enhancement of IL-8 production when either TLR1 or TLR6 was co-expressed (Figure 2). As expected, the TLR4/MD2/CD14 expressing cell line did not respond to FSL-1 stimulation. These results confirmed the specificity of the responses. Thus, *M. pulmonis* stimulated these cells using TLR2, but not TLR4, to produce the chemokine IL-8.

Impaired cytokine production by BMDC generated from TLR2 KO mice

In order to determine if TLR2 stimulation mediates the release of cytokines, we generated BMDC from TLR2 KO and wild-type C57BL/6J mice.

The BMDC were then stimulated with viable *M. pulmonis* for 24 hours, and their IL-12p40 production was noted as a marker of stimulation. The BMDC from the TLR2 KO mice had less than a 2 fold increase in IL-12p40 production when compared to unstimulated controls, while BMDC from the WT mice had a greater than 20 fold increase in IL-12p40 production in response to *M. pulmonis (*Figure 4*)*. Both populations of BMDC responded well to LPS. Thus, TLR2 was found to be important in the cytokine production by BMDC in response to *M. pulmonis.*

Impact of TLR2 on resistance to *M. pulmonis* infection

To determine the impact of TLR2 on control of mycoplasma infection, CFU numbers were determined in the nasal passages and the lungs in TLR2 KO C57BL/6 and WT mice. Each group was composed of 3 mice, and the experiment was done in duplicate (n=6 animals). Each mouse was infected with *M. pulmonis.* At 72 hours post-infection, the mice were sacrificed, and the lungs and nasal washes were collected from each animal.

Upon harvesting, the lungs were analyzed for the presence of CFU. No clinical signs of disease or gross inflammatory lesions were seen at 72 hours post-infection. We show that at 72 hours the TLR2 KO mice had approximately 2-log higher CFU counts in the nasal passages (Figure 5). We also show that the TLR2 KO mice had a 2-log higher CFU count in the lungs than recovered form wild-type mice (Figure 6). Thus, TLR2 KO mice were impaired in their ability to control mycoplasma infection as compared to the wild-type in the upper and lower respiratory tracts

Discussion

In this study, we demonstrate that cellular recognition of viable *M. pulmonis* is mediated by TLR2, and enhanced with the co-expression of TLR1 or TLR6. The TLR2, TLR1, and TLR6 mediated recognition of mycoplasma lipoproteins, derived from several mycoplasma strains, is well documented in the literature [23, 24, 96, 111]. The current paradigm suggests that TLR2 dimerizes with TLR1 to recognize triacylated lipoproteins [26] and with TLR6 to recognize diacylated lipoproteins [94, 120]. However, recent reports suggest that the discrimination in recognition of bacterial lipoproteins might be more complex [120]. To our knowledge, these are the first studies which investigate the TLR recognition of viable *M. pulmonis*. In the recognition studies, utilizing the HEK cell lines, it is seen that viable *M. pulmonis* is recognized by TLR2 alone. However, there is a much stronger cellular response when TLR2 dimerizes with either TLR1 or TLR6, ultimately gaining the largest response from the TLR2/1 expressing cell line. Recently, a dipalmitoylated lipoprotein from *M. pneumoniae* was shown to activate NF-Kβ through TLR2, TLR6, and TLR1 [24]. Thus, these studies demonstrate that *M. pulmonis* recognition is mediated by TLR2 and suggests that *M. pulmonis* expresses both di- and triacylated lipoproteins on its surface, which mediate these responses.

TLR2 recognition of viable *M. pulmonis* likely directs the host's immune responses by mediating the release of pro-inflammatory cytokines. MRM is characterized by an exaggerated inflammatory response which ultimately damages the host's respiratory tract. This source of inflammation is propagated by the release of pro-inflammatory cytokines at the site of infection as well as the

recruitment of inflammatory cells. The mycoplasma stimulation of BMDC generated from WT and TLR2 KO C57BL/6 mice showed that the production of IL-12p40 production was TLR2-dependent. IL-12p40 is a pro-inflammatory cytokine which skews naïve T cells towards a Th1 lineage. This TLR2 dependent cytokine production agrees with recent reports that show that TLR2 stimulation plays a role in directing the downstream adaptive immune response through either direct cell stimulation or through mediating the release of pro-inflammatory cytokines such as IL-12p40 in other conditions such as asthma [121-123]. When stimulated with whole organism *M. pulmonis,* the BMDC from the WT strain had a greater than 18-fold increase of IL-12p40 production over unstimulated controls, while the TLR2 KO strain had a increase in IL-12p40 production. Although there might be other TLR2-independent mechanisms involved in the recognition of *M. pulmonis*, these results highlight the critical role of TLR2 in the recognition of *M. pulmonis.* However, the results clearly highlight the role of TLR2 in mediating the host's inflammatory response to *M. pulmonis.* To further reinforce this point, in the HEK data set we see that the TLR2 mediated recognition of *M. pulmonis* is sufficient to produce the pro-inflammatory cytokine IL-8. IL-8 is a human pro-inflammatory cytokine involved in the recruitment of neutrophils, and is not expressed in the murine system. However, recent reports indicate that the murine homologues KC, LIX and MIP-2 are expressed in the lungs of mice suffering with acute MRM [113].The limiting factor to the HEK cell stimulation experiments is that the murine TLRs have been transfected into a human cell line. However, the production of pro-inflammatory cytokines as a

result of TLR activation is well established [79, 86]. Thus, this TLR2 mediated pro-inflammatory cytokine production indicates that TLR2 is the pivotal TLR responsible for the recognition of *M. pulmonis*, and this recognition is complemented with the expression of either TLR1 or TLR6.

Resistance to infection from invading mycoplasmas is a complex genetic trait. Research into this area has indicated that the intrapulmonary clearance of organisms is mediated by agents of the innate immune system. Specifically, current evidence indicates the AMs as the cell population most responsible for this clearance [34]. AM compose >95% of the cells within the BAL fluid [15]. The ability of AM to bind, ingest and kill *M. pulmonis* has been determined [15, 33, 34]. Studies focusing on resistance to *M. pulmonis* have led to the discovery of mice which are either resistant (e.g. C57BL/6) or susceptible to infection. It's been shown that depletion of AM prior to infection with *M. pulmonis,* in an acute infection model, results in the loss of resistance for the C57BL/6 mice. These same studies show that depletion of the AM population renders[both resistant and susceptible strains to similar organism loads at 48 hours post-infection [34]. AMs recognize pathogens via pathogen associated molecular patterns (PAMPs) through pattern recognition receptors (PRR) such as the TLR family. This leads to receptor mediated endocytosis and the eventual display of pathogenic particles in conjunction with MHC molecules. In this study, we sought to determine if this intrapulmonary clearance of organisms mediated by AM is dependent on TLR2. Recently, TLR activation has been linked to the enhanced phagocytosis of microbes [124]. In these studies, we show the relative primary

transcript levels of TLR1, TLR2, and TLR6 within the BAL of C57BL/6 mice. Our HEK cell recognition data indicate TLR2 as the pivotal TLR necessary to mediate the host's responses to *M. pulmonis*. We expected TLR2 to have a higher expression when compared to TLR1 or TLR6 due to its known role in the recognition of all classes of bacterial lipopeptides. However, in the BAL cells, TLR6 had higher levels of expression in all samples tested. Currently, the only known ligands for TLR6 are bacterial lipoproteins it recognizes as it dimerizes with TLR2. This high level of TLR6 expression suggests that it might play a more substantial role in intrapulmonary defense. To determine what role TLR2 has in disease severity we determined the CFU counts in the lungs and nasal passages of WT and TLR2 KO C57BL/6 mice at 72 hours post-infection. Previous reports indicate that infection of WT C57BL/6 mice results in the intrapulmonary clearance of 83% of organisms within 72 hour post-infection. In both the lungs and the nasal passages, the TLR2 KO mice had a statistically significant 2-log higher bacterial load when compared to their WT counterparts. Thus, the intrapulmonary clearance of organisms utilizes a TLR2-dependent mechanism.

The clearance of organism from the nasal passages must occur independently of the AM due to their confinement to the lower respiratory tract. Therefore, the TLR2 mediated intranasal clearance of the organisms must be mediated through different means. While the response of the lower respiratory tract to an *M. pulmonis* infection seems to be a cell mediated propagation of an inflammatory response, the reaction of the upper respiratory tract appears to lead to the production of antimicrobial compounds, such as the β-defensins. Recent

reports indicate that TLR2-expressing respiratory epithelial cells located at different locations within the respiratory tract elicit different responses when stimulated with the same TLR agonist [91]. Recently, TLR2 expression was confirmed in oral, tongue, salivary gland, pharyngeal, and esophageal epithelial cells [91]. Stimulation of these epithelial cells, *in vitro*, with known synthetic TLR2 agonists led to the production of the anti-microbial peptide β-defensin 2, without producing pro-inflammatory cytokines [91]. Recent reports indicate β-defensin 2 as having anti-microbial activity towards *M. pneumoniae* [125, 126]. Thus, our data suggest that the intranasal clearance of organisms occurs independently of AM and may possibly be due to the production of antimicrobial peptides.

The purpose of this study was to link two areas of ongoing research: the cell-mediated intrapulmonary clearance of organisms from the lungs, and the TLR mediated recognition of mycoplasmas during mycoplasma respiratory infections. Primarily we determined what TLRs are responsible for the recognition of viable *M. pulmonis* and what effect this recognition has on the propagation of the inflammatory response during the infection. Initially, we determined the expression of TLR1, TLR2 and TLR6 within the BAL cells of C57BL/6 mice. We then demonstrated that TLR2 mediates the recognition of viable *M. pulmonis* and this recognition is enhanced via TLR1 or TLR6 co-expression. Furthermore, we demonstrated that recognition of *M. pulmonis* by TLR2 mediated the release of pro-inflammatory cytokines such as IL-12p40. In summary, we show that TLR2 recognition of *M. pulmonis* confers protection for the host during mycoplasma respiratory infections. We hypothesize that AM within the lungs recognize *M.*

pulmonis through TLR2, and this recognition mediates the intrapulmonary clearance organisms. We further hypothesize that the intranasal clearance of organisms is due to the production of anti-microbial peptides. However, due to the widespread expression of TLR2 throughout the respiratory tract, more work is needed to understand the cooperative signaling networks which collectively coordinate the host's innate response to invading mycoplasmas.

Figures.

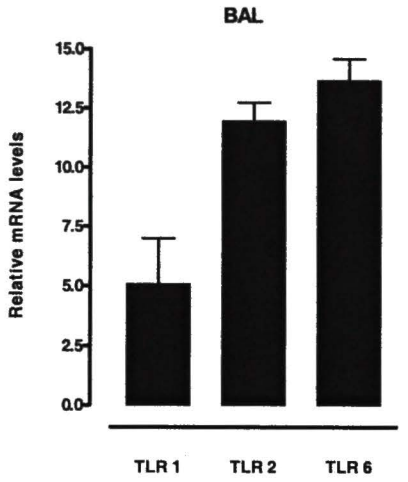

Figure 1. TLR1, TLR2, and TLR6 mRNA are expressed in BAL from C57BL/6J mice

The BAL fluid of two C57BL/6J mice was collected and pooled. The cells were pelleted, RNA isolated, and cDNA was generated. The primary transcript levels of TLR1, TLR2, and TLR6 were determined using sybr-green real-time PCR. The values were normalized to the housekeeping gene GAPDH and the experiment was done a total of three times. An arbitrary value of 10 was added to all data points.

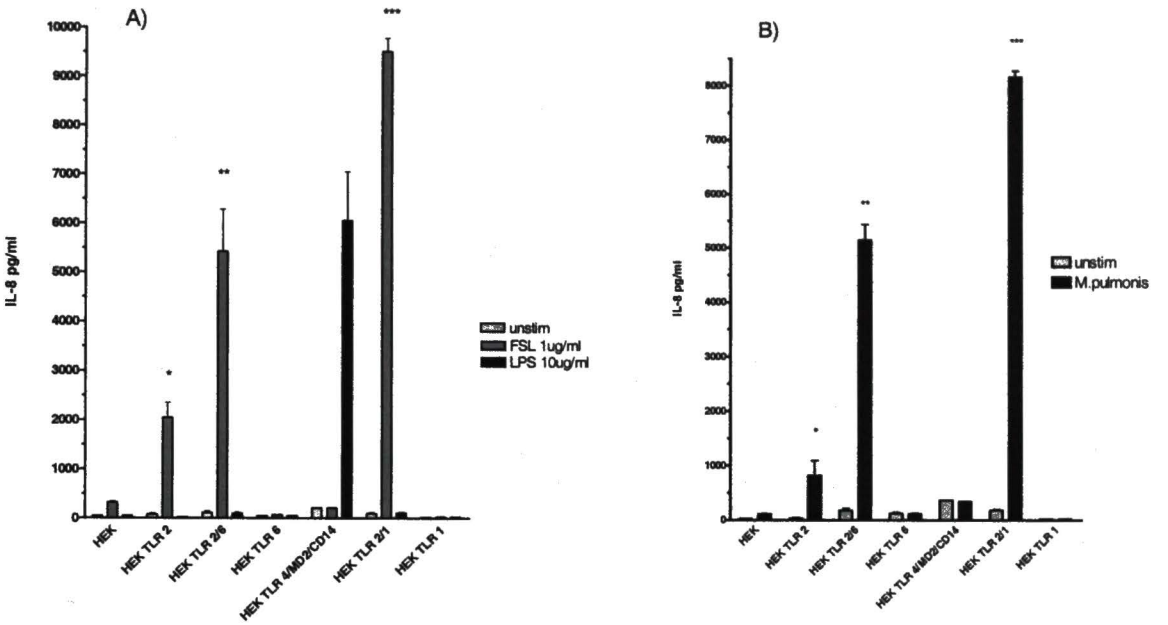

Figure 2. Viable *M. pulmonis* recognition is mediated by TLR2 and enhanced when TLR1 or TLR6 are co-expressed

HEK293 (HEK) or those stably transfected to express individual TLRs and their adaptor proteins (TLR2 or TLR4/md2/cd14) were stimulated for 6 hours with (A), an *E. coli* derived Ultrapure LPS preparation at 10 μg/ml, the TLR2/6 agonists FSL-1 at 1 μg/ml or 24 hours with (B) viable *M. pulmonis* at 0.7 MOI. The supernatants were then collected and assayed for their IL-8 content. Data are expressed as the fold change of IL-8 pg/ml production of each agonist normalized to unstimulated controls. An asterisk "*" denotes a p value ≤ 0.05.

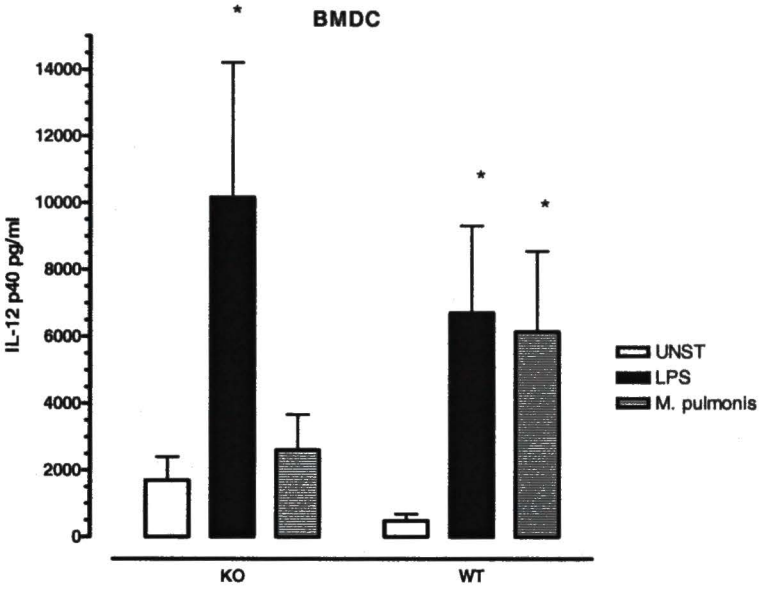

Figure 3. Impaired production of IL-12p40 by BMDC generated from TLR2 KO mice following stimulation with *M. pulmonis*

BMDC were stimulated for 24 hours with an Ultrapure LPS at 10ug/ml or viable *M. pulmonis* at 0.7 MOI. The supernatants were then collected and assayed for their IL-12p40 content. Data are expressed as the IL-12p40 pg/ml production. An asterisk "*" denotes a p value ≤ 0.05.

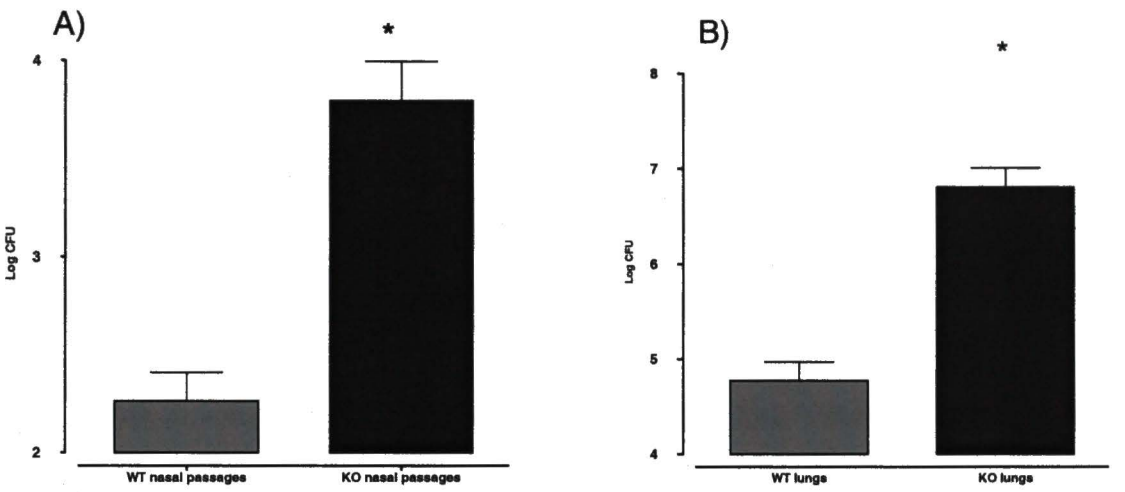

Figure 4. TLR2 KO mice have higher CFU numbers in the upper and lower respiratory tract following mycoplasma infection

CFU numbers were determined in the nasal passages and the lungs in TLR2 KO C57BL/6 and WT mice. Each mouse was infected with *M. pulmonis.* At 72 hours post-infection, the mice were sacrificed, and the lungs and nasal washes were collected from each animal. Each group was composed of 3 mice, and the experiment was done in duplicate (n=6 animals). An asterisk "*" denotes a p value ≤ 0.05.

Chapter IV

ACUTE RESPONSES OF BRONCHOALVEOLAR LAVAGE CELLS ISOLATED FROM C57Bl/6 MICE FOLLOWING STIMULATION WITH MYCOPLASMA PULMNOIS

Introduction

Work in the previous chapters demonstrated that the recognition of *M. pulmonis* is mediated by TLR2. HEK cell lines stably transfected with TLR2 were able to respond to the *in vitro* stimulation with *M. pulmonis* by producing the cytokine IL-8. Previous reports in the literature have indicated that AMs are responsible for the intrapulmonary clearance of mycoplasma organisms from the lungs [15, 17, 33, 34]. The most abundant phagocytic cell population, residing in the airways of the lungs, are the AMs which have been determined by bronchoalveolar lavage samples to constitute ~95% [13]. The purpose of this study was to determine the acute response of the AM following interaction with *M. pulmonis*. BAL were isolated from C57BL/6 mice and stimulated *in vitro* with *M. pulmonis* for six hours. Following stimulation, the cells were collected, RNA isolated, and a microarray analysis was performed to monitor changes in gene transcription.

Materials and Methods

Mice

C57BL/6J and B6.129S1-*TLR2*^{tm1Dgen}/J (TLR2$^{-/-}$) mice, tested to be viral and mycoplasma free, were obtained from The Jackson Laboratory (Bar Harbor, MN). Mice were housed in sterile microisolator cages supplied with sterile bedding, food and water given *ad libitum*. Mice used in the studies were between 9-15 weeks of age. Female mice were used in all studies. Before experimental infection, mice were anesthetized with an intramuscular injection of ketamine/xylazine.

Isolation of BAL from C57BL/6 **mice**

The BAL was performed as previously described [127]. Briefly, two pathogen-free C57BL/6J mice were killed by lethal injection, and their tracheas were exposed and ligated distal to the larynx. A sterile 22G ProtectIV Plus catheter (MEDEX, Carlsbad, CA) was inserted approximately 2 to 3mm into the lumen, and the lungs were then lavaged 3 times with 1 ml aliquots of phosphate-buffered saline solution (Hyclone). The lavages were pooled, and cells pelleted using centrifugation at 200 x g for 10 min at 4°C. BAL cell pellets were resuspended in 500 μl of Trizol (Invitrogen Life Technologies, Carlsbad CA).

In vitro cell stimulations

BAL cells were seeded at 5 x 10^4 cells well^{-1} were plated in a 96-well tissue culture treated plates. The cells were washed and seeded in serum-free DMEM high glucose cell culture medium (Hyclone). The cells were stimulated with whole organism, *M. pulmonis* [multiplicity of infection (MOI) of 0.7 cfu cell^{-1}], suspended in serum-free DMEM high glucose (Hyclone) and added to the wells for a total volume of 200 μl well^{-1}. At 6 hours, supernatants were removed, and RNA was

isolated using Trizol RNA isolation reagent, as recommended by the manufacture (Invitrogen) [119]. The homogenates were frozen at -80°C until further use.

Microarray experiments

Control and experimental samples (n=4) were shipped on dry ice to Miltenyi Biotech (Auburn, CA). Linear amplification of RNA was done and PIQOR immunology microarrays were performed.

Results

Gene expression profiling of BAL stimulated with *M. pulmonis in vitro*

To determine if TLR2-related signaling cascades were induced in AMs following stimulation with *M. pulmonis*, the BAL of C57Bl/6 mice were collected. The cells were stimulated *in vitro* with *M. pulmonis*, and the RNA collected and subjected to microarray analysis screening for immunology related signaling cascades. Our primary focus was the expression of mRNA linked to TLR2 signals through the MyD88 dependent signaling cascade. Several protein kinases associated with the MyD88 dependent signaling pathway were induced in AMs following the acute exposure to *M. pulmonis* (Fig. 1). Also, the TLR related protein kinases TRAIP, and the MAP3K family member TPL2 were induced in the AMs. In addition, a number of TNF related genes, known products of the MyD88 pathway were induced, including TNF, TNFRSF7, and TNFSF9.

Discussion

TLRs signal through a conserved IL-1R signaling pathway that leads to NF-κβ activation through either a MyD88 dependent pathway, which mediates

early phase activation of NF-κβ, or a MyD88 independent/TRIF dependent pathway, which mediates the late phase activation of NF-κβ. The induction of the MyD88 dependent signaling cascade results in the induction of inflammatory cytokines [65, 67]. The TRIF dependent signaling cascade also results in the induction of inflammatory cytokines, co-stimulatory molecules, IFN-β, and other IFN-inducible gene products [65, 67]. As our previous studies have demonstrated that the recognition of viable *M. pulmonis* is mediated through TLR2, these studies aimed to identify the *M. pulmonis* induced, TLR2 mediated, responses of AM following acute exposure to *M. pulmonis*.

Our results demonstrate that the stimulation of AM with viable *M. pulmonis* results in the activation of the MyD88 dependent signaling pathway. The MyD88 dependent signaling cascade utilizes MyD88 to activate the NF-κβ transcription pathway which leads to the production of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 [66]. TLR ligation causes conformation changes in the TIR domain of the TLR receptor complex which recruits MyD88. MyD88 then interacts with the adaptor molecule TIRAP which was induced in the AMs following stimulation with *M. pulmonis*. TIRAP is specifically involved in the TLR2 and TLR4 mediated activation of the MyD88 dependent signaling cascade [67]. The role of TIRAP in the MyD88 dependent signaling pathway was identified in studies which focused on the LPS-TLR4 signaling cascade utilizing TIRAP KO mice. TLR4 is unique among the TLRs in that its ligation activates both the MyD88 dependent and TRIF dependent pathways. TIRAP KO mice stimulated with LPS were found defective in the production of inflammatory cytokines, but

the expression of IFN-inducible genes and the delayed NF-κβ response remained [67]. These results indicated TIRAP as contributing to the MyD88 dependent signaling cascade. In addition, TIRAP deficient mice showed an impaired ability to produce inflammatory cytokines in response to TLR2 agonists. Although TLR4 ligation activates both signaling cascades, the activation of the MyD88 dependent cascade is sufficient to induce inflammatory cytokine production in response to TLR2 agonists [67]. After TIRAP interacts with MyD88, members of the IL-1R associated kinase (IRAK) family are recruited which then interact with TRAF6. The TRAF6-IRAK complex then interacts with members of the mitogen-activated protein kinase kinase kinase (MAP3K) family (e.g. TAK1/MAP3K7) which lead to NF-κβ through the IKK and MAPK signaling cascades [65-67]. Other MAP3K family members have been linked to TLR signaling. The MAP3K family member MAP3K8 (cot/TPL2) was found to play a role in the TLR4-mediated activation of the ERK pathway [128]. In response to TLR4 ligands, TPL2 KO mice showed impaired TNF production and activation of ERK. The TRAF-interacting protein (TRIP) and the MAP3K family member TPL2 was induced in AMs following the *in vitro* stimulation with *M. pulmonis.* Further supporting the MyD88 dependent signaling by AMs in response to *M. pulmonis,* is the number of TNF related genes that were induced. Production of the inflammatory cytokine TNF is indicative of the MyD88 dependent pathway and it was induced by the AMs [65-67]. The inability of mycoplasma lipoproteins to activate NF-κβ and produce inflammatory cytokines in cells isolated from TLR2 KO and MyD88 KO mice has been demonstrated [129]. To our knowledge, this is

the first study which demonstrates that viable mycoplasma organisms signal through the MyD88 dependent pathway *in vitro*. Also, these are the first studies to indicate the specific adaptor molecules utilized in the *M. pulmonis* signaling cascade. However, future studies are needed to confirm the signaling cascade.

Although TLR2 mediated signaling is believed to act solely though MyD88 dependent mechanisms, several genes were induced that are linked to the MyD88 independent signaling pathway; the type I IFN inducible genes GARG16. However, the induction of these genes could be byproducts of autocrine mechanisms taking place *in vitro*. Several gene combinations induced resembled known autocrine mechanisms, such as the inflammatory cytokine GM-CSF and its transcription factor STAT5a. In previous reports, several IFN inducible genes were shown to be induced by autocrine mechanisms [130, 131]. Macrophages isolated from mice deficient in the IFNα/β receptor subsequently showed that production of the IFN inducible chemokine CXCL10 as the result of an autocrine mechanism utilizing IFN-β [130, 131]. In addition, as TLR signaling occurs through conserved IL-1R signaling pathways, it is also worth mentioning that the IL-1R agonist IL-1α was also induced. Thus, these data suggest other signaling mechanisms might be involved in the acute responses of AM to *M. pulmonis*.

The limitation of this study is that the microarray analysis has yet to be confirmed utilizing realtime-PCR or by detecting phosphorylated proteins involved in the signaling cascades suggested by the array. Future studies should aim at confirming the results of the microarray and work to identify the possibility of autocrine mechanisms which might take place *in vitro* and *in vivo*.

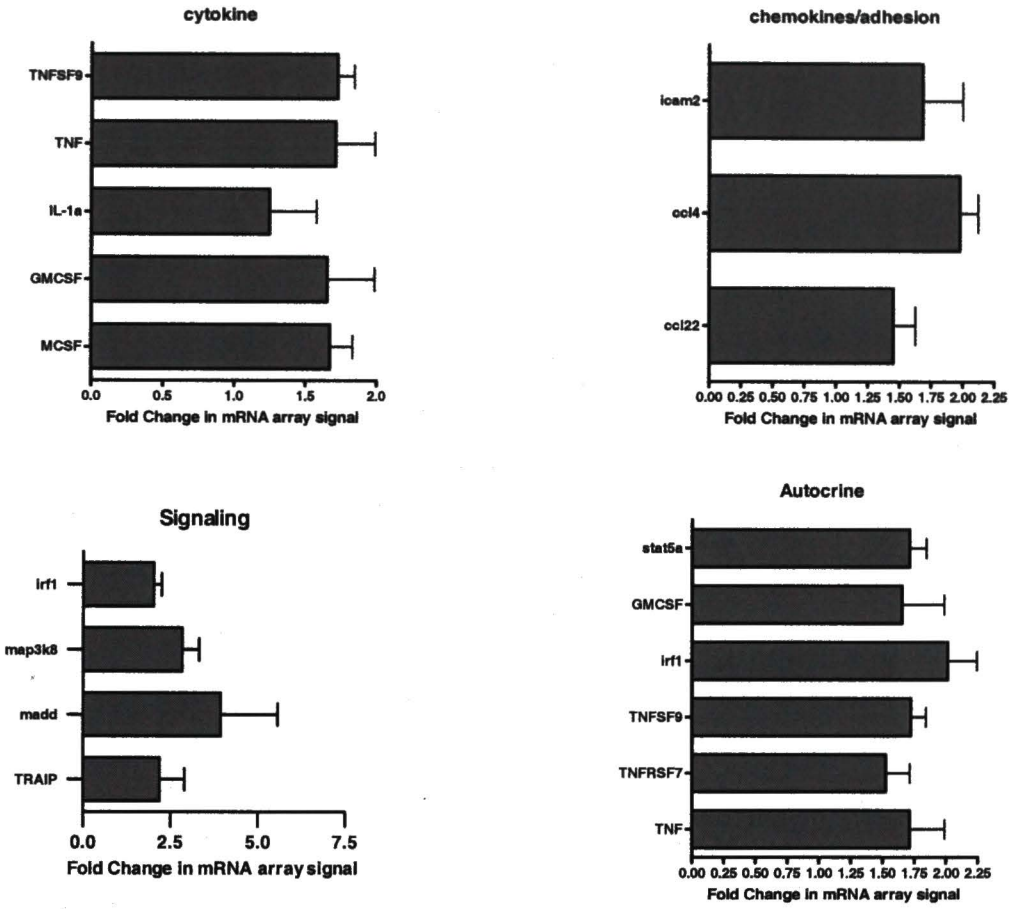

Figure 1. Gene induction by BAL cells following short term exposure to *M. pulmonis*

BAL were isolated from C57Bl/6 mice and stimulated *in vitro* with viable *M. pulmonis* for 6 hours. The genes induced are expressed as fold changes of induction compared to unstimulated controls (n=4).

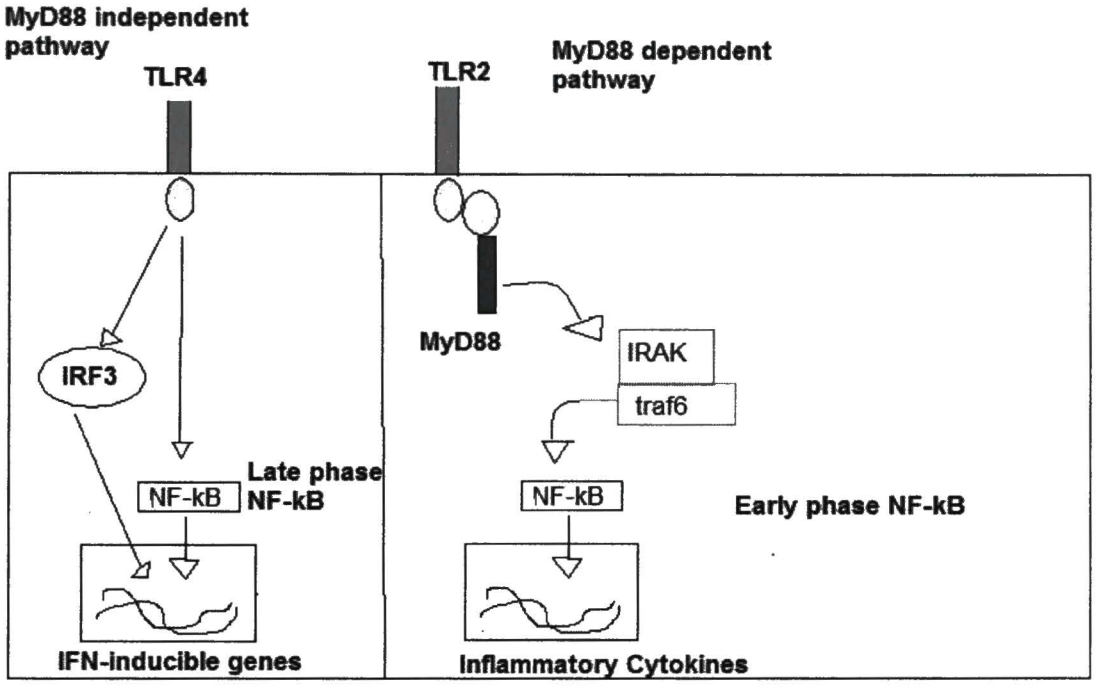

Figure 2. MyD88-dependent and MyD88-independent signaling pathways

Stimulation of TLRs facilitates the activation of two signaling pathways: the MyD88-dependent and MyD88-independent pathways.

Chapter V

Discussion

These studies demonstrated critical roles for the TLRs in the host responses to *M. pulmonis*. I have demonstrated that TLR2, most likely in concert with TLR1 and/or TLR6, contribute to the defense from *M. pulmonis* in the upper and lower respiratory tract, influence the host's cytokine response, and mediate the recognition of viable *M. pulmonis*. I also found that TLR2 specific signaling cascades do appear to be activated in response to *M. pulmonis,* but other signaling events may occur. This information will aid in the development of the elusive mycoplasma vaccine and provide additional insights necessary to understand the host's response to *M. pulmonis*.

These are the first studies which show the recognition of viable *M. pulmonis* by TLRs. TLRs clearly have a role in the recognition of mycoplasmas. Current reports in the literature use purified lipoproteins from various mycoplasma species to determine which TLRs play a role in recognition. In these studies, I demonstrated that viable *M. pulmonis*, a native murine pathogen, is recognized by a HEK cell line stably expressing the human homologue of TLR2. I also demonstrate the ability of a human pulmonary epithelial cell line, A549, to respond directly to *M. pulmonis* by secreting the inflammatory cytokine IL-8. In addition, in HEK cells stably expressing the murine homologues of the TLRs, I showed that the murine homologue of TLR2 mediated the recognition of *M.*

pulmonis, and this recognition was enhanced when TLR1 or TLR6 was co-expressed resulting in the production of IL-8. Although IL-8 is a human cytokine, protein or mRNA levels of the murine homologues of human IL-8, e.g. KC, MIP-2, and LIX, are elevated in the lungs of mice infected with *M. pulmonis [38, 113]*. I also demonstrated the TLR2 dependent production of the inflammatory cytokine IL-12p40 in BMDC generated from WT and TLR2 KO mice. Furthermore, I established that the primary transcripts of TLR1, TLR2 and TLR6 are expressed in BAL cells isolated from C57Bl/6 mice. In conclusion of the TLR2 recognition studies, I demonstrate that TLR2 KO mice have a 2-log higher incidence of organism in the upper and lower respiratory tract 72 hours post-infection. These studies demonstrate that TLR2 mediates the recognition to *M. pulmonis* which confers resistance to infection for the host and augments the cytokine response. After establishing that TLR2 mediates recognition of *M. pulmonis,* I began characterizing the signaling pathways which occur in AM stimulated with *M. pulmonis*. Using microarray analysis, our data strongly suggest that the MyD88 dependent pathway, downstream of TLR2, is utilized in response to *M. pulmonis*. Many of the genes produced as a result of an activated MyD88 pathway, such as TNF family members, chemokines, and adhesion molecules, were strongly induced. In addition to the MyD88 pathway, our data suggests that other signaling pathways, possibly autocrine in nature, are also activated in response to *M. pulmonis*. Thus, the recognition of viable *M. pulmonis* is mediated by TLR2. Furthermore, these studies highlight the role that the TLR2 mediated recognition of viable *M. pulmonis* can have in contributing to the inflammatory response.

Anti-mycoplasma responses in the upper and lower respiratory tracts most likely occur through different mechanisms. As stated earlier, TLR2 KO mice have 2-log higher number of mycoplasmas in the upper and lower respiratory tracts when compared to WT controls. I believe that the epithelium, which is morphologically different in the upper and lower respiratory tracts, plays a role in the responses to infection. *M. pulmonis* is a mucosal pathogen, and the first stage of a mycoplasma respiratory infection is believed to be the interaction between the mycoplasma and the epithelia lining the mucosal surface. Investigations of the respiratory epithelia with *M. pneumoniae* demonstrate the ciliostasis of the epithelial cells along the luminal surface [132, 133]. Recent reports in the literature demonstrate that epithelial cells of the upper and lower respiratory tract react differently when stimulated with the same TLR2 agonists. When stimulated with TLR2 agonist, epithelial cells of the upper respiratory tract produced antimicrobial peptides (e.g. β-defensin) whereas those of the lower respiratory tract promoted an inflammatory response through the release of inflammatory cytokines [89, 91, 134, 135]. Differences in the initial responses to infection by mycoplasma in the upper and lower respiratory tracts could influence the subsequent development of innate and adaptive immune responses. In fact, previous findings in our laboratory also noted different anti-mycoplasma responses which existed between the upper and lower respiratory tracts after immunization [19]. These studies demonstrated that the local immunization of the upper respiratory tract confers protection from mycoplasma infection but required the adjuvant cholera toxin (CT). The localized immunization resulted in higher

IgA titers and an increase in serum IgG titers [19]. Mucosal immunity has previously been suggested to play a role in the protection from mycoplasma infection. Although serum antibody production was induced, this likely plays a limited role in protection from the organisms as the upper respiratory tract exists in a different compartment from the lungs and the circulation [19]. Thus, the responses to *M. pulmonis*, a TLR2 agonist, likely differ between the upper and the lower respiratory tract which could influence the development of protective and inflammatory responses. Future studies should further explore these different mechanisms in hopes to develop a vaccine against *M. pulmonis*.

The anti-mycoplasma responses, of the lower respiratory tract, are a complex balance between inflammatory and protective immune responses. As mentioned earlier, the AM population in the lower respiratory tract is demonstrated to be responsible for the intrapulmonary clearance of the organisms. Depletion of this population of cells exacerbates respiratory mycoplasmosis in resistant C57Bl/6 mice strain [34]. However, the AM may act in concert with the respiratory epithelium as recent reports indicate that the epithelium of the lower respiratory tract is capable of responding to mycoplasma infection by secreting inflammatory cytokines [60, 62, 63, 136]. In fact, the TLR mediated release of cytokines by the respiratory epithelia has been demonstrated [27, 81, 137]. To recall, in our studies, we show the ability of the alveolar type II cell line A549 to directly respond to *M. pulmonis* by secreting the inflammatory cytokine IL-8. In addition to promoting an inflammatory response through cytokine production, the lower respiratory tract can generate protective

immunity to mycoplasma infection. The localized nasal-pulmonary immunization provides greater resistance to infection than the localized immunization of the nasal cavity alone. Immunization of the nasal-pulmonary region resulted in a greater than 10,000 fold fewer number of mycoplasma after infection than immunization of the upper respiratory tract alone [19]. This immunization increased the number of IgA antigen forming cells in both the upper and lower respiratory tract and resulted in higher serum antibody titers in all classes tested (e.g. IgA, IgM, IgG). Furthermore, *M. pulmonis* membrane preparations, likely TLR2 agonist, were shown to be B cell mitogens capable of facilitating their maturation to plasma cells [138-141]. In retrospect, it is possible that the before mentioned studies could be due to TLR2 mediated mechanisms. Thus, the innate and adaptive immune mechanisms, in the lower respiratory tract, play a large role in resistance to mycoplasma infection, and it is likely that TLR2 mediated mechanisms contribute to both. Ongoing efforts in our laboratory are investigating the response that the respiratory epithelium has in contributing the host's defense from infection. Future studies should focus on the ability of the respiratory epithelium to direct the host's immune response to invading mycoplasmas and begin to investigate their role in the innate immune response, and determine whether these effects are mediated by TLR interactions.

There are likely other host factors that can modulate the response of the host to mycoplasma infection. Opsonins within the respiratory tract can also contribute to the anti-mycoplasma responses, altering interactions with the host cell including alveolar macrophages. The interactions of mycoplasmas with

opsonins such as the surfactants (e.g. SP-A, SP-D), and complement is established in the literature [22, 49, 50, 142]. The surfactant proteins SP-A and SP-D bind *M. pneumoniae* where they inhibit its growth and affect its viability [47-49, 125]. In addition, SP-A enhances the phagocytosis of mycoplasmas by AM *in vivo* [17]. In these studies, we have demonstrated that the interaction between *M. pulmonis* and TLR2 is sufficient to induce the production of inflammatory cytokines. Interestingly, recent findings in the literature report that SP-A can interacts with TLR2 and downregulate inflammatory cytokine production [46]. These findings demonstrate that surfactants may have an impact on the host's response to mycoplasma infection and indicate that TLR2 mediated interactions may also be involved. In addition to surfactants, *M. pulmonis* is susceptible to killing by complement [52, 53, 143]. *M. pulmonis* expresses a class of surface antigens known as variable surface antigens (Vsa). There are seven classes of these vsa proteins and all are capable of undergoing size variation. They contain a constant N-terminal region which is connected to one of seven variable C-terminal domains which are characterized by having a number of terminal repeats. These repeats can go as high as 40 or the regions can contain none at all. The length of these vsa proteins, determine the susceptibility of the *M. pulmonis* strain to complement killing. The longer the protein, i.e. the more terminal repeats, the less susceptible to complement killing the strain is. *M. pulmonis* strains with short vsa proteins on their surface are easily killed by complement [52, 53, 143].Complement killing of mycoplasmas occur through the alternative complement pathway and the formation of the membrane attack

complex (MAC) [53]. The longer vsa proteins are believed to prevent the opsonization of the membrane by complement and thus prevent the formation of MAC. Thus, the opsonins surfactant and complement play a role in the antimycoplasma activities of the lung. Any interactions between the surfactants and the vsa proteins have yet to be investigated. Furthermore, future studies should focus on the possible synergistic interactions which might occur between opsonins and the phagocytic cell population within the lungs.

Mycoplasma lipopeptides are recognized by TLRs. However, there is a current gap in the literature pertaining to discrepancies which exist between different strains of mycoplasmas in regards to the class/types of lipoproteins they express. Most reports involving mycoplasma lipopeptides as TLR agonist utilize purified mycoplasma lipoproteins stemming from several species. These reports often utilize MALP-2, a 2-kDa diacylated lipoprotein, isolated from *M. fermentans* [84, 144]. The N-terminal lipopeptide moiety of MALP-2 is believed to be responsible for its activity as lipase treatment abolishes the activity. It is currently known that diacylated lipopeptides are recognized by heterodimers composed of TLR2/6, while triacylated lipopeptides are recognized by TLR2/1 heterodimers [23, 26, 120]. Current reports demonstrate that lipoproteins from *M. fermentans* have demonstrated the ability to active the heterodimer TLR2/6, while lipoproteins from *M. penetrans* activated the TLR2/1 pathway [25]. Furthermore, current reports in the literature indicate that some species of mycoplasmas, e.g. *M. pneumoniae*, express surface lipoproteins capable of stimulating all three TLRs adding an exception to the rule [24]. Mycoplasmas are known to have vsa

[145]. These vsa can undergo size and phase variations. In addition, genetic sequences related to lipoprotein acylation have been discovered in the *M. pulmonis* mycoplasma genome [145]. Furthermore, mycoplasmas are known to mutate at a high rate and culture of the organism often results in mixed populations [53]. Our results from the *in vitro* stimulations of HEK cells suggest that *M. pulmonis* expresses both di- and triacylated lipoproteins on its surface. In addition, utilizing transposon mutants of *M. pulmonis*, recent data in our laboratory has shown that deletion of the MALP-2 homologue of *M. pulmonis* does not diminish its recognition by TLR1, 2 and 6 (data unpublished). Thus, the recognition of *M. pulmonis* by TLRs could vary as the organisms changes the surface antigens it expresses. As studies begin investigating the role of TLRs in mediating immune responses to mycoplasmas, it will be necessary to characterize the antigens expressed by the strain being used. Future studies should use well characterized strains of *M. pulmonis* when conducting recognition studies and take into account the possibility of heterogeneous populations.

The future directions of this project should investigate the role of TLR2 in susceptible strains of mice. In these studies, we utilized C57Bl/6 mice which are resistant to mycoplasmas. The effect of TLR2 in a susceptible strain might differ. Also, the recognition of the vsa proteins by TLRs needs to be investigated. As these are a major class of antigens which are surface expressed by *M. pulmonis*, their role in mediating its recognition by TLRs needs to be investigated. Recently, *M. pulmonis* was suggested to produce biofilms *in vitro* which depended on the

size of the vsa proteins produced [146]. Perhaps studies involving their recognition by TLRs could provide insight into the global problem of microbe avoidance of the immune system through biofilm formations. In conclusion, the surface antigens expressed by the *M. pulmonis* strain utilized in these studies need to be fully characterized. Thus, future studies should focus on the molecular moieties responsible for the host-bacterial interactions discovered in this thesis.

The purpose of these studies was to identify the role of TLRs during mycoplasma respiratory infections. I found that TLR2 mediated the recognition of *M. pulmonis*. I also found that this recognition mediated the protection of the host from infection in the both the upper and lower respiratory tracts. In addition, primary transcripts of TLR1, TLR2 and TLR6 are expressed in cells of the BAL and the expression of TLR2 augments the host's cytokine production. In conclusion, my data strongly suggests that the TLR2 mediated, MyD88 dependent signaling pathway is induced in AM following the acute exposure to *M. pulmonis*. This research will further the current understanding of mycoplasma immunology. This research will not only expand mycoplasma research but provide a foundation to unraveling the immune response generated in microbial respiratory infections.

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