
UNIVERSITY OF NORTH TEXAS
HEALTH SCIENCE CENTER AT FORT WORTH
1970

Spear, Marcia. The Impact of the *Mycoplasma pulmonis* MALP-2 Homologue on Disease Progression. Master of Science (Biomedical Sciences), April 2008. 64 pp., 3 tables, 8 illustrations.

Using *Mycoplasma pulmonis,* this project looked at a possible critical component in mycoplasma disease, the MALP-2 homologue lipoprotein. Studies demonstrated other lipoproteins besides the MALP-2 homologue were critical for *in vivo* disease progression and *in vitro* macrophage IL-6, IL-12, and TNF-α cytokine production. This trend was also seen human endothelial kidney (HEK) cells transfected with toll-like receptor 2 (TLR2) and the heterodimer TLR2/6. An increase in IL-8 cytokine production seen in all stimulated HEK cell lines, indicating the lipoproteins involved in cell interactions are TLR2 mediated.

This project suggests the *M. pulmonis* MALP-2 homologue is not the main lipoprotein involved in disease progression and cell interactions, indicating the MALP-2 homologue may not be an ideal target for vaccines or antibiotics.

THE IMPACT OF THE *Mycoplasma pulmonis* MALP-2 HOMOLOGUE ON DISEASE PROGRESSION

THESIS

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

University of North Texas Health Science Center at Fort Worth

in Partial Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

By

Marcia G. Spear

Fort Worth, Texas

April of 2008

THE IMPACT OF THE *Mycoplasma pulmonis* MALP-2 HOMOLOGUE ON DISEASE PROGRESSION

Marcia G. Spear, B.S.

APPROVED:

Major Professor

Committee Member

Committee Member

University Member

Chair, Department of Molecular Biology and Immunology

Dean, Graduate School of Biomedical Sciences

ACKNOWLEDGEMENTS

When I started my master's project, I had very little research experience. With the help of numerous people I acquired a toolbox filled with lab techniques, knowledge, protocols, and friendships.

I would first like to thank my mentor, Dr. Jerry W. Simecka. His support, guidance, and bleeding ink comments have steered me through the obstacles towards graduation. Thanks for all the encouragement and advice. I would also like to thank my graduate thesis committee members Drs. Lisa Hodge, Porunelloor Mathew, and Rustin "Rusty" Reeves. Their input and guidance have been invaluable.

Of course I cannot forget my fellow lab mates; they have prodded, encouraged, and taught me ways to improve my research. Lab conversation and discussion were always an interesting experience. I appreciate the support you guys offered. Wherever I go I will always remember my friends and colleagues at UNTHSC. Thanks for being there and for laughing at my sometimes corny jokes.

Last but not least I would like to thank my family. They have been a great source of encouragement and support. I appreciate their diligence to help me through my graduate career.

One can never remember to thank all the people who offered advice along the way or shared tidbits of their own experiences as a student. Thanks to those who questioned, laughed, wondered, remarked, and listened to my bemusements.

TABLE OF CONTENTS

LIST OF TABLES

Table Page

CHAPTER 4

LIST OF LLUSTRATIONS

Figure Page

CHAPTER 4

CHAPTER 1

INTRODUCTION

Mycoplasmas lack a cell wall and are one of the several genera within the class Mollicutes. They require sterols such as cholesterol to maintain cytoplasmic membrane stability, and can be major contaminants in cell culture studies and lab work (62). These bacteria also contain one of the smallest genomes found in bacteria. Even with what could be considered an evolutionary disadvantage, its genome undergoes a wide array of mutations in its surface lipoproteins, enhancing microbial survival and possibly pathogenesis (111, 93, 64, 116). Though they have been shown to activate macrophages, they lack classical lipoprotein stimulators such as lipopolysaccaharide (LPS) or lipoteichoic acid (62). Other studies have identified possible key lipoproteins that facilitate macrophage interaction (74, 66, 73, 83).

Mycoplasma mycoides was the first mycoplasma to be identified as a causative agent for contagious bovine pleuropnuemonia, which in 1898 was a widespread and problematic disease in cattle herds (65). Today there are over one hundred recognized species of mycoplasma, of which the bacteria that infect humans cause pulmonary or urogenital diseases (52), such as *Mycoplasma pneumoniae*. Disease pathogenesis of *M. pneumoniae* is studied using the murine strain *Mycoplasma pulmonis,* which was among the first bacteria to have its genome completely sequenced due to its small genome size (19).

M. pneumoniae and *M. pulmonis*

M. pneumoniae is a common cause of both upper and lower respiratory infection in humans and causes 30% of the United States' pneumonia cases (15). Some studies have shown that *M. pneumoniae* is present in more than half of asthmatics studied; this microbe is responsible for exacerbation of symptoms seen with both acute and chronic asthma (57, 41). *M. pneumoniae* has also been indicated in playing a role in walking pneumonia as well as other diseases, like AIDS, asthma, and arthritis, and chronic obstructive pulmonary disease (2, 4, 68, 27, 118). While some antibiotic treatments can alleviate acute symptoms, they are not very effective in treating chronic symptoms (3).

M. pulmonis causes mycoplasma respiratory mycoplasmosis in rats and mice. Signs of disease include dyspnea, snuffling and chattering, ocular and nasal discharge, eye rubbing, and head tilt. In severe cases, weight loss and reduced fertility may occur (56). The severity of disease is dependent upon the interaction of host, pathogen and environmental factors (56). The hosts' age, strain, immune status, and presence of concurrent infections can exacerbate the disease (56). *M. pulmonis* is a commensal organism, where disease is frequently accompanied and exacerbated by other bacteria, such as the cilia-associated respiratory bacillus and *Corynebacterium kutscheri*, as well as viral infections like Sialodacryoadenitis (89, 88, 29). Both *M. pulmonis* and *M. pneumoniae* have similar disease pathogenesis where they can induce both acute and chronic inflammatory lung disease (66, 97). Evidence from human and animal studies have shown both innate and adaptive immunity are major contributors (15).

Mycoplasmas have been implicated in associating with toll-like receptor 2 (TLR2) through lipoprotein interactions (65). The impact of lipoproteins in mycoplasma disease progression is not completely understood. Recent data from our laboratory demonstrate that *M. pulmonis* interactions with TLR2 are important in clearance by innate immunity, presumably macrophages. While it is likely mycoplasma lipoproteins play an important role through recognition by TLR2, the specific *M. pulmonis* lipoproteins involved in this interaction are not known (65). One possible lipoprotein is the MALP-2 homologue. The *Mycoplasma fermentans* MALP-2 lipopeptide can stimulate phagocytes through TLR2 and interfere with clearance (41). This project will focus on examining the possible roles of the *M. pulmonis* MALP-2 homologue in mycoplasma disease and macrophage stimulation.

Innate Immunity in Mycoplasma Disease

In the innate immune response, alveolar macrophages (AM) are the primary phagocyte involved in mycoplasma defense and disease. In *M. pulmonis* studies, two strains of mice encompass the extremes of the clearance model: C57BL/6N (C57) mice, which after 72 hours post infection (p.i.) have been shown to have decreased mycoplasma numbers by more than 83%; and C3H/HeN (C3H) mice, which have a more susceptible immune system, with mycoplasma numbers increasing up to 18,000% by day 3 p.i (44).

One of the major differences between these strains is the activity of alveolar macrophages (AM) since significant mycoplasmacidal activity occurs within 4 hours,

long before recruitment of other cell types or antibodies (21, 24, 78). Studies involving AM depletion in C57 mice observed AM depletion prior to infection reduced killing of *M. pulmonis*, and organism numbers rose to a level comparable to mycoplasma counts seen in C3H mice (44). When C3H AMs were depleted, no altered killing of mycoplasma was seen. This indicated AMs were major contributors to early pulmonary clearance.

Macrophages can be activated by cytokines, antibodies, and proteins, as well as through T-cell interactions (105). Surfactant protein A (44) has been shown to interact with macrophages and increase mycoplasma killing. This 650 kDa glycoprotein is found in abundance in pulmonary fluids and has been shown to bind to macrophages and induce nitric oxide release as well as enhance phagocytosis (78, 105, 44, 75). By 6 hours p.i., AMs isolated *in vitro* showed an 83% decrease was seen in *M. pulmonis* colony forming units (CFUs). No CFU clearance was seen with AMs stimulated with mycoplasma outside the presence of SP-A, demonstrating SP-A is an important macrophage stimulator in the *M. pulmonis* clearance process (44).

Mast cells are primarily associated with IgE and allergic responses. They can also be activated by inflammatory peptides and ligands of complement and Toll-like receptors (TLRs) (65, 71). Mast cells have also been implicated in playing a protective role in the early mycoplasma infection immune response. In mast cell deficient (Wsh) infected mice, greater mycoplasma numbers were seen in infected mice, accompanied by more severe pneumonia and inflamed bronchial characteristics than wild type mice. Angiogenesis and airway vascular remodeling was greater as well (117).

Another component of the innate immune response that contributes to mycoplasma infections are neutrophils. In infected Wsh mice histamine levels greatly increased in parallel with a rise in the neutrophil population, indicating neutrophils are also important contributors of histamine, as seen with mast cells. When neutrophils were depleted, a decline in histamine production was also seen (117). These findings suggest neutrophils can greatly expand their capacity to produce histamine in the presence of mycoplasma, allowing them to contribute to lung and airway inflammation.

Neutrophils have also been shown to contribute in severity of airway lesions since they are one of the first responding cells (16). Indeed, in BALB/c IL-12 KO mice, a decrease in pulmonary histologic inflammation, particularly in the neutrophil infiltrates, was seen (87). IL-23 induced IL-17 has also been shown to play a critical role in neutrophil recruitment. In BALB/c mice, blocking of IL-23 produced a significant reduction in IL-17 mRNA expression in alveolar macrophages, leading to a reduction in neutrophil recruitment and lesion severity (115, 35).

Adaptive Immunity in Mycoplasma Disease

The adaptive immune response also plays a role in mycoplasmosis. Both C57 SCID and C3H SCID mice, which lack B and T cell responses, show an increase in overall systemic infection, as seen with histopathologic evaluation of major extrapulmonary organs, which was not seen in wild type control mice (15). Surprisingly, SCID mice also had reduced lung lesion severity (15). Indeed, results were similar in

studies where nude mice were inoculated intranasally with *M. pulmonis* (17), as well as immunosuppression studies using antithymocyte sera (104). These studies indicate the adaptive immune system was associated in keeping the mycoplasma infection from spreading past pulmonary tissue.

In chronic infections, C3H mice harbored significantly greater numbers of *M. pulmonis* CFUs in their lungs, as well as an increase in the severity of lesions and higher titers of antibody responses in the serum (16). Studies comparing numbers of mycoplasma in the pulmonary tissues of SCID mice and immunocompetent mice revealed no differences (15). This would suggest that lymphoid responses, instead of an uncontrolled infection, might promote inflammatory lesions. Indeed, histologically, one of the characteristics of chronic mycoplasma pulmonary disease is the accumulation of large populations of lymphoid cells trafficking into the respiratory tract (16, 123). Initial changes in lymphocyte numbers were found in Th cells while the numbers of B and CD8+ T cells did not significantly vary (52), which indicated Th cells were the major T cell population responding at the initial onset of mycoplasma disease. Indeed, even though the lung is primarily a Th2 environment, studies have shown during mycoplasma infection the environment shifts to a mixed Th1/Th2 response (111).

Another characteristic of chronic bacterial infections influenced by the adaptive immune response is vascular remodeling of the airways. In an *M. pulmonis* infection, bacteria are able to attach themselves to the epithelial cells lining the airways. Once attached they proliferate, which leads to inflammation of the alveolar spaces (53, 52, 84).

Characteristics of airway remodeling are proliferation of submucosal glands, subepithelial fibrosis, collagen deposition, and reorganizing of the epithelial barrier and local vasculature (72, 108, 125). *M. pulmonis* infection causes additional changes in airway architecture, such as substantial angiogenesis and lymphangiogenesis (4). In C57 Rag1 -/- mice these additional airway remodeling characteristics were greatly reduced, revealing both immunologic and immunopathologic components exist in the adaptive immune response in *M. pulmonis* infections.

Toll-Like Receptors

TLRs are homologues to the Toll receptor found in *Drosophila*, and have been shown to play an important role in recognizing microorganisms (102). These pattern recognition receptors have been found in both vertebrates and invertebrates. There are eleven human and thirteen mice receptors that have been identified and shown to be activated by pathogen associated molecular patterns, such as LPS or lipoproteins (102, 101, 21, 30). The greatest variety of TLRs has been found in phagocytes, such as macrophages. These receptors initiate direct antimicrobial activity and cytokine production through activation of the NF-kB signaling pathway. The toll-like receptor 2 (TLR2) pathway can lead to proinflammatory cytokine transcription, such as TNF-α and IL-12, while TLR4 and others can lead to production of IFN-γ (75).

TLRs are type I membrane proteins with an extracellular domain containing 19-25 tandem copies of leucine-rich repeats. They also have a cytoplasmic Toll/IL-1

receptor domain that is similar to the IL-1 receptor. The proposed structure is a horseshoe-shaped solenoid with a beta sheet and numerous insertions on the concave surface. These numerous insertions and beta sheet may be associated with the binding site. (76). TLRs have been thought to influence antigen specific immune responses by regulating cytokines and costimulatory signals (81).

Of the thirteen reported TLR family members, only TLR2 has been implicated in interacting with mycoplasmas (123, 101, 21, 30). This receptor recognizes peptidoglycan, lipoarabinomannan, zymosan, and lipoproteins from Gram-positive bacteria and mycobacteria (44, 106, 123). Studies have shown that TLR2 forms heterodimers with TLR1 and TLR6 to discriminate further between bacterial proteins. The TLR2/1 heterodimer recognizes triacylated lipoproteins whereas the TLR2/6 heterodimer recognizes diacylated lipoproteins (101). Recent studies indicated MALP-2 could stimulate spleen cells in C57Bl/6 TLR6 deficient mice at low levels, indicating a possibility TLR2 could homodimerize with itself in the absence of TLR6 (12).

A closer look at the TLR6 domain revealed the leucine rich repeat (LRR) region 6-17 contains the functional activity in diacylated lipoprotein recognition (77). In a similar pattern, the TLR1 functional activity for recognition of triacylated lipoproteins was located in the LRR region 9-17. These LRR regions are located on the extracellular domain of the receptor, with each LRR forming a short β strand and loop structure (8).

Mycoplasma Lipoproteins

Production of bacterial triacylated lipoproteins occurs through the addition of a diacylglycerol group to the sulfhydryl group of the cysteine. This is followed by cleavage of the signal sequence and addition of a third acyl chain to the now free N terminal group of the cysteine (77). In many bacterial species, such as mycoplasma, genes do not encode the last enzyme in the pathway, therefore producing diacylated lipoproteins which lack the third acyl chain (77).

Mycoplasma lipoproteins contain two ester-linked fatty acids bound to glycerol cysteine and are anchored into the membrane by an N-terminal (118). Studies have shown that both the lipid and peptide/protein components are needed for signaling through TLR interaction (93). The biochemical nature of macrophage activators from mycoplasma is vague, resulting in controversy over which lipoprotein components are involved in stimulating macrophages. The fact that mycoplasma express several lipoproteins only complicates the problem (74). Mycoplasma lipoproteins have been implicated in triggering inflammatory signaling and dendritic cell maturation through TLRs on macrophages (82, 109).

Lipoproteins are also phase variable proteins (31, 59). Phase variation is thought to aid in microbial survival by providing diverse subpopulations that can respond quickly to changing environmental conditions (21). These surface-bound phase variable lipoproteins are encoded by the VSA genes (46). Indeed, studies concluded there was more VSA phase variation in mycoplasma recovered from C57 wild-type mice than

immunodeficient C57 RAG knockout mice. This data strongly indicated mycoplasma phase variation is used as a mechanism for avoidance of the immune system (27).

M. fermentans MALP-2 Lipopeptide

In 1993, a macrophage activating material isolated from *M. fermentans* was discovered using TX-114 phase fractionation (87). Shortly after, this material was purified, revealing a macrophage activating lipopeptide designated MALP-2 (73). Further studies revealed the single copy *malp* gene of *M. fermentans* contained one open reading frame encoding the sequences for two membrane anchored products: MALP-404, the fully mature lipoprotein product, and MALP-2, which corresponded to the N-terminal region of MALP-404 (13, 24). The hypothesized pathway for MALP-2 formation included the proteolytic cleavage of a larger product to generate the 14 amino acid long lipopeptide. Indeed, extracellular proteolytic cleavage was seen in the sequencing of *M. fermentans* cultures subject to TX-114 phase fractionation and immunoprecipitation (24). Two released fragments were identified, the smaller one only containing the N-terminal portion of MALP-404 (24).

Interestingly, different strains of *M. fermentans* expressed different ratios of the two MALP-404 products despite consistent presence of full-length transcripts of the *malp* gene (25), perhaps indicating a role in lipoprotein variability and random proteolytic cleavage. Indeed, *in vitro* studies comparing MALP-2 and FLS-1, a synthetic lipopeptide derived from *Mycoplasma salivarium* that shares the same structure as MALP-2, showed

size and amino acid placement play a role in TLR interaction. Different FSL-1 lipopeptides with different amino acid substitution and lengths were used to determine whether a dipeptide structure was necessary for full biological activity. Lipopeptides containing 2 to 5 amino acids still exhibited strong stimulatory activity in HEK293 cells transfected with TLR2 and TLR6 whereas lipopeptides with only one amino acid showed marginally reduced activity (76). A substitution change made from the end Phe to Arg also reduced stimulatory activity and NFκB activity, suggesting hydrophobic interaction was also needed in TLR recognition (76). Thus, these studies show evidence that size, amino acid placement, and hydrophobicity could play major roles in MALP-2 lipoprotein variations and TLR interaction.

The chimeric orientation has also proven to be important in MALP-2 recognition. Generally, because of the Cahn-Ingold-Prelog rules where assignment of substituents of an asymmetric atom depends on the atomic mass of neighboring substituents, the molecule designation changes from S to R when lipid moiety is transferred to lipoprotein (65). Indeed, the R stereoisomer of MALP-2 has been found to be more than 100 times more active than the S stereoisomer in stimulating release of cytokines, chemokines, and nitric oxide (65).

Later studies confirmed this lipopeptide interacted with macrophages via the TLR2/6 heterodimer (74, 101, 102). Experiments stimulating alveolar macrophages from TLR 6 $^{-/-}$ mice with the lipopeptide MALP-2 showed some TNF-α production. In contrast, cytokine production was significantly higher in stimulated alveolar macrophages from

mice with functional TLR6 (102). This data indicates the TLR2/6 heterodimer is critical for MALP-2 recognition.

Other studies with MALP-2 have shown up-regulated MHC class II and stimulatory molecules during maturation of dendritic cells, as well as increased cytokine and stimulatory activity of T cells (62). Granulocyte function in neutrophils has also been shown to be up-regulated in *in vitro* studies (110). However, Pam3CysCK4, a synthetic triacylated lipopeptide that interacts with TLR2/1 showed the same neutrophil stimulatory effect but at higher concentrations (110).

MALP-2 has also been studied for use as an adjuvant. Efficient mucosal delivery of the HIV-1 Tat protein was seen when coupled to a synthetic MALP-2 lipopeptide, as evidenced by higher IgA antibody titers in inoculated Balb/c mice (10). Generally, antigens delivered mucosally alone are prone to degradation and rapid clearance, resulting in a poor immunogenic response (10). Further studies revealed the MALP-2 mucosal adjuvant promoted a T cell independent activation and maturation of mature B cells, mainly in the follicular zone. However, immunization of Balb/c RAG-1 -/- mice showed MALP-2 mediated stimulation of TLR2/6 was not able to circumvent the need for T cell help for efficient antigen specific B cell activation (11). Interestingly, B cell activation markers were increased in wild type Balb/c mice, as well as proliferation in the presence of the MALP-2 adjuvant (11), suggesting B cells are a potential target for MALP-2 mediated activation *in vivo*.

Improved intranasal immunization was also seen with co-inoculation of the lipopeptide MALP-2 and the live-attenuated measles virus (63). After intranasal/intrabronchial inoculation in cotton rats, the use of MALP-2 led to improved immunization of the live-attenuated measles virus through increased recruitment and infiltration of leukocytes in lung tissue (63).

MALP-2 has also been studied for adjuvant use with LPS since LPS use is limited by its toxicity and pyrogenicity (26). MALP-2 was shown to induce cross tolerance to LPS in pretreated MALP-2 macrophages; however, this protection is incomplete. This could be because NK cells are known to play an important role in LPS toxicity, and express very little to no TLR2 (36, 49). Thus, as seen with the HIV, measles virus, and LPS studies, MALP-2 could be an ideal adjuvant candidate.

MALP-2 Homologues

MALP-2 homologues have since been discovered in other species, *Mycoplasma agalactiae*, *Mycoplasma hyorhinis,* and *Mycoplasma gallisepticum* (74, 83, 66). *M. agalactiae* and *M. hyorhinis* MALP-2 homologues have only been compared to the *M. fermentans* MALP-2 through amino acid analysis and other structural comparisons (74, 66). It is unknown whether these homologues contain any functional homology with the MALP-2 lipopeptide.

Only *M. gallisepticum* has been shown to not play a critical role in the infection process (66). Ciliostasis and mycoplasma numbers were compared between a *M.*

gallisepticum strain lacking the homologue gene and the wild type in chicken embryo tracheal organ cultures. Studies indicated there was no difference for the time taken for ciliostasis to occur within the tracheal rings or in mycoplasma numbers, indicating the *M. gallisepticum* MALP-2 homologue is not a critical lipopeptide in typical *M. gallisepticum* disease pathogenesis (66).

A MALP-2 homologue was also derived from *M. pneumoniae* but its cytokine inducing activity was much weaker than MALP-2 or FSL-1 in stimulated HEK293 cells transfected with murine TLR 2 and TLR6 and THP-1 cells (50).

The macrophage activating lipoprotein moieties discovered in *Mycoplasma arthritidis* have been shown to act independently of the superantigen mitogen and stimulate macrophages through a TLR2 dependent pathway (22). These active lipoprotein moieties are present at higher concentrations in virulent rather than avirulent strains of *M. arthritidis*. They also induce dendritic cell maturation with increased expression of MHC II in C57Bl/6 and C3H/HeJ mice. However, these moieties may not be analogous to MALP-2 since MALP-2 has been shown to activate TLR2 independent of CD14 and the *M. arthitidis* components are CD14 dependent (22). CD14 has an analogous function to CD36, which serves as a facilitator or co-receptor for activation through TLR2/6. MALP-2 recognition has been shown to be CD36 dependent (48).

Dr. Kevin Dybvig from the University of Alabama recently isolated MALP-2 homologue transposon mutants of *M. pulmonis*. Mutants were isolated from a library of random transposon mutants in the site of transposon insertion identified by sequencing.

Colonies were singled out by tetracycline resistance from the transposon. Using wild type and transposon mutants of *M. pulmonis*, this project looks at the possible roles of the MALP-2 homologue in disease pathogenesis. Since the *M. fermentans* MALP-2 peptide plays a critical role in the infection process, it is hypothesized that the MALP-2 transposon mutants, FC1 and FC2, will have altered survival, disease pathogenesis, and/or macrophage stimulatory activity as compared to the parental *M. pulmonis* strain, CT.

CHAPTER 2

MATERIALS AND METHODS

M. pulmonis strains

M. pulmonis MALP-2 homologue transposon mutants were recently isolated thru transposon mutagenesis. Few strategies exist to genetically manipulate mycoplasmas. Plasmids have only been found to work in *Mycoplasma mycoides* and *Mycoplasma capricolum* (32). Of the two gram-positive bacterial transposons that can transpose into all mycoplasma species, Tn4001T was chosen for its smaller size (4.7 kb). The smaller size allows this transposon to be used as a cloning vehicle. Transformation with plasmids containing Tn4001T results in insertion of the transposon into the mycoplasma genome at various sites. Tetracycline served as the antibiotic marker to ensure the transposon was inserted into the mycoplasma genome (33).

Mutants were isolated from a library of random transposon mutants with the site of transposon insertion identified by sequencing. Colonies were singled out by tetracycline resistance from the transposon. The *M. pulmonis* strains used in this study are:

FC1, FC2—transposon Tn4001T is inserted into the MALP-2 homologue gene.

R40— the hsd and vsa site-specific recombinase, designated HvsR, is needed to catalyze DNA inversions that create the variability seen in lipoproteins (40). Transposon Tn4001T

was inserted into the variable surface antigen (VSA) locus for HvsR recombinase, which allows this strain to only produce 40 tandem repeat lipoproteins (40). This strain serves as a control.

CT— parent strain of R40 and FC1, FC2

WT—UAB CT strain of *M. pulmonis*. This virulent strain was included to serve as a positive control.

Mycoplasma cultures

Each *M. pulmonis strain* was grown in 500 ml of Hayflick's broth medium and allocated into 1 ml tubes. The mycoplasma were then frozen in 1 ml aliquots at -80° C. CFU counts were determined through serial dilutions (1/10) plated on mycoplasma Hayflick's agar plates. Plates were stored in a 37°C incubator. After 7 days, colonies were counted and CFUs/ml were calculated (47).

Mycoplasma media

The Hayflick's broth (250 ml) consisted of: 5.25 g of PPLO broth (Acumedia, Lansing, MI), 0.05 g DNA from herring sperm (Sigma-Alderich, St. Louis, MO), 500 μl phenol red, 50 ml donor equine serum (Hyclone, Logan, UT), 2.5 ml of 50% dextrose, and 192.25 ml of ultra pure water. Broth used for growth curve experiments was made with and without the antibiotic tetracycline (Sigma-Alderich). Also, 2.5 g of noble agar (Sigma-Alderich) was added when making mycoplasma Hayflick's agar plates for the

CFU counts in a 250 ml volume (45). 65 μl of 250 mg/ml Cefoperazone sodium salt (Sigma-Alderich) was added to the agar mixture to ensure only mycoplasma grew on the plates. All media was brought up to physiological pH.

PCR

For confirmation studies, growth curves, and CFU studies, PCR was used to confirm the transposon was still inserted in the MALP-2 homologue gene. Two end primers, 3460R and 3460F were used, as well as Tet6, the primer which bound to the transposon (Table 1). Primer sequences were obtained by Dr. Kevin Dyvbig from the University of Alabama in Birmingham, and were synthesized by Invitrogen (Carlsbad, CA). The primers were oriented in such a way that when the transposon stayed in the MALP-2 homologue gene, a 300bp band formed with primers Tet6 and 3460F. If the transposon was not inserted correctly or was absent, a 556bp band formed using the two end primers, 3460R and 3460F. Individual mycoplasma colonies were selected from the agar plates and placed in 0.5 ml tubes containing 20 μl of distilled water. A metal loop was used to retrieve colonies and was sterilized by passing through a flame between each colony. The tubes were then boiled in water for 5 minutes. From these samples, 2 μl was taken and added to another 0.5 ml Eppendorf tube containing 2 ul of each primer for a total of 4 ul, 20 μl of HotStar Taq Plus Master Mix (Qiagen, Mississauga, Canada), and 26 μl of sterile water. After thoroughly vortexing these tubes, they were placed in the Eppendorf Mastercycler (Westbury, NY). The protocol used for PCR amplification was:

5 minutes at 94°C, 30 seconds at 94°C, 45 seconds at 50°C, and 1 minute and 30 seconds at 65°C, repeat from step 2 for 39 cycles, 5 minutes at 65°C, and hold at 4°C. The polyacrylamide gel consisted of 1.2%-1.5% agarose I (Midwest Scientific, Valley Park, MO). One microliter of ethidium bromide was added to the gel mix consisting of 0.6g agar and 50 ml of 1x TBE. 1x TBE served as the running buffer. Tris-Glycine SDS Sample Buffer was mixed with mycoplasma samples as well as a 100 bp DNA ladder (Invitrogen).

RT-PCR

For confirmation studies, RT-PCR was used to determine whether the transposon interrupted the MALP-2 homologue gene function on an RNA level. For the preparation of samples, 50 ml of FC1, FC2, and CT were spun down at 10,000g for 20 minutes. Medium was poured off and the pellets were placed in 0.5 ml tubes. 100 μl of chloroform was added to all samples, before these samples were vortexed carefully for 30 seconds. The tubes were then allowed to sit at room temperature for 2-3 minutes. The tubes were then centrifuged for at 14,000 rpm for 30 minutes at 4°C. The aqueous phase was pipetted off and 500 μl of isopropyl alcohol was added to the tubes. Samples were then incubated at 80 °C for 15 minutes. After this incubation, the samples were centrifuged again at 14,000 rpm for 30 minutes at 4°C. At this point an RNA pellet formed at the bottom of the tubes. The aqueous phase was pipetted off and the pellet was washed with 75% ethanol. After washing, 1 ml of 75% ethanol was added to the sample and the tube was

inverted gently to break up the pellet. The tubes were then centrifuged again at 14,000 rpm for 30 minutes at 4°C before the 75% ethanol was pipetted off. The tubes were inverted with caps open under a hood for 20-30 minutes to allow the samples to dry. The pellets were then resuspended in 10 μl of DEPC water. The quantity of RNA was then measured through absorption at 260 nm and 280 nm by the GeneQuant II Spectophotometer (Pharmacia Biotech, Piscataway, NJ). The amount of RNA was calculated in μg/ml. The samples were then mixed with RNAse free DNAse (Ambion, Austin, TX) inactivated by phenol chloroform. These samples were placed in the Eppendorf Mastercycler at 37°C for 1 hour, followed by 75°C for 5 minutes before a 4°C hold.

For the RT-PCR reaction, 1 μg of sample was added to PCR tubes, along with 10.5 μl of DEPC water. These samples were placed in the Eppendorf Mastercycler at 70°C for 5 minutes. After five minutes, the RT reaction mix was added to the samples. The RT reaction mix consisted of: 5 μl of 5x reaction buffer (Promega, Madison, WI), 5 μl of 2.5 mM of dNTPs (Promega), 1 μl of RNasin (Promega), 1.5 μl of 100 μM oligo d(T) (Promega), and 1 μl of 200 u/μl MMLV RT enzyme (Promega). An extra tube with just DEPC water and the RT reaction mix was added to the experiment for a negative control. The protocol used for RNA conversion to cDNA using the Eppendorf Mastercycler was: 42°C for 15 minutes, then 37°C for 45 minutes, followed by 99°C for 5 minutes before a 4°C hold.. Once the RNA was converted to cDNA, PCR was used to

amplify the cDNA. This PCR protocol and placing the samples in a polyacrylamide gel were the same as for the PCR experiments mentioned previously.

Gene sequence analysis

The *M. fermentans* MALP-2 gene and the *M. pulmonis* MALP-2 homologue gene protein and DNA sequences were compared using the DNAMAN program (Quebec, Canada) (43). The peptide protein sequences were compared to other mycoplasma known to have MALP-2 homologues: *M. agalactiae, M. hyorhinis,* and *M. gallisepticum* (83, 74, 66). All sequences were found in the PubMed protein and nucleic acid database.

Animals

Female BALB/c mice from Harlan Sprague-Dawley (Indianapolis, IN) were used in all experiments and were between 8 to 12 weeks old. The animals were housed in sterile cages with sterile food and water provided without restriction. Before experiments mice were anesthetized with 0.1 to 0.3 ml of a mixture of ketamine and 0.5 ml of xylazine intramuscularly. Mice were infected intranasally with 20 μl of 2 x 10^5 CFUs/ml.

In vivo CFU counts

Lungs and spleens were minced and placed in 1 ml of Hayflick's broth. Nasal washes were collected by flushing the nasal passages with 1 ml of Hayflick's broth using a syringe. Lung samples were sonicated for 1 minute, while the spleen and nasal washes

were sonicated for 45 seconds. Serial dilutions (1/10) were made for each tissue sample and plated on Hayflick's agar plates. Plates were then placed in a 37°C incubator for seven days before colonies were counted.

Mycoplasma preparation for cell culture stimulation

30 ml of Hayflick's medium was inoculated with approximately 5 x 10^5 CFUs/ml of each mycoplasma strain. Media was placed in a 37° incubator and gently spun at 120 rpm for three hours. After this incubation, the media centrifuged for 10,000g for 20 minutes. Media was pippetted off, and the mycoplasma pellet was resuspended in cell culture media.

Cell culture

Two murine macrophage cell lines, J774A.1 (28), which are peritoneal macrophages, and MH-S (70), which are alveolar macrophages, and human endothelial kidney (HEK) cells transfected with TLR2 or the TLR2/6 heterodimer (31) were stimulated with whole organism from the mutant and wild type mycoplasma strains. A luciferase reporter plasmid containing the cDNA of TLR2 or TLR2 and TLR6 was used to transfect the cells. Levels of IL-6, IL-12, and TNF-α were measured using ELISA (BD Biosciences, San Diego, CA). All media used was acquired from Hyclone (Logan, UT). Both macrophage cell lines were stimulated with 5 x 10^5 CFUs/ml per *M. pulmonis*

strain, and 20 μg/ml of crude LPS extract from *Eschericha coli* (Sigma-Alderich). HEK cell lines were stimulated with 5 x 10^4 CFUs/ml as well as 20 μg/ml of crude LPS.

The MH-S cell line was grown with sterile RPMI-1640 w/ L-glutamine medium with 10% fetal bovine serum (FBS). In MH-S cell culture and maintenance, flasks were washed with sterile 1x Dulbecco's Phosphate Buffered Saline (PBS) followed by a 5 minute incubation with 1x Trypsin. After this incubation, RPMI medium was added to the flask and placed back in the incubator. For cell culture experiments the cells were instead transferred to a 50 ml conical tube instead of placed back in the incubator. The conical tube was then spun in a swinging bucket rotor for 10 minutes at 130 rpm. A white pellet of cells formed at the bottom of the tube. After the spin, medium was poured off and the cells were resuspended in 30 ml of medium. Cells were counted using a 1:10 dilution with a 0.4% solution of Trypan Blue (Sigma-Alderich). Cell count per ml per conical tube was calculated by multiplying the cell count by the dilution factor (1/10), then by the hemocytometer factor, 10^4. A 6 well plate system was used for stimulation; 100,000 cells were placed in each well. These plates were incubated for 24 hours at 37°C. Mycoplasma strains as well as LPS were added to the cells before plates were at spun for 900 rpm for 15 minutes at 28°C. The plates were then incubated again for 24 hours at 37°C.

The J774A.1 cell line was grown with sterile High Glucose DMEM consisting of 4mM L-glutamine, 4500 mg/L glucose with 10% FBS. For J774A.1 cell culture and maintenance, these cells were scraped off the flask before being transferred to a 50 ml

conical tube. Spinning and counting protocols were the same as the MH-S. A 6 well plate system was also used, except 1 million cells were placed in each well. Plates were incubated for two hours before adding agonists. Plates were then incubated again for 24 hours.

For HEK cell culture and maintenance, High Glucose DMEM medium with 10% FBS was used. Flasks were incubated for five minutes and washed with sterile PBS. Cell culture protocol was the same as used for the MH-S cells. IL-8 cytokine production was measured for HEK cell lines.

ELISA

The ELISA assay from BD Biosciences is based on the antibody sandwich approach (61). A capture antibody specific to the cytokine of interest (IL-6, IL-12, TNF-α, or IL-8) was diluted at 1:250 and bound to a 96 well plate with coating buffer. After an overnight incubation at 4°C, a series of washes with 1x PBS containing 0.05% Tween removed any unbound capture antibody. Blocking buffer was then added to the wells, binding to unbound sites in the wells not occupied by capture antibody. After another overnight incubation followed by a series of washes, standards and samples were added to the wells before another overnight incubation. Cytokine standards were diluted at 1/10, ranging from 1000 pg/ml to15.6 pg/ml. During this incubation, the capture antibody bound to its specific cytokine in the sample and standard. After another series of washes, a working detector was added to the wells. The working detector consists of a detection

antibody, which binds to the captured cytokine, and horseraddish peroxidase (HRP), a fluorescence tag that binds to the detection antibody. A substrate solution, tetramethylbenzine (TMB) peroxidase substrate from Moss Inc. (Pasadena, CA), was then added, reacting with the HRP and causing bound cytokine to glow blue. Absorbance was read and used to calculate the concentration of cytokine within each well at 630nm. Hydrochloric acid was then used to stop the TMB reaction with HRP, allowing the ELISA plate to be read at 430nm.

Statistical Analysis

Data were transformed using logarithmic transformation, and compared using one-way analysis of variance. Group differences were compared with a Tukey's Post Test. A p value of ≤ 0.05 was considered statistically significant. The Prism program (GraphPad, San Diego, CA) was used for its graphing and statistical functions.

CHAPTER 3

RESULTS

Transformation and confirmation of the FC1 and FC2 MALP-2 homologue mutants.

M. pulmonis MALP-2 homologue transposon mutants were recently isolated thru transposon mutagenesis by Dr. Kevin Dybvig's lab (University of Alabama, Birmingham, Ala.). Mutants were constructed by transforming the *M. pulmonis* strain CT with the transposon Tn4001T, which contained the plasmid pIVT-1 (33). pIVT-lac encodes the *arcA* gene, which encodes arginine deiminase in mycoplasma, along with a *lacZ* operon (96). Mycoplasma which were transformed resulted in tetracycline resistant colonies (96). Inverse PCR was used to determine which mycoplasma colonies had taken up the transposon into the MALP-2 homologue gene (33).

Once these mutants were singled out and sent to our lab, the transposon mutation was confirmed in the FC1 and FC2 mycoplasma strains by PCR and reverse transcriptase PCR (RT-PCR). In the PCR experiments, while strains CT and R40 had a single band at 556 bp, indicating an intact MALP-2 homologue gene (Figure 1A), only the 300 bp band was seen in the mutants, indicating the transposon was correctly inserted in the MALP-2 homologue gene (Figure 1B). While there was some reversion to the CT genotype seen in 66% of FC1 and FC2 bacterial colonies tested, this reversion was minor and could be eliminated with the addition of tetracycline to the medium (Figure 1C). PCR was done on

separate samples of the primer sets, mastermix, and the sterile water to rule out possible contamination of the parental strain CT in FC1 and FC2 samples. PCR revealed no contamination in the reagents (Figure 1D).

Results from the RT-PCR showed no MALP-2 expression at the RNA level in the FC1 and FC2 mutants (Figure 2). Thus, both FC1 and FC2 mutants were confirmed to have the transposon inserted in the MALP-2 homologue gene.

High homology exists between the M. pulmonis MALP-2 homologue and the M. fermentans MALP-2 peptide.

Homology studies were used to determine how analogous the *M. pulmonis* MALP-2 homologue was to the *M. fermentans* MALP-2 lipopeptide at both the DNA and protein level. If the *M. pulmonis* MALP-2 homologue has a high homology with the *M. fermentans* MALP-2, this could indicate a possible connection to analogous function. 50% analogous homology was seen in the DNA comparison of the MALP-2 gene (Figure 3), while protein homology was found to be lower at 25% (Figure 4). Both DNA and protein sequences were compared using the DNAMAN multiple alignment program. The *M. fermentans* MALP-2 lipopeptide was then contrasted with different strains of mycoplasma found to have a MALP-2 homologue (Table 2). All MALP-2 and MALP-2 homologue sequences were obtained from the PubMed nucleic acid database or the PubMed protein sequence database. Two of the strains compared, *M. agalactiae* and *M. hyorhinis,* had to have some analogous homology with the *M. fermentans* MALP-2

lipopeptide through sequential and amino acid sequence analysis. (74, 83). *M. gallisepticum* was also compared even though its MALP-2 homologue has been found to not play a critical role in pathogenecity in tracheal organ cultures (66). Protein homology between *M. gallisepticum* and the MALP-2 peptide was the lowest of any compared mycoplasma species. The protein homology for *M. pulmonis* was higher than *M. gallisepticum*, *M. agalactiae* and *M. hyorhinis*. However, there were gaps between the peptide's amino acid sequences in most mycoplamsa strains, as evident by one or two extra amino acids within the homologue peptides. The largest gap was found in the *M. pulmonis* strain, which measured six amino acids long (Table 2). The *M. pulmonis* MALP-2 homologue was also compared to the other MALP-2 homologues. The closest homology was with *M. hyorhinis* at almost 24% (Table 3). Thus, comparisons between the *M. fermentans* MALP-2 lipopeptide and the *M. pulmonis* MALP-2 homologue indicate greater homology than MALP-2 homologues from other mycoplasma species.

The MALP-2 homologue does not play a critical role in pulmonary clearance

To determine if the *M. pulmonis* MALP-2 homologue had an impact on survival after infection, three and fourteen day CFU studies were conducted with five Balb/c mice per group for each mycoplasma strain. Five mice were infected with broth to serve as the control group for both day three and day fourteen studies. CFU numbers were determined for lungs, spleen, and nasal passages from both infected and non-infected mice. The parental mycoplasma CT strain was compared in a three day CFU study with the

extensively studied UAB CT strain, also known as the WT strain (2-4, 15-20,23,24,27,33-35,40-42, 44-47,52-54, 59, 65,68,72,76,78,81,87-89,92,95-98,104,105,111-115,121,123,125), in order to determine whether the CT strain caused similar CFU counts as the WT. There was no difference seen between the CFU numbers from the CT and WT mycoplasma strains recovered from the lungs or nasal passages three days after infection (Figure 5A and 5B).

On day three, there was no difference seen between CFUs recovered from CT, R40, FC1, and FC2 lung and nasal wash samples after infection, indicating the MALP-2 homologue may not impact the rate of clearance (Figure 5A). No colony growth was seen in the spleen samples. Day fourteen studies revealed only mycoplasma growth in lung and nasal passages for the WT strain, indicating the parental CT strain, and thus the mutants derived from it, were not as virulent as the WT strain (Figure 5B and 5C). PCR analysis revealed the transposon was not spontaneously lost in mice infected with mutant strains FC1 and FC2 (Figure 6). Thus, the MALP-2 homologue may not affect CFU mycoplasma numbers in an *in vivo* environment after three days of infection.

Macrophage cytokine production is not impacted by the absence of the MALP-2 homologue

Cytokine production can be a major component in disease pathogenesis. In order to determine whether the *M. pulmonis* MALP-2 homologue was critical in stimulating cytokine production by macrophages, two different macrophage cell lines, J774A.1 and

MH-S cells, were inoculated with the mutant and wild type strains at approximately 5 x 10^5 CFUs/ml and incubated overnight. This experiment was repeated three times. Higher statistical significance for IL-6, IL-12, and TNF-α was seen with J774A.1 cells stimulated with FC1 and FC2 as compared to the parental CT and the mutant control R40 *M. pulmonis* strains.However, these cells were inadvertently stimulated with significantly more FC1 and FC2 CFUs than WT, CT, or R40. In these experiments, the average CFUs/ml used for stimulation for each mycoplasma strain was 2.76 x 10^5 CFUs for WT, 1.34 x 10^5 CFUs for R40, 8.02 x 10^4 CFUs for CT, 3.85 x 10^5 CFUs for FC1, and 2.00 x 10^5 CFUs for FC2. This could account for the increase in FC1 and FC2 cytokine production. This trend was seen with J774A.1 IL-6, IL-12, and TNF-α cytokine production (Figure 7A, 7B, and 7C). In MH-S cells, there was no difference seen in IL-6 cytokine production (Figure 7D). However, in the J774A.1 cells the absence of the MALP-2 homologue, viable *M. pulmonis* stimulated cytokine production was seen, indicating the MALP-2 homologue is not critical in stimulation of macrophage cell lines.

The M. pulmonis MALP-2 homologue is not critical for HEK TLR2 interactions

Recent studies from Dr. Simecka's lab have shown *M. pulmonis* interacts with TLR2 to mediate disease pathogenesis. To determine whether the MALP-2 homologue was required for TLR2 mediated stimulation of cells, HEK cells transfected with either TLR2 or the TLR2/6 heterodimer were inoculated with 5 x 10^4 CFUs/ml and incubated for 24 hours. Since IL-8 is produced by the human epithelial layer (117), the levels of this

cytokine in cell culture supernatants were measured. A reporter plasmid containing the cDNA of TLR2 or TLR2 and TLR6 was used to transfect the cells. There was a significant increase in IL-8 production between the HEK, TLR2, and TLR2/6 cell lines in all mycoplasma strains as well as in LPS (Figure 8). A crude form of LPS was used that included other lipoproteins besides LPS, allowing it to stimulate other receptors outside of TLR4. Thus, increased IL-8 production seen in TLR2 and TLR2/6 cells for all mycoplasma strains indicates the *M. pulmonis* MALP-2 homologue is not a critical lipoprotein in TLR2 interactions.

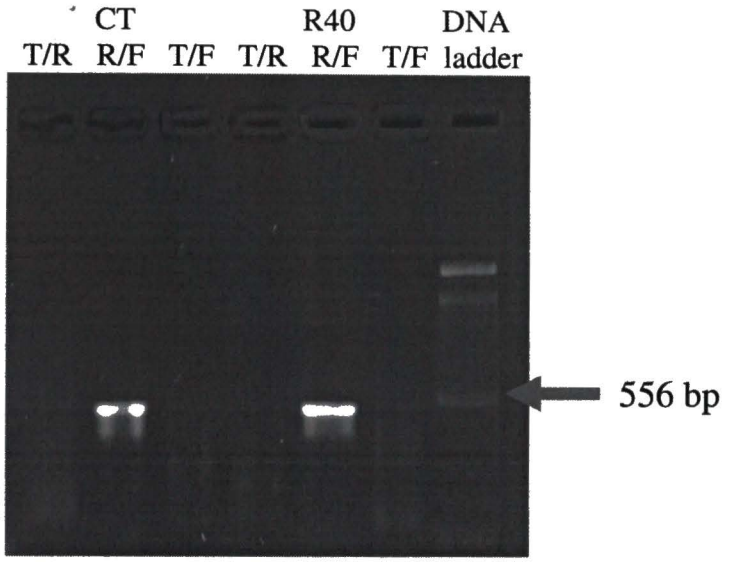
CHAPTER 4

ILLUSTRATIONS

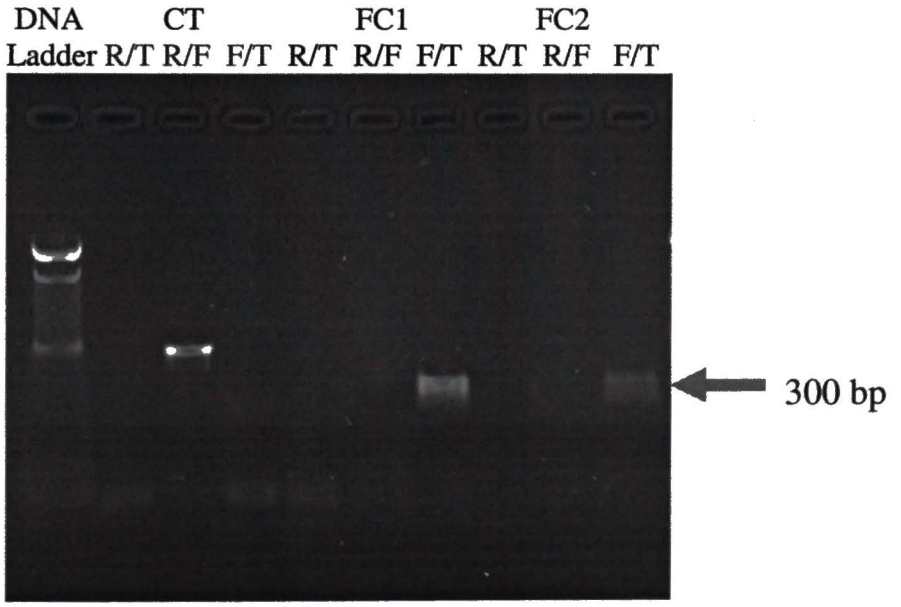
Location	Primer	Sequence
	3460R	AAATTATTGGAGTTGATTGAGCC
transposon Tn4001T	Tet6	AGGACTGCATAACATCTTCCGCAG
	3460F	AGAATTTGGTAAACAGGAAAAGC

Table 1. Primer design for detection of transposon. Three primers were used to determine whether the transposon maintained its position in the *M. pulmonis* MALP-2 homologue gene. The Tet6 primer bound to the transposon sequence. The 3460R primer bound to a MALP-2 homologue gene DNA sequence that was upstream of where the transposon would insert while the 3460F primer bound to a MALP-2 homologue gene DNA sequence that was downstream of where the transposon would insert. A 556 bp product was seen using primers 3460R and 3460F and indicated the transposon was not inserted in the MALP2 homologue. When the transposon was inserted a product of 300 bp was seen using primers Tet6 and 3460F.

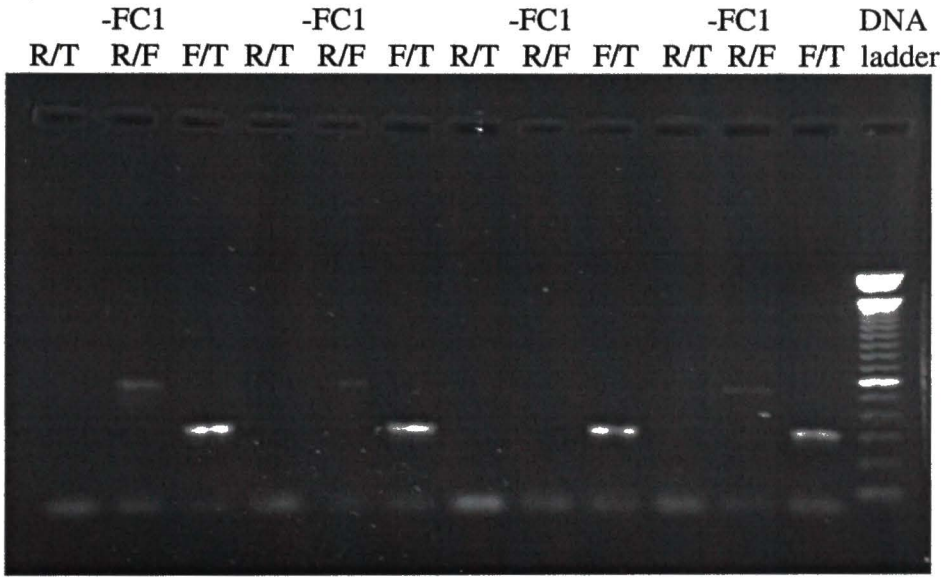
A)


CT
R40
DNA
T/R R/F T/F T/R R/F T/F ladder
556 bp

B)


DNA
CT
FC1
FC2
Ladder R/T R/F F/T R/T R/F F/T R/T R/F F/T
300 bp

C)



D)

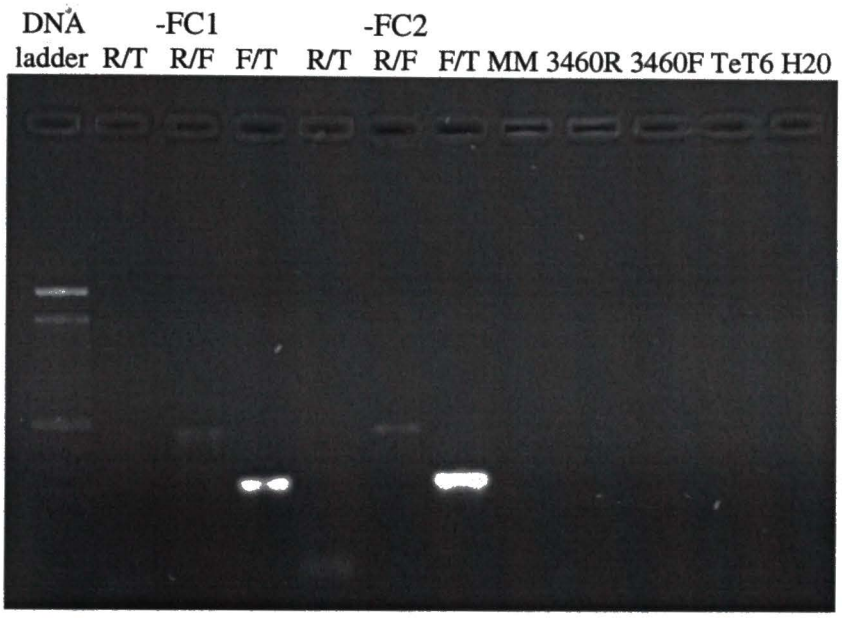


Figure 1. PCR results for confirmation of transposon insertion in the MALP-2 homologue gene. R stands for the 3460R primer, F stands for the 3460F primer, and T stands for the Tet6 primer. A) CT and R4 bands using the R/F primer combination as indicated by arrow at the 556 bp mark. B) A 300 bp band was seen using the Tet6 and 3460F primers, indicating the transposon was correctly inserted in the *M. pulmonis* MALP-2 homologue gene. C) FC1 samples represent FC2 bands as well. A faint 556 bp band was seen using the R/F primers along with the 300 bp F/T band in 66% of the bacterial colonies tested, but this reversion was minor and could be eliminated with the addition of 5 μl of 3 μg/μl tetracycline per 50 ml of medium D) Gel shows FC1 and FC2 representative samples as well as reagents run alone in order to check for contamination. N=12 for FC1 and FC2 mycoplasma strains.

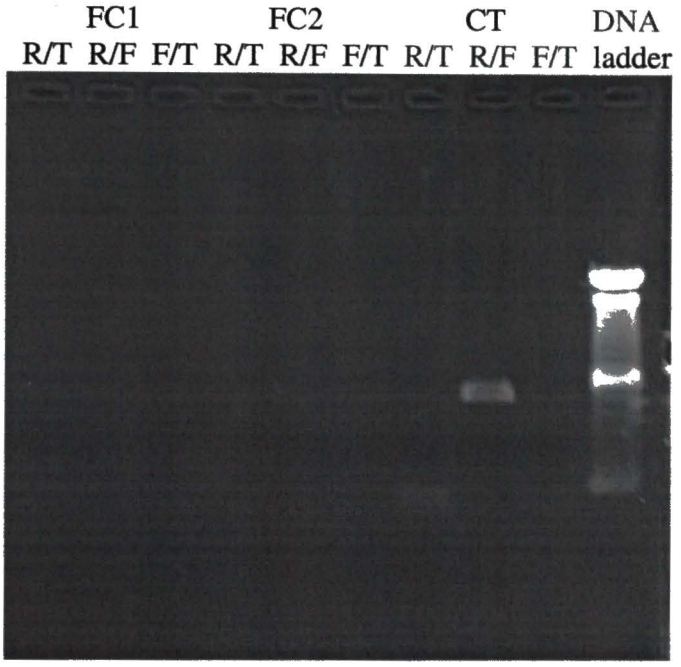
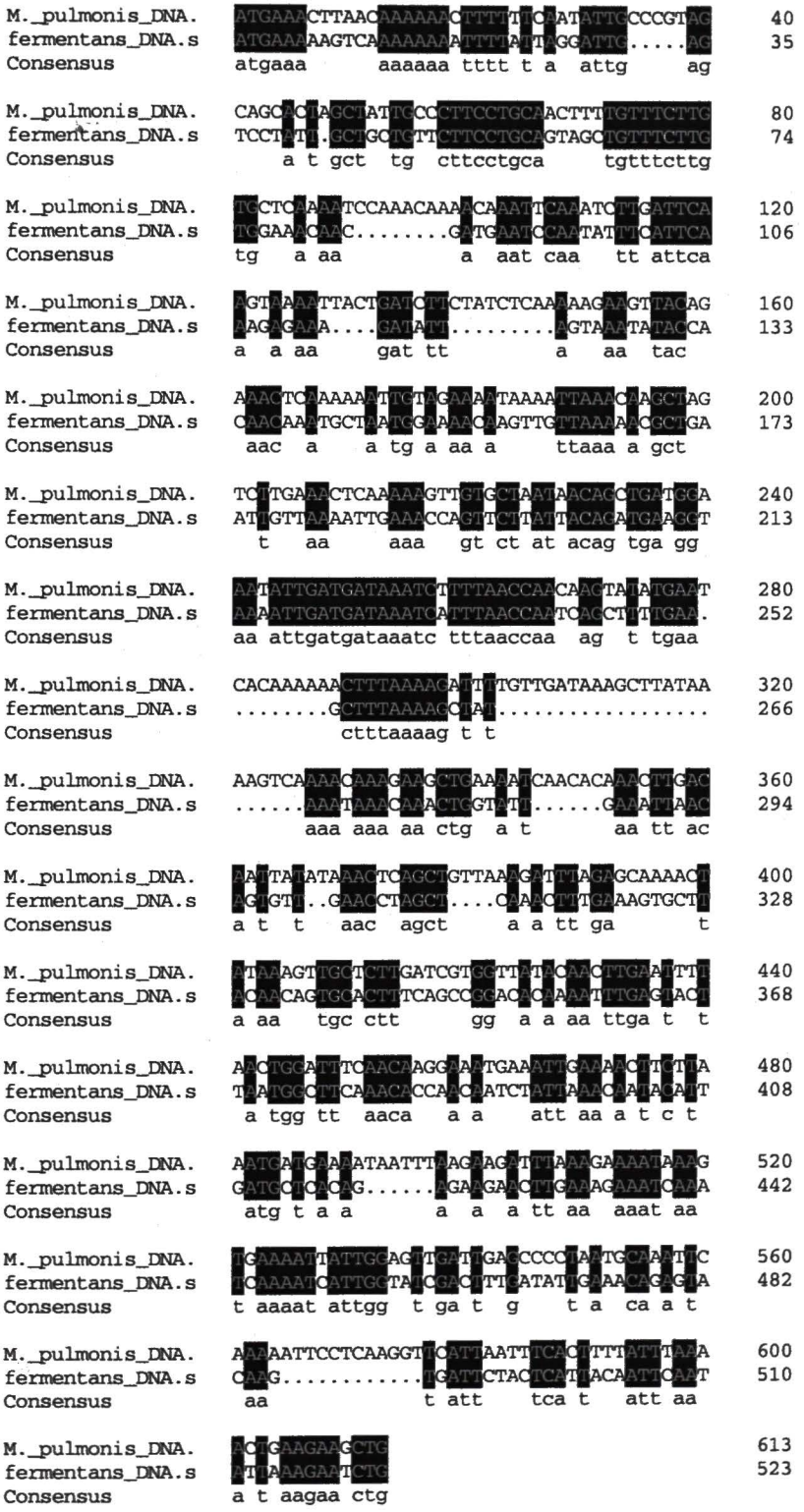


Figure 2. RT-PCR confirmation of the transposon interrupting MALP-2 homologue gene function in FC1 and FC2 mycoplasma strains. After FC1 and FC2 mycoplasma strains were confirmed through PCR, RT-PCR was used to determine whether the transposon effectively interrupted the MALP-2 homologue gene function. RT-PCR of FC1 and FC2 did not show the 300 bp band, comfirming the transposon inserted into the MALP-2 homologue gene, completely interrupting gene function at the RNA level.


M._pulmonis_DNA.
fermentans_DNA.s
Consensus
atgaaa aaaaaa tttt t a attg ag
a t gct tg cttcctgca tgtttcttg
tg a aa a aat caa tt attca
a a aa gat tt a aa tac
aac a a tg a aa a ttaaa a gct
t aa aaa gt ct at acag tga gg
aa attgatgatgataaatc tttaaccaa ag t tgaa
ctttaaaag t t
aaa aaa aa ctg a t aa tt ac
a t t aac agct a a tt ga t
a aa tgc ctt gg a a aa ttga t t
a tgg tt aaca a a att aa a t c t
atg t a a a a a tt aa aaat aa
t aaaat attgg t ga t g t a ca a t
aa t att tca t att
t aaagaagctg
a t aagaa ctg

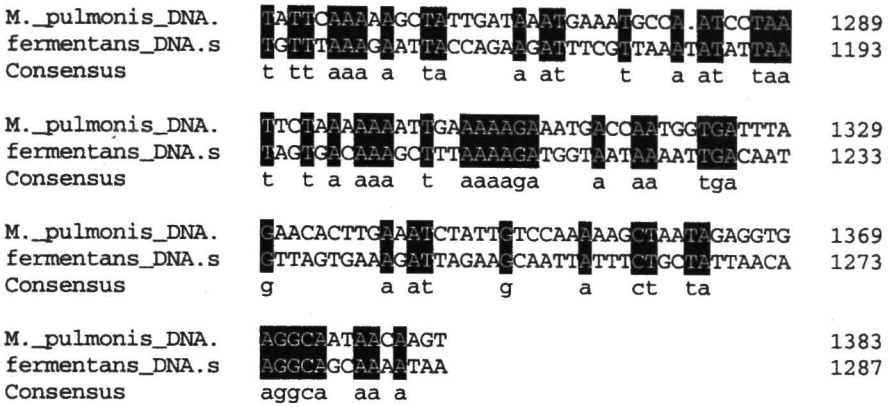


Figure 3. DNA homology between *M. pulmonis* MALP-2 homologue gene and *M. fermentans* MALP-2 gene. DNA homology comparison was achieved using the DNAMAN program. Gene sequences were retrieved through the PubMed nucleic acid database. *M. pulmonis* homology was at 50.07%.

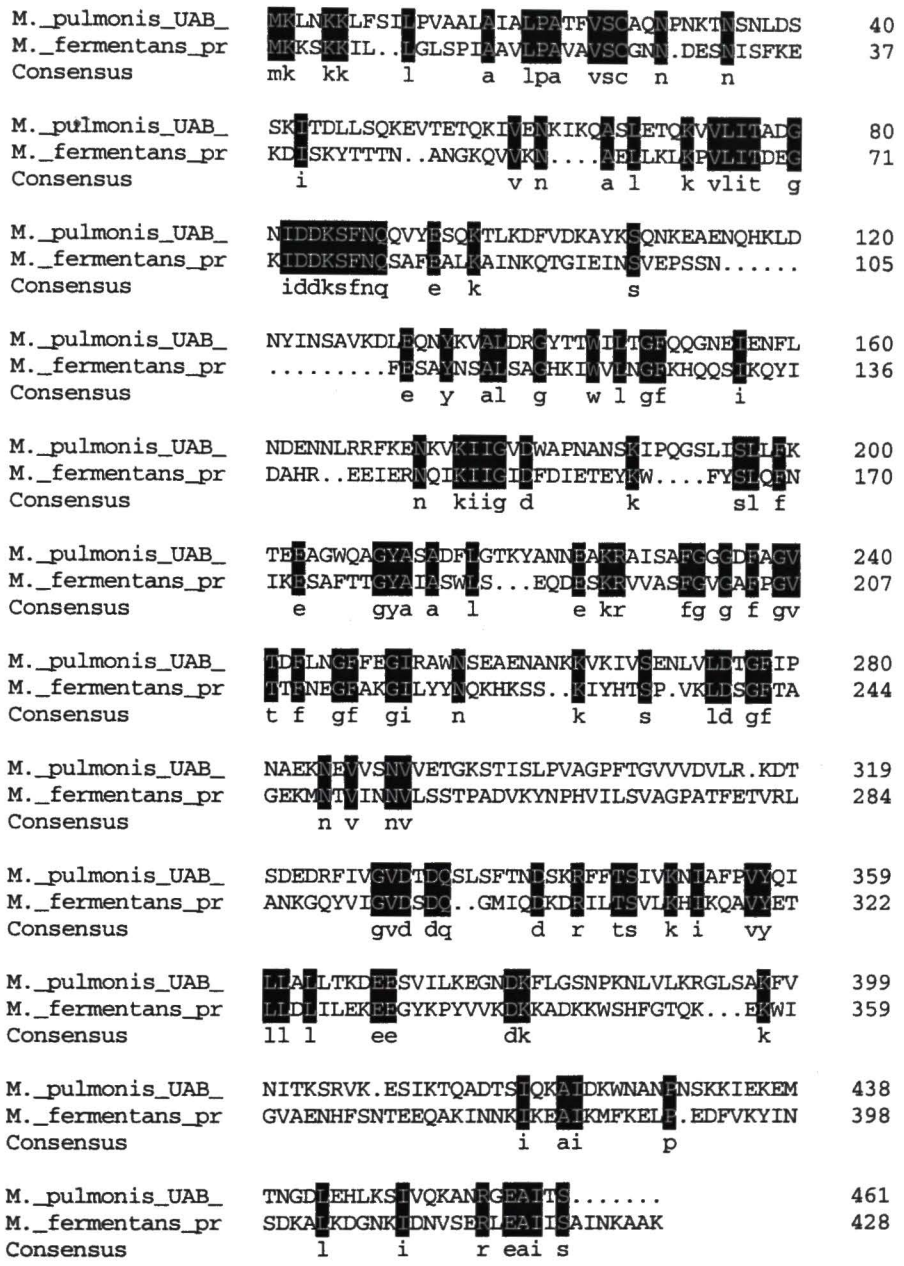


Figure 4. Protein Homology between *M. pulmonis* MALP-2 homologue gene and *M. fermentans* MALP-2 gene. Using the DNAMAN sequencing program, amino acid sequences were compared between the two strains. *M. pulmonis* protein homology was found to be 25.53%. Gene protein sequences were retrieved from the PubMed protein sequence database.

M. pulmonis 42.86%

```
Malp-2_peptide_a    CGN.....NDESNIS.FKEK    14
M._pulmonis_MALP    QGNEIENFLNDENNLRRFKEN    21
Consensus             gn       nde n   fke
```

M. hyorhinis 35.71%

```
Malp-2_peptide_a    CGNNDESNISFKEK    14
M._hyohinis_MALP    CKNLEHSEILYRLK    14
Consensus           c n   s i   k
```

M. agalactiae 26.67%

```
Malp-2_peptide_a    CG.NNDESNISFKEK    14
M._alagatia_MALP    CGFTHQASLVGLDEN    15
Consensus           cg     s     e
```

M. gallisepticum 18.75%

```
Malp-2_peptide_a    CGNNDESNISFKEK..    14
M._gallisepticum    CG..AASSLTYESSVQ    14
Consensus           cg    s
```

Table 2. Protein sequence homology between MALP-2 activating peptide and homologues. MALP-2 peptide sequences from different mycoplasma species were compared to the *M. fermentans* MALP-2 peptide using the DNAMAN program. Of the four, *M. pulmonis* was shown to have the highest homology. Peptide sequences were obtained from the PubMed protein sequence database.

M. fermentans 42.86%

```
M._pulmonis_MALP    QGNEIENFLNDENNLRRFKEN    21
M._fermentans_MA    CGN......NDESNIS.FKEK    14
Consensus            gn       nde n   fke
```

M. hyorhinis 23.81%

```
M._pulmonis_MALP    QGNEIENFLNDENNLRRFKEN    21
M._hyorhinis_MAL    ....CKN.LEHSEILYRLK..    14
Consensus                n l     l r k
```

M. gallisepticum 19.05%

```
M._pulmonis_MALP    QGNEIENFLNDENNLRRFKEN    21
M._gallisepticum    CGAASS..LTYESSVQ.....    14
Consensus            g       l   e
```

M. agalactiae 14.29%

```
M._pulmonis_MALP    QGNEIENFLNDENNLRRFKEN    21
M._alagatia_MALP    .....CGFTHQAS.LVGLDEN    15
Consensus                 f       l    en
```

Table 3. Protein sequence homology of *M. pulmonis* MALP-2 homologue activating peptide. MALP-2 peptide sequences from different mycoplasma species were compared to the *M. pulmonis* MALP-2 homologue peptide using the DNAMAN program. Of the four, *M. pulmonis* was shown to have the greatest homology with the *M. fermentans* MALP-2 peptide at 42.86%. *M. hyorhinis* has the second highest analogous homology sequence with the *M. pulmonis* homologue at 23.81%. Peptide sequences were obtained from the PubMed protein sequence database

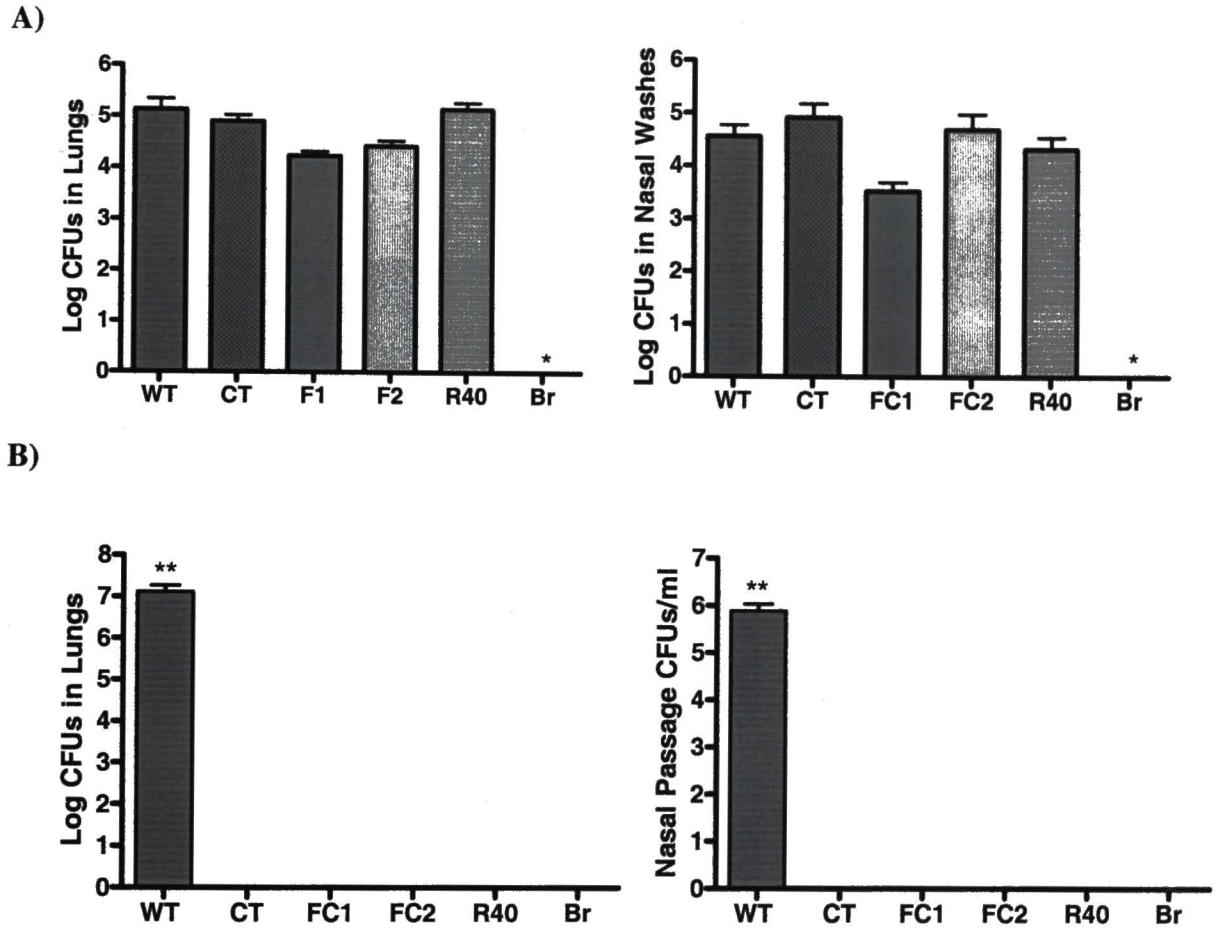


Figure 5. Lung and nasal passage CFUs in Balb/c mice infected with mycoplasma strains after 3, 7, and 14 days. Mice were infected with 2 x 10^5 CFUs/ml. A) Lung and nasal passage samples were taken after 3 days and B) 14 days in order to determine any differences in disease between the strains. No significance was seen in CFU growth between the *M. pulmonis* strains 3 days after infection. CFU growth was detected only for the WT strain at 14 days, indicating the CT mycoplasma strain and its mutant derivatives were not as virulent. Br = broth control group. * indicates all mycoplasma strains are significant when compared to the broth control group; ** indicates all mycoplasma strains are significant when compared to WT CFUs. Data was transformed using logarithmic transformation, and compared using one-way analysis of variance. Group differences were compared with a Tukey's Post Test. $p \leq 0.05$; N = 5 for 3 day studies; N = 10 for day 14 studies.

A)

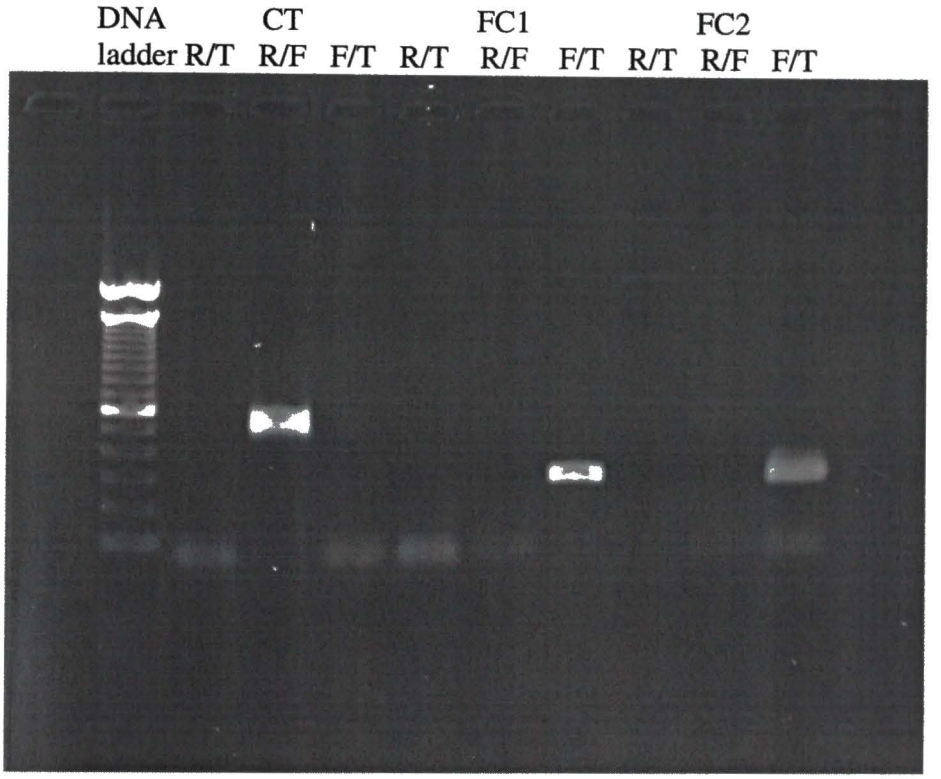


Figure 6. PCR analysis of day 3 lung CFUs. No reversions to the parental CT strain were found in FC1 and FC2 colonies grown from lung samples as seen with the growth curve *in vitro* colonies, indicating the transposon was not spontaneously lost in the MALP-2 homologue gene.
N = 8.

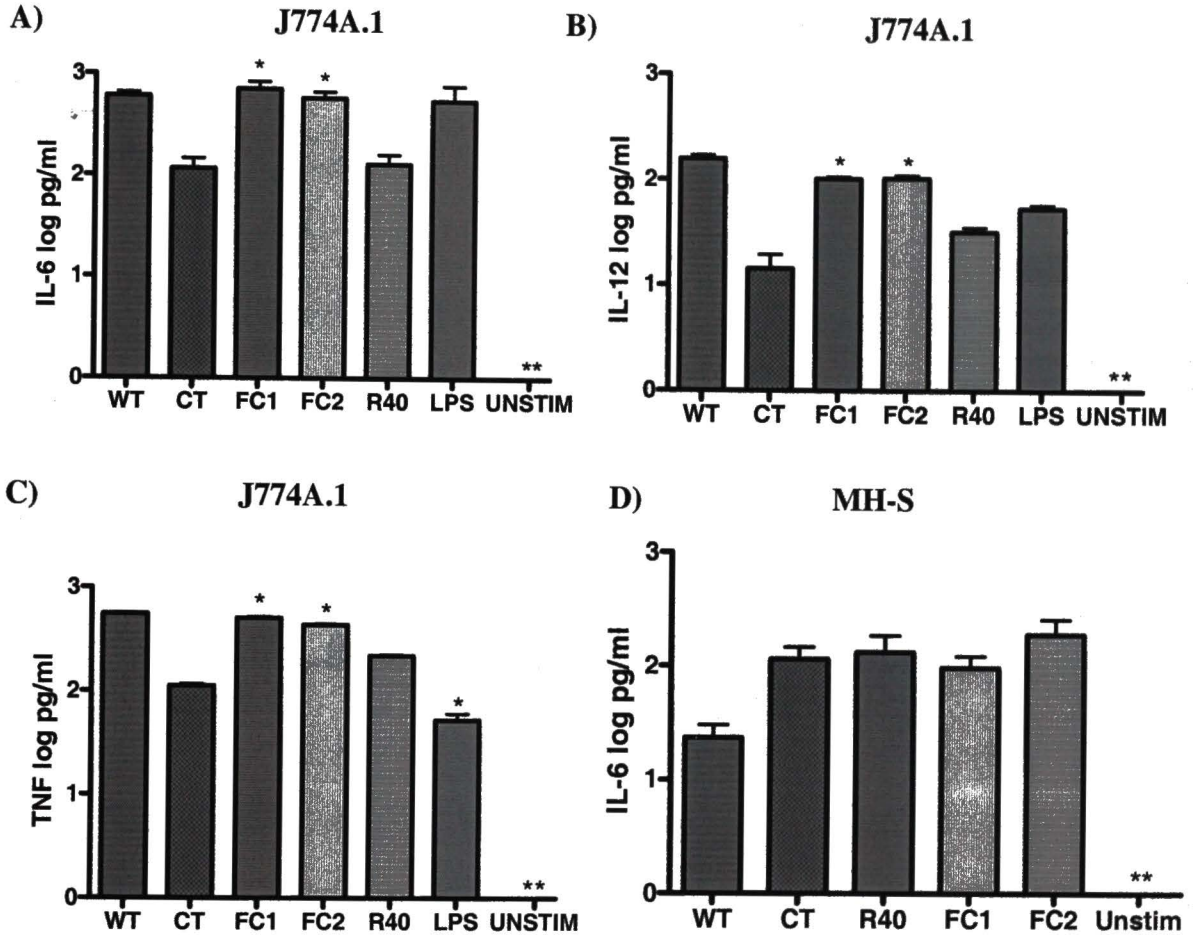


Figure 7. Cytokine production in J774A.1 and MH-S cells. $p \leq 0.05$. Both cell lines were inadvertently stimulated with significantly higher FC1 and FC2 CFUs. While this accounts for higher cytokine production, the fact there is any cytokine production at all is relevant. * indicates significance when compared to CT and R4; ** indicates significance when compared to all other experimental groups; *** indicates significance when compared to WT. J774A.1 cells stimulated with FC1 and FC2 have significantly higher A) IL-6, B) IL-12, and C) TNF-α cytokine production than cells simulated the parental strain CT. D) MH-S cells stimulated with FC2 had higher IL-6 cytokine production as compared to cells stimulated with CT. Both macrophage cell lines were stimulated with 5 x 10^4 CFUs per mycoplasma strain. Data was transformed using logarithmic transformation and compared using one-way analysis of variance. Group differences were compared with a Tukey's Post Test. N = 3

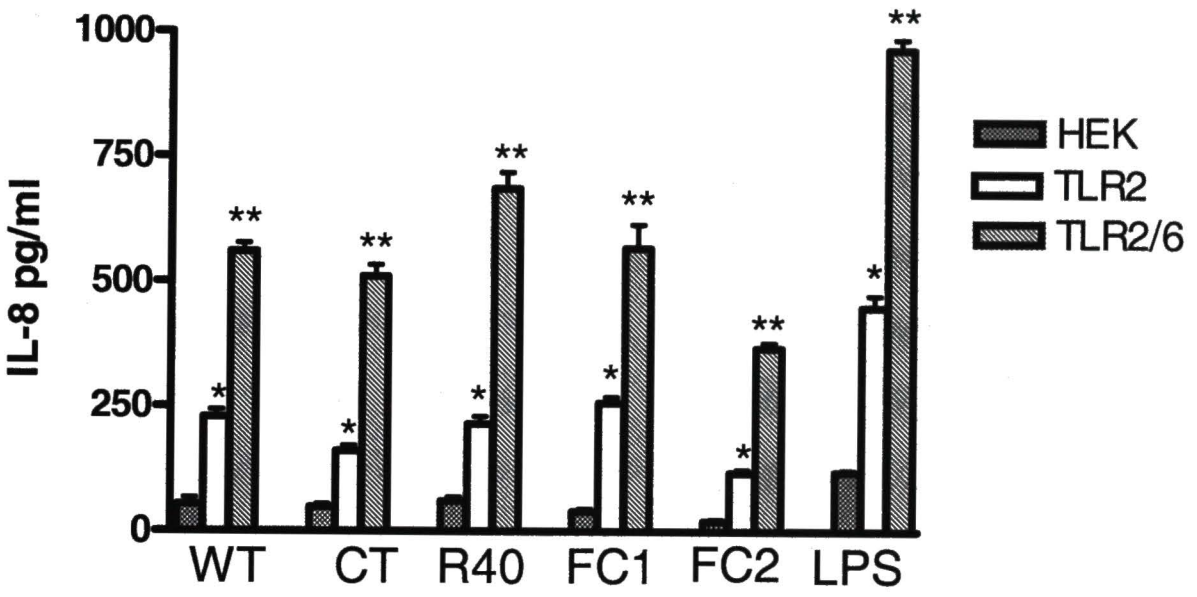


Figure 8. IL-8 cytokine differences between various mycoplasma strains. HEK cells were transfected with either TLR2 or the TLR2/6 heterodimer. Cells were inoculated with 5 x 10^4 CFUs and incubated overnight. An increase in IL-8 cytokine production was seen between the three cell lines for all the mycoplasma strains as well as LPS. * indicates significantly higher cytokine production as compared to HEK cells; ** indicates higher cytokine production as compared to HEK and TLR2 cells. Data was transformed using logarithmic transformation, and compared using one-way analysis of variance. Group differences were compared with a Tukey's Post Test. N = 3. $p \leq 0.01$

CHAPTER 5

DISCUSSION

Mycoplasma lipoproteins are thought to be a critical component to infection and disease progression. While phase variation might be used as a mechanism for avoidance of the immune system (21), the impact of lipoproteins in mycoplasma disease progression is not completely understood. Previous studies indicate membrane bound lipoprotein phase variation might aid microbial survival by providing diverse subpopulations that could respond quickly to changing environmental conditions (31, 59). While it is likely mycoplasma lipoproteins play an important role through recognition by TLR2, the specific *M. pulmonis* lipoproteins involved in this interaction are not known (65).

In 1995, a macrophage activating lipopeptide (MALP-2) from *M. fermentans* was discovered, and later studies confirmed this lipopeptide interacted with macrophages via the TLR2/6 heterodimer (73). MALP-2 homologues have since been discovered in other species, *M. agalactiae, M. hyorhinis,* and *M. gallisepticum.* (66, 74, 80). Studies involving *M. agalactiae* and *M. hyorhinis* have only focused on sequence homology, providing no evidence for a functional correlation with the *M. fermentans* MALP-2 lipopeptide (74, 80). In contrast, studies with *M. gallisepticum* showed the MALP-2 homologue may not play a critical role in the disease process, as evidenced by the lack of change in CFU growth in tracheal organ cultures (66). However, we are not aware of any studies examining the *in vivo* role of any mycoplasma MALP-2 homologue. Since the *M. fermentans* MALP-2 lipopeptide can stimulate phagocytes and cause cytokine production

(102), this project focused on examining the possible roles of the *M. pulmonis* MALP-2 homologue in mycoplasma disease and macrophage stimulation. This project is the first to examine the *M. pulmonis* MALP-2 homologue and determine any functional roles. Based on the known *in vitro* functions of the *M. fermentans* MALP-2 lipopeptide, we hypothesized the *M. pulmonis* MALP-2 homologue would have a role in infection and contribute to inflammatory responses through macrophage TLR2 interaction and cytokine production.

In this project, DNA and protein sequence comparison studies revealed considerable MALP-2 lipopeptide homology between *M. pulmonis* and *M. fermentans*. There was 50% homology between the *M. pulmonis* and *M. fermentans* MALP-2 DNA sequences while there was only a 25.53% protein match between the *M. fermentans* MALP-2 gene and the *M. pulmonis* homologue gene. Importantly, the *M. fermentans* MALP-2 amino acid peptide sequence, which has been shown to be the active part of the lipoprotein(25), had higher homology with the *M. pulmonis* homologue peptide sequence than with other mycoplasma species' MALP-2 homologues. However, in this project, the *M. pulmonis* MALP-2 homologue lipopeptide appears to not be critical for cell interactions and *in vivo* disease progression.

In vivo studies revealed the parental CT strain and the mutants derived from it were less virulent than the WT strain. *M. pulmonis* CFUs of either FC1, FC2, and R40 mutants or the CT parental strain were not recovered from lung, nasal passage, or spleen samples at day 14 after infection. CFUs from day 3 after infection studies comparing the

CT and WT strains revealed no differences in mycoplasma growth. However, CT is a mycoplasma strain derived from WT, and was originally passaged 12 times before used to create the FC1, FC2, and R40 mutants (40). During this passaging, the CT strain likely acquired mutations resulting in the loss of virulence.

Despite CT and WT strain differences, the *M. pulmonis* MALP-2 homologue may not be important in the infection process. In this study, mycoplasma strains FC1 and FC2 CFU numbers did not change in lung or nasal passage samples at day 3 after infection. PCR determined the transposon was still inserted in the MALP-2 homologue gene in 90% of the FC1 and FC2 colonies tested. Indeed, this project's *M. pulmonis* CFU studies were more consistent with previous *in vitro* CFU studies done with mutant *M. gallisepticum* strains, which did not have a functional MALP-2 gene (66). This would indicate the *M. pulmonis* MALP-2 homologue may not play an important role in mycoplasma infection in a pulmonary environment.

In this project, alveolar macrophages (MH-S) and peritoneal macrophages (J774A.1) were stimulated with both mutant and wild type strains of *M. pulmonis* in order to determine any impact the MALP-2 homologue may have on *in vitro* cytokine production. IL-6, IL-12, and TNF- α production were measured. In previous studies, IL-6 has been shown to mediate lymphocyte activation, antibody production, and class switching to IgG2b (55). IL-12 can induce NK activation as well as play a role in maturation of Th cells to Th1 cells, which can lead to enhancement of macrophage phagocytosis (54). Increase in TNF-α production activates the vascular endothelium and

permeability; this allows leukocytes, antibody, and complement to easily pass through tissue and reach the inflammation site (65). All three are implicated in the inflammation process.

The absence of the MALP-2 homologue seemed to affect IL-6, IL-12, and TNF-α production. Even though there was no significance seen with MH-S cells stimulated with the mycoplasma strains, significantly higher IL-6, IL-12, and TNF-α cytokine production was seen in J774A.1 cells stimulated with FC1 and FC2 as compared to CT and R40. However, these cells were inadvertently stimulated with significantly more FC1 and FC2 CFUs than WT, CT, or R40. This may account for the increase in FC1 and FC2 cytokine production. The possibility of no difference in cytokine production between CT, FC1, and FC2 may implicate the MALP-2 homologue is not crucial for cytokine production in an *in vitro* system, leading to the possibility other lipoproteins might have a more prominent role in macrophage cytokine production.

Stimulated human endothelial kidney (HEK) cells transfected with either TLR2 or TLR2 and TLR6 showed the MALP-2 homologue was not the primary lipopeptide that interacted with the TLR2 receptor. There was a significant increase in IL-8 production between HEKs, HEK TLR2 (TLR2), and HEKs TLR2/6 (TLR2/6) in all mycoplasma strains. IL-8 is another proinflammatory cytokine that recruits neutrophils to the site of infection (117). Cells stimulated with LPS also had higher IL-8 production, but this was expected since the crude form of LPS used in these experiments contained other bacterial proteins besides lipopolysaccaharide. These findings were indicative to previous studies

showing the *M. fermentans* MALP-2 lipopeptide interacted with the TLR2/6 heterodimer, which led to an increase in IL-6 and TNF-α production (102). This would suggest the primary *M. pulmonis* lipoproteins involved in TLR2 interactions are diacylated. Overall, the higher increase in IL-8 production from stimulation of all strains of mycoplasma used in the study indicates loss of the MALP-2 homologue does not affect TLR2 mediated cell stimulation, leading to the conclusion that other components, such as lipoproteins, are involved in TLR2 mediated cell stimulation.

Future studies include synthesizing the *M. pulmonis* MALP-2 homologue for use in stimulating macrophage cell lines; this would confirm whether the MALP-2 homolgue is indeed not capable of stimulating cytokine production by cells. In order to confirm the *M. pulmonis* MALP-2 homolgue is not needed for TLR2 interaction, 3 day infection studies should be done using TLR2 $^{-/-}$ mice. Growth curve studies should also be addressed to determine whether the absence of the MALP-2 homologue affects bacterial growth. *M. agalactiae* and *M. hyorhinis* CFU studies should also be undertaken in order to see whether their MALP-2 homologues have any functional attributes akin to the *M. fermentans* MALP-2 lipopeptide, or whether they follow the trend set by the *M. gallisepticum* and *M. pulmonis* MALP-2 homologues.

In summary, this project is the first to study the potential roles the *M. pulmonis* MALP-2 homologue might have in disease pathogenesis. Studies revealed no differences in lung and nasal passage mycoplasma numbers or *in vitro* cytokine production, suggesting the MALP-2 homologue did not play a critical role in the *M. pulmonis*

infection process. HEK cell culture studies further indicated this with a significant increase in cytokine production for all strains of mycoplasma in the presence of TLR2 and TLR2/6. These studies implicate other lipoproteins, besides the MALP-2 homologue, are needed for TLR2 interactions and cytokine production during the infection process.

CHAPTER 6

REFERENCES

1 **Ashman, R., Kay, P., Lynch, D., and J. Papadimitriou.** *Murine candidiasis*: sex differences in the severity of tissue lesions are not associated with the levels of serum C3 and C5. Immun. Cell Biol., 1991, 69, 7-10.

2 **Aurora, A., Baluk, P., Zhang, D., Sidhu, S., Dolganov, G., Bashaum, C., and McDonald, D., and N. Killeen**. Immune complex-dependent remodeling of the airway vasculature in response to a chronic bacterial infection. J. of Immunology, 2005, 175, 6319-6326.

3 **Bakshi, C., Malik, M., Carrico, P., and T. Sellati.** T-bet deficiency facilitates airway colonization by *Mycoplasma pulmonis* in a murine model of asthma. J. of Immunology, 2006, 177, 1786-1795.

4 **Baluk, P., Tammela, T., Ator, E., Lyubynska, N., Achen, M., Hicklin, D., Jeltsch, and M., P., T., Pytowski, B., Stacker, A., et al.** Pathogenesis of persistent lymphatic vessel hyperplasia in chronic airway inflammation. J. Clin. Invest., 2005, 115, 247-257.

5 **Bas., S., Neff, L., Vuillet, M., Spenato, U., Seva, T., Matsumoto, M., and C. and Gabay**. The proinflammatory response to *Chlamydia trachomatis* elementary bodies in human macrophages is partly mediated by a lipoprotein, the macrophage infectivity potentiator, through TLR2/TLR1/TLR6 and CD14. J. of Immunology, 2008, 180(2), 1158-1168.

6 **Baseman, J.B.a.J.G.T.** Mycoplasmas: sophisticated, reemerging, and burdened by their notoriety. Emerg Infect Dis., 1997, 3(21).

7 **Bebear, C., and S. Pereyre.** Mechanisms of Drug Resistance in *Mycoplasma pneumoniae*. Curr. Drug Targets Infect. Disord., 2005, 5(3), 263-271.

8 **Bell, J., Mullen, G., Leifer, C., Massoni, A., Davies, R., and D. Segal.** Leucine-rich repeats and pathogen recognition in Toll-like receptors. Trends Immunol., 2003, 164, 554-557.

9 **Beutler, B.** Inferences, questions, and possibilities in Toll-like receptor signaling. Nature, 2004, 430, 257-263.

10 **Borsutzky, S., Fiorelli, V., Ebensen, T., Tripiciano, A., Rharbaoui, F., Scoglio, A., Link, C., Nappi, F., Morr, M., Butto, S., Cafaro, A., Muhlradt, P., Ensoli, B., and C. Guzman.** Efficient mucosal delivery of the HIV-Tat protein using the synthetic lipopeptide AMLP-2 as adjuvant. Eur. J. Immunology, 2003, 33, 1548-1556.

11 **Borsutzky, S., Kretschmer, K., Becker, P., Muhlradt, P., Kirschning, C., Weiss, S., and C. Guzmen.** The mucosal adjuvant macrophage-activating lipopeptide-2 directly stimulates B lymphocytes via the TLR2 without the need of accessory cells. J. Immunology, 2005, 174, 6308-6313.

12 **Buwitt-Beckmann, U., Heine, H., Weismuller, K., Jung, G., Brock, R., Akira, S., and A. Ulmer.** TLR1- and TLR6-independent recognition of bacterial lipopeptides. J. Biological Chemistry, 2006, 281(14), 9049-9057.

13 **Calcutt, M., Kim, M., Karpas, A., Muhlradt, P., and K. Wise.** Differential posttranslational processing confers intraspecies variation of a major lipoprotein and a macrophage-activating lipopeptide of *Mycoplasma fermentans*. Infection and Immunity, 1998, 67(2), 760-771.

14 **Callaham, C., and F. Wolinsky.** Hospitalization for pneumonia among older adults. J. Gerontol. Ser., 1996, 51, M276-M282.

15 **Cartner, S., Lindsey, R., Gibbs-Erwin, J., Cassell, G., and J. Simecka.** Roles of Innate and Adaptive Immunity in Respiratory Mycoplasmosis. Infection and Immunity, 1998, 66(8), 3485-3491.

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

17 **Cassell, G., and J. McGhee.** Pathologic and immunologic response of nude mice following intranasal inoculation of *Mycoplasma pulmonis*. J. Reticuloendothel. Soc., 1975, 18(342).

18 **Cassell, G., and J. Davis.** Protective effect of vaccination against *Mycoplasma pulmonis* respiratory disease in rats. Infect Immun, 1978, 21, 69-75.

19 **Chambaud, I., Heilig, R., Ferris, S., Barbe, V., Samson, D., Galisson, F., , Moszer, I., Dybvig, K., Wroblewski, H., Viari, A., Rocha, E., and A. and Blanchard.** The complete gemone sequence of the murine respiratory pathogen *Mycoplasma pulmonis*. Nucleic Acids Research, 2001, 29(10), 2145-2153.

20 Chan, S., Perussia, B., Gupta, J., Kobayashi, M., Pospisil, M., Young, H., Wolf,S., Young, D., Clark, S., and G. Trinchieri. Induction of interferon γ production by natural killer cell stimulatory factor: characterization of the responder cells and synergy with other inducers. J. Exp. Med., 1991, 173, 869.

21 Chuang, T.H., and Ulevitch, R.J. Cloning and characterization of a sub-family of human toll-like receptors: hTLR7, hTLR8 and hTLR9. Eur. Cytokine Netw., 2000, 11, 372-378.

22 Cole, B., Mu, H., Pennock, N., Hasebe, A., Chan, F., Washburn, L., and M. Peltier. Isolation and purification of macrophage- and dendritic- cell-activating components from *Mycoplasma arthitidis*: association with organism virulence and involvement with toll-like receptor 2. Infection and Immunity, 2005, 73(9), 6039-6047.

23 Davidson, M., Davis, J., Lindsey, R., and G. Cassell. Clearance of Different Strains of *Mycoplasma pulmonis* from the Respiratory Tract of C3H/HeN Mice. Infection and Immunity, 1988, 56(8), 2163-2168.

24 Davis, J.K., Parker, R.F., White H., Dziedsic D., Taylor G., Davidson M.K., Cox N.R., and G.H. Cassell. Strain differences in susceptibility to murine respiratory mycoplasmosis in C57BL/6N and C3H/HeN mice. Infect. Immun., 1985, 50, 647-654.

25 Davis, K., and K. Wise. Site-specific proteolysis of the MALP-404 lipoprotein determines the release of a soluble selective lipoprotein-associated motif-containing fragment and alteration of the surface phenotype of *Mycoplasma fermentans*. Infection and Immunity, 2001, 70(3), 1129-1135.

26 Deiters, U., Gumenscheimer, M., Galanos, C., and P. Muhlradt. Toll-like receptor 2-and 6- mediated stimulation by macrophage-activating lipopeptide 2 induces lipopolysaccaharide (LPS) cross tolerance in mice, which results in protection from tumor necrosis factor alpha but in only patial protection from lethal LPS doses. Infection and Immunity, 2003, 71(8), 4456-4462.

27 Denison, A., Clapper, B., and K. Dybvig. Avoidance of the Host Immune System through Phase Variation in *Mycoplasma pulmonis*. Infection and Immunity, 2005, 73(4).

28 Dietz, J., and B. Cole. Direct activation of the J774.1 Murine macrophage cell line by *mycoplasma arthritidis*. Infection and Immunity, 1982, 37(2), 811-819.

29 **Dobutsu, J.** Seromonitoring of laboratory mouse and rat colonies for common murine pathogens. Lab Anim. Sci., 1979, 28(2), 297-306.

30 **Du, X., Poltorak,A., Wei,Y., and Beutler,B.** Three novel mammalian toll-like receptors: gene structure, expression, and evolution. Eur. Cytokine Netw., 2000, 11, 362-371.

31 **Duesburg, U., von dem Busscue, A., Kirschning, C., Miyake, K., Sauerbruch.** T Cell activation by synthetic lipopeptides of the hepatitis C virus (HCV)—core protein is mediated by toll like receptors (TLRs) 2 and 4. Immunology Letters, 2002, 84, 89-95.

32 **Dybvig, K., and L. Voelker.** Molecular Biology of Mycoplasmas. Annu. Rev. Microbiol, 1996, 50, 25-27.

33 **Dybvig, K., French, T., and L. Voelker.** Construction and use of derivatives of transposon Tn4001T that function in *Mycoplasma pulmonis* and *Mycoplasma arthritidis*. J. Bacteriology, 2000, 182(15), 4343-4347.

34 **Faulkner, C., Simecka, J., Davidson, M., Davis, J., Schoeb, T., Lindsey, J., and M. Everson.** Gene expression and production of tumor necrosis factor alpha, interleukin 1, interleukin 6, and gamma interferon in C3H/Hen and C57BL/6N mice in acute *Mycoplasma pulmonis* disease. Infection and Immunity, 1995, 63, 4084-4090.

35 **Ferretti, S., Bonneau, O., Dubois, G., Jones, C., and A. Trifilieff.** IL-17, produced by lymphocytes and neutrophils, is necessary for lipopolysaccharide-induced airway neutrophilia: IL-15 as a possible trigger. J. Immunology, 2003, 170, 2106-2112.

36 **Flo, T., Halaas, O., Torp, S., Ryan, L., Lien, E., Dybdahl, B., Sundan, A., and T. Espevik.** Differential expression of toll-like receptor 2 in human cells. J. Leukoc. Biol., 2001, 69, 474-481.

37 **Fraser, C.M., J. D. Gocayne, O. White, M. D. Adams, R. A. Clayton, R. D. Fleischmann, C. J. Bult, A. R. Kerlavage, G. Sutton, J. M. Kelley, et al.** The minimal gene complement of *Mycoplasma genitalium*. Science, 1995, 270, 397-403.

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

39 Gordon, H., and G. Rosenthal. The relationship of gender and in-hospital death: increased risk of death in men. Med. Care, 1999, 37, 318-324.

40 Gumulack-Smith, J., Teachman, A., Tu, H., Simecka, J., Lindsey, R., and K. Dybvig. Variation in the surface proteins and restriction enzyme systems of *Mycoplasma pulmonis* in the respiratory tract of infected rats. Mol. Microbiol., 2001, 40, 1037-1044.

41 Haas, H., Faclone, F., Holland, M., Schramm, G., Haisch, K., Gibbs, B., Bufe, A.,and M. Schlaak. Early interleukine-4: its role in the switch towards a Th2 response and IgE-mediated allergy. Int. Arch. Allergy Immunol, 1999, 119(86).

42 Henderson, I.R., Owen, P., and J.P. Nataro. Molecular switches—the ON and OFF of bacterial phase variation. Mol. Microbiol., 1999, 33, 919-932.

43 Herron, P., Hughes, G., Fielding, S., and P. Dyson. Transposon Express, a software application to report the identity of insertions obtained by comprehensive transposon mutagenesis of sequences genomes: analysis of the preference for in vitro Tn5 transposition into GC-rich DNA. Nucleic Acids Research, 2004, 32(14), e113.

44 Hickman-Davis, J., Lindsey, R., Zhu, S., and S. Matalon. Surfactant protein A mediated mycoplasmacidal activity of alveolar macrophages. American J. of Physiology, 1998, 274, 270-277.

45 Hickman-Davis, M., S. M., Gibbs-Erwin, J., and J.R. Lindsey. Depletion of Alveolar Macrophages Exacerbates Respiratory Mycoplasmosis in Mycoplasma-Resistant C57BL Mice but Not Mycoplasma-Susceptible C3H Mice. Infection and Immunity, 1997, 65(6).

46 Hodge, L., Marinaro, M., Jones, H., McGhee, J., Kiyono, H., and J. Simecka. Immunoglobulin A (IgA) responses and IgE-associated inflammation along the respiratory tract after mucosal but not systemic immunization. Infection and Immunity, 2001, 69(4), 2328-2338.

47 Hodge, L., and J. Simecka. Role of upper and lower respiratory tract immunity in resistance to mycoplasma respiratory disease. J. of Infectious Diseases, 2002, 186, 290-294.

48 Hoebe, K., Rutschmann, S., Du, X., Mudd, S., Crozat, K., Sovath, S., Shamel, L., Hartung, T., Zahringer, U., and B. Beutler. CD36 is a sensor of diacylglycerides. Nature, 2005, 433, 523-527.

49 Hornung, V., Rothenfusser, S., Britsch, S., Krug, A., Jahrsdorder, T., Giese, T., Endres, S., and G. Hartmann. Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. J. Immunology, 2002, 168, 4531-4537.

50 Into, T., Dohkan, J., Inomata, M., Nakashima, M., Shibata, K., and K. Matsushita. Synthesis and characterization of a dipalmitoylated lipopeptide derived from paralogous lipoproteins of *Mycoplasma pneumoniae*. Infection and Immunity, 2007, 75(5), 2253-2259.

51 Johansson, K.-E., and B. Pettersson. Taxonomy of Mollicutes. In Razin, S., and R. Herrmann ed. Molecular Biology and Pathogenicity of Mycoplasmas, pp. 1-30 (Kluwer Academic, Plenum, New York, 2002).

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

53 Jones, H., and J. Simecka. T lymphocyte responses are critical determinants in the pathogenesis and resistance to mycoplasma respiratory disease. Front. Biosci., 2003, 8, D930-D945.

54 Kaliński P, H.C., Snijders A, Snijdewint FG, and M. Kapsenberg. IL-12-deficient dendritic cells, generated in the presence of prostaglandin E2, promote type 2 cytokine production in maturing human naive T helper cells. J. Immunology, 1997, 159(1), 28-35.

55 Kishimoto T, A.S., Narazaki M, and T. Taga. Interleukin-6 family of cytokines and gp130. Blood, 1995, 86, 1243-1254.

56 Kohn, D., and C. Clifford. Biology and Disease of Rats. Laboratory Medicine, 2nd Ed., pp. 121-165 (Academic Press, N.Y., 2002).

57 Kraft, M., Cassell, G., Henson, J., Watson, H., Williamson, J., M armion, B., and Gaydos, C., and R. Martin. Detection of *Mycoplasma pneumoniae* in the Airways of Adults with Chronic Asthma. Am. J. Respir. Crit. Care Med., 1998, 158(3), 998-1001.

58 Kwon, D., and C. Lu. Polyamine effects on antibiotic susceptibility in bacteria. Antimicrobial Agents and Chemotherapy, 2007, 51(6), 2070-2077.

59 Lai., W., Linton, G., Bennet, M., and S. Pakes. Genetic control of resistance to *Mycoplasma pulmonis* infection in mice. Infection and Immunity, 1993, 61(11), 4615-4621.

60 Lein, E., Sellati, T.J., Yoshimura, A., Flo, T.H., Rawadi, G., Finberg, R.W., Carroll, J.D., Espevik, T., Ingalls, R.R., Radolf, J.D., and D.T. Golenbock. Toll-like receptor 2 functions as a pattern recognition receptor for diverse bacterial products. J. Bio. Chem., 1999, 274.

61 Lewis, V., Thacker, W., and S. Mitchell. Enzyme-linked immunosorbant assay for chlamydial antibodies. . J. of Clinical Microbiology, 1977, 6(5), 507-510.

62 Link, C., Gavioli, R., Ebensen, T., Canella, A., Reinhard, E., and C. Guzman. The toll-like receptor ligand MALP-2 stimulates dendritic cell maturation and modulates proteasome composistion and activity. Eur. J. Immunology, 2004, 34, 899-907.

63 Luhrmann, A., Tschernig, T., Pabst, R., and S. Niewiesk. Improved intranasal immunization with live-attenuated measles virus after co-inoculation of the lipopeptide MALP-2. Vaccine, 2005, 23, 4721-4726.

64 Lysynyansky, L., Rosengarten, R., and D. Yogev. Phenotypic switching of variable surface lipoproteins in *Mycoplasma bovix* involves high-frequency chromosomal rearrangements. J. Bacteriol., 1996, 178, 5395-5401.

65 Malaviya, R., Ikeda, T., Ross, E., and S. Abraham. Mast cell modulation of neutriphil influx and bacterial clearance at sites of infection through TNF-α. Nature, 1996, 381, 77-80.

66 Markham, P., Kanci, A., Czifra, G., Sundquist, B., Hains, P., and G. Browning. Homologue of Macrophage-Activating Lipoprotein in *Mycoplasma gallisepticum* Is Not Essential for Growth and Pathogenicity in Tracheal Organ Cultures. J. Bacteriol., 2003, 185(8), 2538-2547.

67 Marmion, B.P. Eaton agent--science and scientific acceptance: a historical commentary. Rev Infect Dis., 1990, 12, 338-353.

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

69 Mathematics, U.O.B.C.D.O. Initial Value Problems for Growth and Decay. 2008.

70 Mbawuike, I., and H. Herscowitz. MH-S, a murine alveolar macrophage cell line: morphological, cytochemical, and functional characteristics. J. Leukocyte Biology, 1989, 46(2), 119-127.

71 McCurdy, J., Olynych, T., Maher, L., and J. Marshall. Cutting edge: distinct toll-like receptor 2 activators selectively induce different classes of mediator production from human mast cells. J. Immunology, 2003, 170, 1625-1629.

72 McDonald, D. Angiogenesis and remodeling of airway vasculature in chronic inflammation. Am. J. Respir. Crit. Care. Med., 2001, 164, S39-S45.

73 Muhlradt, P., Kiess, M., Meyer, H., Sussmuth, R., and J. Jung. Isolation, structure elucidation, and synthesis of a macrophage stimulatory peptide from *Mycoplasma fermentans* acting at picomolar concentration. J. Exp. Med., 1997, 185(11), 1951-1958.

74 Muhlradt, P., Kiess, M., Meyer, H., Sussmuth, R., and G. Jung. Structure and specific activity of macrophage-stimulating lipopeptides from *Mycoplasma hyorhinis.* Infection and Immunity, 1998, 66(10), 4804-4810.

75 O'Neill, L.A. Signal transduction pathways activated by the IL-1 receptor/toll-like receptor superfamily. Curr. Top. Microbiol. Immunol., 2002, 270, 47-61.

76 Okusawa, T., Fujita, M., Nakamura, J., Into, T., Yasuda, M., Yoshimura, A., Hara, Y., Hasebe, A., Golenbock, D., Morita, M., Kuroki, Y., Ogawa, T., and K. Shibata. Relationship between structures and biological activities of mycoplasmal diacylated lipopeptides and their recognition by toll-like receptors 2 and 6. Infection and Immunity, 2003, 72(3), 1657-1665.

77 Omueti, K., Beyer, J., Johnson, C., Lyle, E., and R. Tapping. Domain exchange between human toll-like receptors 1 and 6 reveals a region required for lipopeptide discrimination. J. Biological Chemistry, 2005, 280(44), 36616-36625.

78 Parker, R.F., Davis, J.K., Blalock, D.K., Thorp, R.B., Simecka, J.W., and Cassell, G.H. Pulmonary clearance of *Mycoplasma pulmonis* in C57BL6N and C3H/HeN mice. Infect Immun, 1987, 55(2631-2635).

79 Pennica D, N.G., Hayflick JS, Seeburg P.H., Derynck R., Palladino M.A., Kohr W.J., and Aggarwal B.B. Human tumour necrosis factor: precursor structure, expression and homology to lymphotoxin. Nature, 1984, 312(5996), 724-729.

80 **Quesniaux, V., Fremond, C., Jacobs, M., Parida, S., Nicolle, D., Yeremeev, V., and Bihl, F., Erard, F., Botha, T., Drennan, M., Soler, M., Bert, M., Schnyder, B., and B. Ryffel.** Toll-like receptor pathways in the immune responses to mycobacteria. Microbes and Infection, 2004, 6(10), 946-959.

81 **Reise Sousa, C.** Activation of dendritic cells: translating innate into adaptive immunity. Curr. Opin. Immunol., 2004, 16, 21-25.

82 **Romics, L., Dolganiuc, A., Velayudham, A., Kodys, K., Mandrekar, P., and Golenbock, E., and G. Szabo.** Toll-like receptor 2 mediates inflammatory cytokine induction but not sensitization for liver injury by Propioni-bacterium acnes. J. Leukocyte Biology, 2005, 78, 1255-1264.

83 **Rosati, S., Pozzi, S., Robino, P., Montinaro, B., Conti, A., Fadda, M., and M. and Pittau.** P48 Major Surface Antigen of *Mycoplasma agalactiae* Is Homologous to a malp product of *Mycoplasma fermentans* and Belongs to a Selected Family of Bacterial Lipoproteins. Infection and Immunity, 1999, 67(5), 6213-6216.

84 **Rottem, S.** Interaction of mycoplasmas with host cells. Physol. Rev., 2003, 83, 417.

85 **Ruschmeyer, D., Thude, H., and P. Muhlradt.** MDHM, a macrophage-activating product of *Mycoplasma fermentans*, stimulated murine macrophages to synthesize metric oxide and become tumoricidal. FEMS Immunol. Med. Microbiol., 1993, 7(3), 223-229.

86 **Sabroe, I., Parker, L., Dower, S., and M. Whyte.** The role of TLR activation in inflammation. J. Pathology, 2008, 214, 126-135.

87 **Salvatore, C., Fonseca-Aten, M., Katz-Gaynor, K., Gomez, A., Mejias, A., and Somers, C., Chavez-Bueno, S., McCracken, G., and R. Hardy.** Respiratory tract infection with *Mycoplasma pneumoniae* in interleukin-12 knockout mice results in improved bacterial clearance and reduced pulmonary inflammation. Infect Immun, 2007, 75(1), 236-242.

88 **Schoeb, T., Juliana, M., Nichols, P., Davis, J., and J. Lindsey.** Effects of viral and mycoplasmal infections, ammonia exposure, vitamin A deficiency, host age, and organism strains on adherence of *Mycoplasma pulmonis* in cultured rat trachea. Lab Anim. Sci., 1993, 43(5), 417-424.

89 **Schoeb, T., Dybvig, K., Keisling, K., Davidson, M., and J. Davis.** Detection of *Mycoplasma pulmonis* in cilia-associated respiratory bacillus isolates and in respiratory tracts of rats by nested PCR. J. Clinical Microbiol., 1996, 35(7), 1667-1670.

90 **Schwandner, R., Dziarski, R., Wesche, H., Rothe, M., and C.J. Kirschning.** Peptidoglycan and lipoteichoic acid-induced cell activation is mediated by Toll-like receptor 2. J. Biol. Chem., 1999, 274, 17406.

91 **Seya, T., and M. Matsumoto.** A lipoprotein family from *Mycoplasma fermentans* confers host immune activation through toll-like receptor 2. International J. of Biochemistry and Cell Biology, 2002, 34, 901-906.

92 **Shen, X., Yu, H., Gumulak, J., French, C.T., Zou, N., and K. Dybvig.** Gene rearrangements in the vsa locus of *Mycoplasma pulmonis*. J. Bacteriol., 2002, 182, 2900-2908.

93 **Shimizu, T., Kida, Y., and K. Kuwano.** Lipid-associated membrane proteins of *Mycoplasma fermentans* and *M. penetrans* activate human immunodeficiency virus long-terminal repeats through Toll-like receptors. Immunology, 2004, 113(1), 121-129.

94 **Shimizu, T., Kida, Y., and K. Kuwano.** *Mycoplasma pneumoniae*-derived lipopeptides iduce acute inflammatory responses in the lungs of mice. Infection and Immunity, 2007, 76(1), 270-277.

95 **Simecka, J., Patel, P., Davis, J., Ross, S., Otwell, P., and G. Cassell.** Specific and nonspecific antibody responses in different segments of the respiratory tract in rats infected with *Mycoplasma pulmonis*. Infect Immun, 1991, 59, 3715-3721.

96 **Sitaraman, R., Denison, A., and K. Dybvig.** A unique, bifunctional site-specific DNA recombinase from *Mycoplasma pulmonis*. Molecular Microbiology, 2002, 46(4), 1033-1040.

97 **Siveke, J., and A. Hammann.** T helper 1 and T helper 2 cells respond differentially to chemokines. J. Immunology, 1998, 160, 550-554.

98 **Sun, X., Jones, H., Hodge, L., and J. Simecka.** Cytokine and chemokine transcription profile during *Mycoplasma pulmonis* infection in susceptible and resistant strains of mice: macrophage inflammatory protein 1β (CCL4) and monocyte chemoattractant protein 2 (CCL8) and accumulation of CCR5+ on Th cells. Infect Immun, 2006, 74(10), 5943-5954.

99 Tabeta, K., Georgel,P., Janssen,E., Du,X., Hoebe,K., Crozat,K., Mudd,S., and Shamel, L., Sovath,S., Goode,J. et al. Toll-like receptors 9 and 3 as essential components of innate immune defense against mouse cytomegalovirus infection. Proc. Natl Acad. Sci. U. S. A., 2004, 101, 3516-3521.

100 Taguchi, T., Aicher, W., Fujihashi, K., Tamamoto, M., McGhee, J., and H. and Kiyono. Novel function for intestingal and intraepithelial lymphocytes: murine CD3+, γδ T cells produce IFN-γ and IL-5. J. Immunology, 1991, 147, 3736.

101 Takeuchi, O., Kaufmann, A., Grite, K., Kawai, T., Hoshino, K., Morr, M., and Muhlradt, P.F., and S. Akira. Cutting edge: preferentially the R-stereoisomer of the mycoplasmal lipopeptide macrophage-activating lipopeptide-2 activates immune cells through a Toll-like receptor 2- and MyD88-dependent signaling pathway. J. Immunology, 2000, 164, 554.

102 Takeuchi, O., Kawai, T., Muhlradt, P.F., Morr, M., Radolf, J.D., Zychlinsky, A., and Takeda, K., and S. Akira. Discrimination of bacterial lipoproteins by Toll-like receptor 6. Jap. Society for Imm., 2001, 13(7), 933.

103 Taub, D., Proost, P., Murphy, W., Anver, M., Longo, D., van Damme, J., and J. and Oppenheim. Monoctye chemotactic protein-1 (MCP-1), -2, and -3 are chemotactic for human T lymphocytes. J. Clin. Investig., 1995, 95, 1370-1376.

104 Taylor, G., Taylor-Robinson, D., and G. Fernald. Reduction in the severity of *Mycoplasma pneumoniae*-induced pneumonia in hamsters by immunosuppressive treatment with antithymocyte sera. J. Med. Microbiol., 1974, 7(3), 343-348.

105 Tino, M., and J. Wright. Surfactant protein A stimulates phagocytosis of specific pulmonary pathogens by alveolar macrophages. Am. J. Physiol. (Lung Cell. Mol. Physiol.), 1996, 270(14), 677-688.

106 Underhill, D.M., Ozinsky, A., Smith, K.D., and A. Aderem. Toll-like receptor-2 mediates mycobacteria-induced proinflammatory signaling in macrophages. Proc. Nat. Acad. Sct. USA, 1999, 96, 14459.

107 University of Kentucky, C.o.A. Agripedia. 2006.

108 Vignola, A.M., G., Costanzo, R., Giorgi, M., Gjomarkaj, M., Bellia, V., and Bonsignore. Airway remodeling in asthma. Chest., 2003, 123, 417S-422S.

109 Weigt, H., Muhlradt, P., Emmendorffer, A., Krug, N., and A. Braun. Synthetic mycoplasma-derived lipopeptide MALP-2 induces maturation and function of dendritic cells. Immunobiology, 2003, 207(3), 223-233.

110 Wilde, I., Lotz, S., Engelmann, D., Starke, A., Zandbergen, G., Solbach, W., and and Laskay, T. Direct stimulatory effects of the TLR2/6 ligand bacterial lipopeptide MALP-2 on neutrophils granulocytes. Med. Microbiol. Immunology, 2006, 196, 61-71.

111 Wise, K.S., M. F. Kim, and R. Watson-McKown. Variant membrane proteins. In Tully, S.R.a.J.G., ed. Molecular and diagnostic procedures in mycoplasmology (Academic Press, New York, N.Y., 1995).

112 Woolard, M., Hardy, R., and J. Simecka. IL-4-independent pathways exacerbate methacholine-induced airway hyperreactivity during mycoplasma respiratory disease. J. Allergy Clin Immunol., 2004, 114, 645-649.

113 Woolard, M., Hodge, L., Jones, H., Schoeb, T., and J. Simecka. The upper and lower respiratory tracts differ in their requirement of IFN-γ and IL-4 in controlling respiratory mycoplasma infection and disease. J. Immunology, 2004, 172, 6875-6883.

114 Woolard, M., Hudig, D., Tabor, L., Ivey, J., and J. Simecka. NK cells in gamma-interferon-deficient mice suppress lung innate immunity against Mycoplasma spp. Infect Immun, 2005, 73(10), 6742-6751.

115 Wu, Q., Martin, R., Rino, J., Breed, R., Torres, R., and H. Chu. IL-23-dependent IL-17 production is essential in neutrophil recruitment and activity in mouse lung defense against respiratory *Mycoplasma pnuemoniae* infection. Microbes and Infection and Immunity, 2007, 9, 78-86.

116 Xu, X., Zhang, D., Lybuynska, N., Wolters, P., Killeen, N., Baluk, P., McDonald, and D., H., S., and G. Caughey. Mast cells protect mice from *Mycoplasma pneumoniae*. American J. of Respiratory and Critical Care Medicine, 2006, 173, 219-225.

117 Xu, X., Zhang, D., Zhang, H., Wolters, P., Killeen, N., Sullivan, B., Locksley, R., and Lowell, C., and G. Caughey. Neutrophil histamine contributes to inflammation in *Mycoplasma pneumoniae*. J. of Experimental Medicine, 2006, 203(13), 2907-2917.

118 Yamamoto, M., Sato, S., Hemmi, H., Sanjo, H., Uematsu, S., Kaisho, T., et al. . Essential role for TIRAP on activation of signaling cascade shared by TLR2 and TLR4. Nature, 2002, 420, 324-329.

119 Yamamoto, Y., Tomioka, H., Sato, K., Yamada, Y., and T. Setogawa. Sex differences in the susceptibility of mice to infection induced by *Mycobacterium intracellulare.* `Am. Rev. Respir. Dis., 1990, 142(430-433).

120 Yamamoto, Y., Saito, H., Setogawa, T., and H. Tomioka. Sex differences in host resistance to *Mycobacterium marinum* infection in mice. Infect Immun, 1991, 59(4089-4096).

121 Yancey, A.L., Watson, H.L., Cartner, S.C., and J. Simecka. Gender is a major factor in determining the severity if mycoplasma respiratory disease in mice. Infect Immun, 2001, 69(5), 2865-2871.

122 Yogev, D., Rosengarten, R., Watson-McKown, R., and K.S. Wise. Molecular basis of mycoplasma surface antigenic variation: a novel set of divergent genes undergo spontaneous mutation of periodic coding regions and 5' regulatory sequences. EMBO J., 1991, 10, 4069-4079.

123 Yoshimura, A., Lien, E., Ingalls, R.R., Tournanen, E., Dziarski, R., and D. Golenbock, E., and G. Szabo. Cutting edge: recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2. J. Immunology, 1999, 163(1).

124 You, X., Zeng, Y., and Y. Wu. Interactions between mycoplasma lipid-associated membrane proteins and the host cells`. J. Zhejiang University Science B., 2006, 7(5), 342-350.

125 Zhu, Z., Homer, R., Wang, Z., Chen, Q., Geba, G., Wang, J., Zhang, Y., and J. and Elias. Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. J. Clin, Invest., 1999, 103, 779-788.