12-29-2014

Role of Mycoplasma gallisepticum and Host Airway Epithelial Cell Interaction in Inflammation

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University of Connecticut, 2014

ABSTRACT

Mycoplasma gallisepticum infection in chickens is associated with severe inflammation of the trachea, air sacs and lungs. *M. gallisepticum* cytadheres to the tracheal epithelium and mediates infiltration of macrophages, heterophils and lymphocytes to the tracheal submucosa but the molecular mechanisms underpinning the severe inflammatory response is not well understood. This research focuses on identifying how *M. galisepticum* and chicken tracheal epithelial cell (TEC) interaction might contribute to this response. The first study identified that *M. gallisepticum* lipid associated membrane proteins (LAMP) from both virulent and non-virulent strains were able to up-regulate several inflammatory genes from tracheal epithelial cells both *in vitro* and *ex vivo* including, but not limited to IL-1β, IL-6, IL-8, IL-12p40, CCL-20 and NOS-2. However live virulent strains were significantly more efficient in not only up-regulating these genes to a greater extent but also differentially regulating several unique genes in TECs. The study also identified that *M. gallisepticum* LAMPs mediate the inflammatory gene up-regulation via TLR-2 ligation in an NF-κB dependent pathway. The second study identified, that interaction of a virulent strain with TECs leads to significantly more macrophage chemotaxis than a non-virulent strain. Macrophages upon co-culture with *M.*

gallisepticum exposed TECs up-regulated expression of IL-1β, IL-6, IL-8, MIP-1β, CXCL-13, CCL-20 and RANTES. Interaction of R_{low} with TECs enabled more efficient gene up-regulation from macrophages compared to R_{high}. Kinetic analysis of these genes identified the peak of expression of cytokine genes to be 6 hours reducing significantly thereafter, whereas expression of chemokine genes remained significantly above control level until 24 hours. These results further our understanding of molecular mechanisms leading to the severe inflammatory response observed during *M. gallisepticum* infection and suggest that interaction of *M. gallisepticum* with chicken tracheal epithelial cells play significant role in this process.

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A Dissertation

Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

At the

University of Connecticut

2014

APPROVAL PAGE

Doctor of Philosophy Dissertation

Role of *Mycoplasma gallisepticum* and Host Airway Epithelial Cell Interaction in Inflammation

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DEDICATION

To my family, for their unconditional love, support and prayers throughout the course of my dissertation research

ACKNOWLEDGEMENTS

From the beginning of my journey in the pursuit of doctoral studies until the completion, I owe an immense debt of gratitude to my advisor Dr. Lawrence K. Silbart. His support, guidance and insights throughout my dissertation research were invaluable. I thank him for providing me with the opportunity to sharpen my knowledge and skills and helping me get one step closer to become an independent researcher.

I am extremely grateful to Dr. Steven J. Geary, Dr. Paulo H. Verardi, Dr. Steven M. Szczepanek and Dr. Salvatore Frasca Jr. for several stimulating discussions and providing their expert views on microbiological, immunological and pathological aspects of my research.

I would also like to thank my colleagues Frank Zappulla, Debra Rood, Jessica Beaudet, Heather Wanckzyk and Dr. Diana Alejo for their constant support, help with the research and providing me with an excellent work environment. I would cherish their friendship all along. I would also like to acknowledge all faculty, friends and well-wishers whose assistance at some stage of my graduate education was invaluable.

I acknowledge my husband Dr. Jaideep Saha, my parents Santosh K. Majumder and Gita Majumder as well as my brother Sugata Majumder for their constant support, affection, motivation and prayers. Their love is the key to my success.

I thank all for making my transition from a naïve student to a PhD scholar become easier.

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LITERATURE REVIEW

1.1 Introduction

Mycoplasmas have gained considerable attention in the world of microbiology as pathogens to a wide array of hosts despite their extremely small size and limited biosynthetic capabilities. They are not considered as typical bacteria and belong to the class of 'Mollicutes' (in Latin: *mollis*, soft; *cutis*, skin) due to the lack of a rigid peptidoglycan cell wall. Other organisms including ureaplasmas, acholeplasmas, entomoplasmas, spiroplasmas, and phytoplasmas are also considered to be mollicutes [1]. Mycoplasma genome size ranges from as low as 580 kb in *Mycoplasma genitalium* [2] up to 1,380 kb for *M. mycoides* subsp. *mycoides* LC [1]. Their extremely small size enabled scientists to not only sequence the genomes, but also led to creation of a synthetic genome of *Mycoplasma mycoides*, and construction of a viable bacterium upon transplantation into *Mycoplasma capricolum* [3]. Mycoplasmas are phylogenetically linked to gram positive bacteria and are believed to have evolved from these bacterial classes via degenerative evolution. Their uniqueness is also reflected by their usage of UGA codon (normally a stop codon) to encode for tryphophan [4].The small genome sizes correspond with their inability to synthesize nutrients and thereby they depend on the host for nutrient acquisition and are considered obligate parasites [5].

Due to their limited biosynthetic capabilities resulting from degenerative evolution, most mycoplasmas are parasitic in nature, exhibiting strict host specificities and are common pathogens of humans, animals, reptiles, fish, insects, and plants [1]. Primarily they are pathogens of respiratory and urogenital tracts, but are also known to cause systemic diseases of the eyes, mammary glands and joints [1]. Mycoplasmas are also increasingly found in unnatural habitats, for example urogenital mycoplasma *M.*

hominis have been isolated from respiratory tract and joints [1, 6, 7]. However there are several commensal mycoplasmas that live innocuously within their host as part of the natural flora, without any detrimental effects. Even though very few mycoplasmas are associated with mortality, economic significance arises from the extreme morbidity they cause in humans, pets, and livestock, especially in poultry.

1.2 History of mycoplasmas

More than a century ago in 1898 a bovine pleuropneumoniae like organism was successfully cultivated by Nocard and Roux which set the stage for mycoplasmology, a new area in science. Even though these organisms have long been known, their taxonomy has been contentious including the simplest classification; bacteria or a virus? In 1930 the virus status of mycoplasma was refuted. In 1935 an L-form (a laboratory strain after partial or complete removal of cell wall) of *Streptobacillus moniliformis* was isolated by Klienenberger, which significantly resembled mycoplasmas in their colony formation, morphology and filterability. The possibility that mycoplasmas represented a stable L form of a yet to be identified parent bacteria led to the coining of their independent taxonomic status. For almost 30 years it was believed that mycoplasmas are nothing but L-form variants of common bacteria until in 1969 the genomic analysis data containing the guanine and cytosine content of mycoplasmas became available, and ruled out this conception [8]. From an evolutionary perspective, mycoplasmas not only have lost their cell wall due to degenerative evolution but also have lost many more genes responsible for various functions including biosynthetic capabilities like de novo biosynthesis of purines, a functional tri-carboxylic acid cycle, and a cytochrome mediated electron transport chain system. This has led to a significantly diminished genome size,

differentiating them from the L-forms [4, 9]. Hence, mycoplasmas are now recognized as the smallest known self-replicating bacterium. The name 'mycoplasma' was derived from Greek *mykes* or fungus and *plasma* for something molded to denote the fungus like morphology of *Mycoplasma mycoides* the first identified mycoplasma. Current classification of mycoplasma was done by Edward and Freundt in 1956 and consists of the order *Mycoplasmatales*, family *Mycoplasmatacae*, genera *Mycoplasma* and several species [9, 10].

1.3 Mycoplasmosis in various species

1.3.1 Mycoplasmosis in humans

Many different species of mycoplasmas are known to infect humans including *Mycoplasma pneumoniae*, *Mycoplasma genitalium*, *Mycoplasma penetrans*, *Mycoplasma hominis*, *Mycoplasma salivarium*, *Mycoplasma fermentans* and others. The most well studied pathogen among them is *M. pneumoniae*, known to cause upper and lower respiratory disease, tracheobronchitis, pharyngitis and community acquired pneumonia or "walking" pneumonia [11]. In children, inflammation of the throat with or without cervical adenopathy, conjunctivitis and myringitis are also observed. However, younger children are more likely to present with coryza and wheezing, and occasionally progress towards pneumonia, but older children and elders are more likely to develop bronchopneumonia involving one or more lobes, sometimes leading to hospitalization. Mild asymptomatic infections are more frequently observed in adults with very few progressing to bronchopneumonia [12]. A significant percentage *M. pneumoniae* infection may lead to extrapulmonary manifestation. Chronic *M. pneumoniae* infections are sometimes associated with asthma and chronic obstructive pulmonary disease likely

due to a Th2 skewed host immune response [13, 14]. Central nervous system complications including encephalitis, polyradiculitis, cerebellar syndrome, encephalomyelitis etc. have also been observed as extrapulmonary manifestations of infection. Although rare, cardiovascular, renal and gastrointestinal complications and even hemolytic anemia have been found to be associated with *M.pneumoniae* infection [12].

Mycoplasma genitalium is another fairly well studied human pathogen known to be associated with urogenital complications. It is known to cause a sexually transmitted non-gonococcal urethritis in men and pelvic inflammatory disease in women [15-17]. *Mycoplasma penetrans*, the first identified invasive mycoplasma has been observed to penetrate eukaryotic cells using their tip structure and is also known to colonize human urogenital tract and have been associated with HIV suggesting a possible co-infection[17-19]. *Mycoplasma hominis* is another known urogenital pathogen associated with bacterial vaginosis and preterm births [17, 20]

1.3.2 Mycoplasmosis in animals

Mycoplasma mycoides subsp mycoides small colony type (MMMSC) and *Mycoplasma bovis* are two well-known pathogens in bovines. MMMSC is highly contagious and cause bovine pleuoropneumoniae that can sometimes be fatal [21]. *M. bovis* is associated with mastitis and pneumonia in dairy cattle [22, 23]. *Mycoplasma capricolum* subsp. caripneumoniae (MCC) causes caprine pleuropneumonia and are often associated with mortality in goats. *Mycoplasma mycoides* subsp. *mycoides* large colony type and *Mycoplasma mycoides* subsp. *capri* can cause mastitis and arthritis in goats [24]. *Mycoplasma agalactiae* is also a well-known caprine mycoplasma that causes mastitis

associated reduced milk production [25]. *Mycoplasma hyopneumoniae* causes highly infectious porcine enzootic pneumonia in swine resulting in significant economic losses [26]. *Mycoplasma hyorhinis* can also occasionally cause serofibrinous to fibrinopurulent polyserositis and arthritis in pigs [27]. *Mycoplasma felis* causes conjunctivitis and pneumonia in felines as well as pleuritis in equines [28-30]. *Mycoplasma pulmonis* is a rodent respiratory pathogen that resembles atypical pneumonia caused by *M.pneumoniae* in humans and frequently serves as an animal model for this disease. *Mycoplasma arthritidis* is commonly found in rats and cause polyarthritis in mice. This mycoplasma possesses a superantigen also known as *Mycoplasma arthritidis* mitogen (MAM), a distinctive feature among all mycoplasmas which is known to polyclonally activate lymphocytes and leads to arthritic disease [31].

1.3.3 Mycoplasmosis in fishes and reptiles

Mycoplasma crocodyli and *Mycoplasma alligatoris* are two reptilian mycoplasmas [32]. *Mycoplasma alligatoris* causes lethal invasive disease in alligators including necrotizing pneumonia, fibrinous polyserositis, polyarthritis, severe pericarditis, necrotizing myocarditis, pyogranulomatous meningitis, and necrotizing synovitis. They can rapidly disseminate into various tissues by degrading extracellular matrix proteins and can cause acute brainstem hemorrhage. They are called flesh-eating mycoplasma and are often associated with mortality [32-34]. *Mycoplasma agassizii* causes rhinitis, palpebral edema as well as nasal and ocular discharge in desert tortoise thereby affecting the animal's ability to breathe and making it difficult for them to survive in the wild. In fish *Mycoplasma mobile* cause severe necrosis of epithelial and gill arches. Name of this

mycoplasma was coined due to their fastest gliding motility, a process of binding and movement on glass surfaces utilizing a polarized tip structure [35].

1.3.4 Avian mycoplasmosis

The first identified avian mycoplasma was *Mycoplasma synoviae*. It causes infectious synovitis in poultry and produces respiratory lesions and airsacculitis [36]. *Mycoplasma iowae* infection is also characterized by airsacculitis, embryo mortality, chondrodystrophy, poor feathering etc. in chicken and turkeys [36]. *Mycoplasma meleagridis* cause respiratory illness in poultry [37]. *Mycoplasma gallisepticum* is the most studied of the avian mycoplasmas and is a well-known respiratory and reproductive tract pathogen of poultry resulting in significant economic losses to the poultry industry. *M. gallisepticum* cause tracheitis, airsacculitis, respiratory rales, coughing, nasal discharge and chronic respiratory disease in chickens especially in the presence of a co-infection [38-40]. It recently has become a major threat to passerine birds and has been isolated from American goldfinches, house finches and purple finches [41, 42]. It causes severe periorbital swelling and conjunctivitis with mucopurulent drainage and nasal exudate in house finches [43] resulting in drastic decline in house finch population.

1.4 Mycoplasma host interaction

Despite their extremely small genome and lack of various metabolic pathways, mycoplasmas are able to evade host immune responses and establish chronic infections within wide array of hosts. However mycoplasma host interaction is a quite complex phenomenon and various strategies employed by mycoplasma in disease pathogenesis and host immune/inflammatory response to infections are discussed in subsequent sections.

1.4.1 Cytadhesion and gliding motility

Mycoplasmas are generally flask shaped with a narrowed tip structure that enables gliding motility and mediates tight attachment to host cell surface. Cytadhesion to host cells is a necessary step towards disease pathogenesis as mutants lacking cytadhesion are avirulent [44, 45]. Their tapered polarized structure of the "tip organelle" is composed of an electron dense core containing adhesion molecules and is well-studied in *M. pneumoniae.* In *M. pneumoniae*, protein P1 is densely aggregated near the tip structure along with an associated molecule P30, and mutants lacking P1 or P30 are associated with reduced cell attachment and virulence *in vivo* [46, 47]. Whereas P1 the primary adhesion molecule, P30 enables P1 to function properly. The P1 gene has highest G+C content in the entire genome and may suggest an unknown exogenous origin [45]. P30 plays a role in cell development as *M.pneumoniae* lacking P30 tend to have morphological abnormalities [46]. Importantly, P30 is known to possess amino acid sequence homology with eukaryotic structural proteins including cross-reactive epitopes shared with fibrinogen, keratin, and myosin, and may be implicated as a basis for molecular mimicry and post infectious autoimmunity[46, 48]. Both P1 and P30 are known to elicit strong immunological responses in convalescent phase sera [46]. Two other proteins closely associated with P1 are found in the tip organelle and named P40 and P90 based on their molecular weight. These are found to be surface localized near the tip organelle but not directly associated with receptor binding and may aid in cytoskeletal organization or clustering of P1 at the tip [46, 47]. Other groups of proteins called HMW1-3 (high molecular weight protein) also help anchor P1 at the tip organelle [49, 50]. These accessory molecules are known to maintain proper distribution of the adhesins

and loss of these proteins lead to loss of attachment capabilities suggesting their significant role in cytadherence. Other proteins including P65 and P200 share similar structural features as HMW1 and HMW3 and probably play important role in *M. pneumoniae* cytoskeletal organization. HMW2 may also play an important role as mycoplasma cytoskeletal protein due to the similarity in conformation with the filamentous portion of myosin heavy chain [45, 47, 51, 52].

Like *M. pneumoniae* P1, *M. genitalium* and *M. gallisepticum* contain homologous cytadhesion molecules named MgPa and GapA. Other mycoplasmas like *M. hominis*, *M. hyopneumoniae*, *M. mobile* and *M. agalactiae* also possess cytadhesion molecules [44, 53-56], although apparently not homologous to *M.pneumoniae* P1 based on sequence comparisons. In *M. gallisepticum,* analysis of the GapA operon revealed the presence of another gene, termed crmA, with sequence homology to P40 and P90 of *M. pneumonia* [53]. Loss of CrmA also reduced virulence and cytadhesion capabilities of *M. gallisepticum* suggesting its association with GapA for cytadhesion [57]. *M. hominis* cytadhesin OppA seems to have an ecto ATPase activity that helps in cytadhesion [54]. Very recently it was identified that cytadherence is not only necessary for attachment to host cell surface but are also involved in initiation of host inflammatory response via ligation to TLR-4 [58, 59].

Gliding motility is yet another unique phenomenon observed in mycoplasma and involves the polarized tip structure, which enables them to bind and move on solid surfaces. Although not all pathogenic mycoplasmas possess gliding motility, some mycoplasma species do including *M. mobile,* the fastest mycoplasma, *M. gallisepticum* and *M. pneumonia*. Energy required for this process is generally obtained via ATP

hydrolysis [60, 61] In *M. gallisepticum* cytadhesion proteins GapA and CrmA were found to be associated with gliding motility as mycoplasmas lacking these genes are unable to move on solid surfaces [62, 63]. Gliding motility allows mycoplasmas to escape mucocilary clearance, enabling them access the mucosal epithelial cells. Therefore these properties across various species of mycoplasma play a significant role in virulence and disease pathogenesis upon infection.

1.4.2 Invasion of Host cells

Mycoplasmas are generally known as extracellular pathogens by virtue of colonizing the mucosal surfaces, however more recent research has identified their capability for invasion and survival within non phagocytic host cells, aiding in evasion of host immune response[19, 64-70]. Initailly only *M. penetrans* was thought to be able to invade host cells, but more recent studies identified *M. gallisepticum*, *M. synoviae, M. suis, M. fermentans*, *M. hyorhinis*, *M. bovis* among others to have invasive capabilities[67, 68, 71-78]. Most of these pathogens were found to invade and reside within non-phagocytic cells. *M. gallisepticum* was found to invade chicken erythrocytes, fibroblasts and HeLa cells, with a low passage virulent strain being significantly more efficient in invasion *in vitro* and systemic spread *in vivo* than a high passage cytadherence deficient avirulent strain [67, 68, 71, 76]. A recent report demonstrated that the ability of *M. gallisepticum* to invade host cells depends on host cell extracellular matrix (ECM) molecules like collagen type IV, plasminogen and fibronectin [77-81]. However, there is no clear evidence that the organism can invade tracheal epithelial cells, as they were predominantly found to colonize the mucosal surface and were only rarely found inside phagocytic vacuoles ([82] and unpublished observations, see appendix).

1.4.3 Host Cell Receptors

Host cell surface sialoglycoconjugate receptors are well established for their role in mycoplasma attachment [44]. *M. pneumoniae* is also known to attach to human erythrocytes via a carbohydrate moiety on an active glycoprotein and a sialic acid free glycoprotein receptor on human lung MRC-5 cells [83-85]. In very recent literature it has been referred that cytadhesion of *M.pneumoniae* occurs via TLR-4 receptors present on host cell [59] whereas it has long been known that mycoplasmal lipoproteins are able to precipitate host innate immune responses via ligation of membrane lipoprotein to host TLR-2 homodimers, or TLR1/2 and TLR2/6 heterodimers [86].

Mycoplasmas are known to bind cilia present on the respiratory epithelial cell surface as has been shown with *Mycoplasma hyopneumoniae* and their binding to porcine ciliated respiratory epithelial cells. Zhang *et al* 1994 reported *M. hyopneumoniae* specifically bind to glycolipid receptors on cilia and suggested these to be the major receptors for mycoplasma to cytadhere ciliated cells [87]. Several proteins from *M. hyopneumoniae* were found to be associated with cilium binding including P97 adhesin, protein P159 and two surface proteins MHP385 and MHP384 [88-90]. A very recent study reported that *M. pneumoniae* colonization of human respiratory epithelial cell requires cilia as a conduit for mycoplasma to access the host cell surface [91]. It is long been known that mucociliary apparatus impairs bacterial colonization on host epithelial cell surface, but mycoplasmas are able to induce ciliostasis which help them avoid mucociliary clearance [92, 93]. During mycoplasma infection squamous metaplasia, a non-cancerous change in the surface lining of ciliated columnar epithelial cells to a squamous morphology is

observed along with loss of cilia. It is believed that both these changes function as a host defense mechanism to avoid mycoplasma attachment on epithelial cell surface [93, 94]

The above discussed ciliary functions are attributed to the motile cilia that helps in clearance of pathogens, whereas non-motile or primary cilia are known to induce intracellular signaling. This signaling often occur in presence of cytokine like IL-1 and leads to production of other cytokines, nitric oxide and prostaglandin E2 that in turn help in clearance of pathogens. However, involvement of primary cilia during mycoplasma infections remains unknown [95].

1.4.4 Mycoplasma lipoproteins

Mycoplasmas do not possess cell walls or outer membranes, and thus lack lipopolysaccharide (LPS), a known endotoxin, as well as other cell wall components like lipotechoic acid, flagella, and peptidoglycan. These cell surface molecules, also known as pathogen associated molecular patterns (PAMP), ligate to specific host cell receptors of the innate immune system known as pattern recognition receptors (PRRs) like Toll like receptors (TLRs) or NOD like receptors (NLRs) among others [96, 97]. TLRs are the most common PRRs to ligate PAMPs and precipitate an inflammatory response [97, 98], for example bacterial lipopolysaccharides ligate to host TLR-4 and flagellin ligate to TLR-5.

Mycoplasma membranes are composed of a single lipid bi-layer with numerous embedded integral and peripheral proteins and membrane anchored lipoproteins [99-101]. Due to the lack of cell wall and outer membranes, mycoplasma lipoproteins are the only PAMP available on mycoplasma cell surface, and are very well known for their inflammatory properties and capability of mounting innate immune responses [102]. A

significant percentage of mycoplasma genomes are dedicated to these lipoproteins; for example in *M. gallisepticum*, VlhA (variable lipoprotein hemagglutinins) encoding genes occupy 10.4% of the total genome [103] suggesting the significance of mycoplasmal lipoproteins for bacterial fitness. Mycoplasmas are also capable of phase variable expression of their surface lipoproteins, aiding in circumvention of host immune responses. The first mycoplasma lipoproteins found to interact with a host TLR was macrophage activating lipopeptide-2 (MALP-2) of *M. fermentans* and a 44 kDa lipoprotein from *M. salivarium*. TLR-2 but not TLR-4 knockout mice remained unstimulated by MALP-2, suggesting that MALP-2 binds to TLR-2 and initiates the inflammatory response [104-107]. Later interaction of mycoplasmas with host TLRs identified ligation of TLR-1/2 heterodimers with tri-acylated lipoproteins and TLR-2/6 heterodimers with di-acylated lipoproteins, sometimes requiring involvement of CD-14. This led to subsequent activation of NF-κB in a MyD88 dependent pathway [86, 100, 107-112]. It was also observed that an R-stereoisomer of MALP-2 was about a 100 times more potent in leukocyte cytokine production than an S- stereoisomer [113]. Several studies that reported inflammatory activities of mycoplasma lipoproteins also identified that the N-terminal lipid moiety rather than the protein moiety was responsible for activation of NF-κB and pro-inflammatory cytokine secretion [86, 100, 105, 107-112]. These reports suggest immense importance of structure and conformation in inflammatory properties of mycoplasma lipoproteins. The NF-κB activation after TLR ligation mediated by mycoplasma lipoproteins was found to induce several chemokines and cytokines including TNF-α, IL-6, MIP-1β [86, 100, 107-112, 114]. These genes were also expressed during *M. gallisepticum* infection, however it is not known if induction of

these genes is mediated by *M. gallisepticum* lipoproteins. A MALP-2 homolog in *M. gallisepticum* has been characterized and was found to have no effect on attachment, growth or virulence of the organism, but potential role of P47 in induction of cytokines is yet to be elucidated [115, 116]. A truncated form of *M. synoviae* lipoprotein MSPB was also found to induce nitric oxide and cytokine expression in chicken monocyte derived macrophages via ligation of TLR-15 [117, 118]. Mycoplasma lipoproteins were not only found to be active *in vitro*, but several *in-viv*o studies also reported their role in inflammation [106, 119, 120] . MALP-2 was found to induce MIP-1α, MCP-1, and MIP-2, thereby promoting leukocyte infiltration in mouse peritoneal cavity after peritoneal injection [106]. TLR-2 was found to play a role in innate resistance against *M.pulmonis* infection [121].

TLR ligation by lipoproteins was also found to be responsible for leukocytic cell death both via apoptosis and necrosis. Lipoproteins from *M. salivarium* and *M. fermentans* were found to preferentially induce necrotic cell death in lymphocytic cell lines MOLT-4 and Raji and one monocytic cell line THP-1 ,whereas induced apoptotic cell death in another monocytic cell line (HL-60) and was dependent on both caspase activation and TLR-2 ligation [122, 123]. Later MALP-2 was found to induce a sequential bifurcate response in HEK-293 cell line via ligation to TLR-2/6 heterodimer. This lead to NF-κB activation and pro-inflammatory cytokine production as an early event, partially mediated by MyD88 and FADD, and at later stages initiated apoptosis regulated by p38 MAPK , MyD88 and FADD [124]. Similar observations were also made with *M. genitalium* lipoproteins that induced pro-inflammatory cytokine secretion as well as apoptosis by activating NF-κB in THP-1 cells. *M. penetrans*

lipoproteins also induced apoptosis in a similar fashion in mouse macrophages [125, 126]. Surprisingly, a study by Gerlic *et al*. reported that *M. fermentans* lipoproteins in fact inhibit TNF-α induced apoptosis in U937 cells [127] and implied that cell lines used by Into *et al*. are uncharacteristically responsive to TLR induced apoptosis, resulting in a skewed dataset. Another study by Hopfe *et al*. reported that *M. hominis* ecto-ATPase OppA induced ATP release and apoptotic cell death of HeLa cells [128], and Into *et al*. also showed that mycoplasma lipoproteins induce ATP release from cells, which in turn binds to the purinergic receptors and mediate cell cytotoxicity [122].This suggests that TLR mediated NF-kB activation may not be the only pathway involved in the induction of apoptosis. Unpublished data from our lab also found apoptosis to be an important factor during *M. gallisepticum* infection of chickens, identified by tunnel assay and pro-apoptotic cytokine gene expression but it is not known if apoptosis were mediated by lipoproteins.

1.5 Modulation of Host Immune System and Immune Response

The initial interaction of mycoplasmas with their host occurs at mucosal surfaces and subsequently leads to a cascade of inflammatory events. This phenomenon is an important aspect of disease pathogenesis and often determines the susceptibility or resistance to the disease. Whereas innate immune responses are critical in early response and control of infection, adaptive immune responses have contrasting roles in control and pathogenesis of the infection. However many mycoplasma diseases lead to persistence of organism and ineffective immune responses against the infection leads to development of chronic inflammation. Interaction between mycoplasma and host immune system is a complex phenomenon, and various aspects of this interaction has been described below

1.5.1 Innate response to mycoplasma infection

The innate response to infectious agents involves cells of the innate immune system like polymorphonuclear leukocytes (PMN), macrophages, NK cells that are able to mediate direct killing via phagocytosis or by releasing antimicrobial peptides like defensins, collectins, complement, lysozyme, reactive oxygen or and/or nitrogen among others. Mycoplasmas are capable of inducing ciliostasis and possess gliding motility thereby escaping clearance by mucocilary action and facilitating adhesion to the respiratory epithelial cells [93, 129]. Mycoplasma host interaction occurs not only by cytadhesion but also by ligation of mycoplasma surface exposed lipoproteins to host toll like receptors (TLRs) leading to activation of NF-κB and downstream production of cytokine and chemokines [1, 59, 86, 100, 102, 108, 120, 130-132].

Danger associated molecular patterns or DAMPs are also known to induce inflammatory responses and involve nuclear or cytosolic proteins and ATP. DAMPs are recognized via intracellular NOD (nucleotide binding oligomerization domain) receptors and mediate activation of inflammasomes and subsequent induction of inflammatory responses. Mycoplasma infection often leads to extracellular release of ATP and activation of inflammasomes via ligation of ATP to P2X7 receptors and subsequent release of IL-1β, thereby contributing to the inflammatory response [58, 133, 134]. Extracellular ATP is also known to enhance macrophage activation and in certain cases apoptosis, mediated by mycoplasmal lipopeptides [122, 135]. Besides release of ATP from cells, mycoplasmas were also found to activate NLRP3 inflammasome promoting migration and invasion of gastric cancer cells [136].

Most studies that examine the interaction of host innate immune system with mycoplasmas utilized *Mycoplasma pulmonis*, a rodent respiratory pathogen. Alveolar macrophages (AMs), were found to be of major importance in antimycoplasmal defense during this infection and directly correlated with increasing numbers of AMs. Those mycoplasma strains that were able to evade phagocytosis by AMs were more successful in survival within the host and frequently resulted in exacerbated disease in comparison with strains which were easily phagocytized [132, 137]. Mycoplasmas on the other hand were found to be phagocytized by polymorphonuclear leukocytes (PMN) without antibody mediated opsonization and are presumed to be either receptor or complement mediated. Phagocytosis without opsonization is often a critical step for intracellular survival; therefore PMNs presumably play no role in anti-mycoplasma defense and in fact, may contribute to dissemination of mycoplasma to other organs [138, 139].

Mycoplasmas are known to resist phagocytosis in absence of opsonins and since *M. pulmonis* is trypsin sensitive, presence of an antiphagocytic protein on their surface is possible. Nonspecific opsonins are suggested to play an important role in mycoplasmacidal activity and might involve complement breakdown product, fibronectin and C-reactive protein. Complement proteins alone were found to be ineffective in mycoplasma killing, and the ability to avoid killing by the complement activation pathway could be a possible virulence mechanism of mycoplasmas [140, 141].

Surfactant proteins were also found to play an important role in mycoplasma clearance. SP-A specifically interacts with alveolar macrophages via SP-A receptor and was shown to affect release of reactive oxygen species, chemotaxis of monocytes/macrophages as well as enhancing their phagocytic capabilities. Reactive

oxygen and nitrogen species including peroxynitrite and nitric oxide generated by macrophages were also implicated in oxidative killing of mycoplasmas [132]. However mycoplasmas were found to be resistant to oxidative attack by inhibiting production of reactive oxygen-nitrogen species via catalase utilization and argininine depletion, thereby evading oxidative killing during phagocytosis [132, 142-145].

Several studies have identified enhancement in NK cell activity induced by various mycoplasma species [132]. MAM was found to augment lytic capability of human NK cells directly or via indirect effect of cytokines produced from T cells [146]. It was also found that intraperitoneal injection of *Mycoplasma pulmonis* increased splenic and pulmonary NK cell activity and NK cell depletion resulted in decreased killing of this pathogen and disease exacerbation [132, 147].

1.5.2 Adaptive response to mycoplasma infection

The adaptive immune response is a highly specialized mechanism involving lymphocytes (B and T cells), antigen presenting cells (dendritic cells, macrophages etc.) and various processes to eliminate specific microorganisms. Adaptive immune responses are generally induced within a few days after initial exposure to pathogenic microbes and create immunological memory leading to enhanced responses upon subsequent encounters. Adaptive immune responses probably have the most significant effect on mycoplasma disease progression. However, whereas some adaptive responses play beneficial role in controlling the disease, while other elements may lead to severe immunopathology as a result of immune dysregulation.

Mycoplasma infections are generally associated with infiltration of PMNs, macrophages, B and T cells in the submucosa. However, the precise role of B and T

cells during mycoplasma infections, bacterial clearance, restoration of tissue morphology and chronicity of infection are poorly described. In any case, lymphocyte recruitment and activation play an important role in mycoplasma pathogenesis. *M. pneumoniae* infection causes acute peribronchial and perivascular lymphoid accumulation and destruction of respiratory epithelium. Increase in pulmonary T cells has also been observed during *M. pulmonis* infection and both B and T cell accumulation in lungs of goats are observed upon mycoplasma infection [148-150]. However lymphocyte infiltration appears to contribute to both immunopathogenesis and control of disease. For example, nude or SCID mice develop significantly less severe lung disease than wild type mice following *M. pulmonis* infection, but these mice are also susceptible to a more widely disseminated systemic disease [151-153]. Reconstitution of SCID mice with lymphocytes led to development of as severe lesions as normal mice and reconstitution with T cells alone promotes development of more severe lesions. But surprisingly these results have little to no effect on number of mycoplasmas in the lungs, suggesting T cells may not play a role in localized resistance [151, 153]. Cell-mediated immunity was also found to be of limited importance during *M. pneumonia* infection, as severity of pneumonia did not increase with T-cell deficiencies in human subjects [151, 154]. T helper cells are known to contribute to inflammatory responses after mycoplasma infection. During *M. pneumoniae*, *M. agalactiae*, *M. pulmonis* and *M. bovis* infection T cells were found to be the major cell type associated with the disease and $CD4^+$ T helper cells were more predominant than $CD8^+$ T cells [150, 153]. However $CD8^+$ T cells are found to play important role during *M. gallisepticum, M. pneumoniae*,

M. agalactiae, *M. pulmonis* and *M. bovis* infection albeit at smaller numbers [39, 150, 153, 155, 156].

Antibody responses also seem to be important in prevention of mycoplasma dissemination to extra pulmonary tissues [151]. Infection with various species of mycoplasmas were found to elicit responses from all type of antibody classes including IgA, IgM, IgD, IgE and IgG. IgM is often the first antibody generated upon mycoplasma infections and later a switch to other classes of antibodies are observed based on the activity of T helper cells and cytokines [153]. IgG response predominate after initial IgM production and is the major antibody class involved in control of mycoplasma infections. IgG can mediate complement activation, and opsonization of mycoplasmas by phagocytes and various subclasses of IgG (and Fc receptors) determine cell types that phagocytize the pathogen [157]. For example IgG1 mediates killing of *M. bovis* via macrophages and IgG2 via both macrophages and neutrophils [153]. It is also known that maternal IgG can provide protective immunity from mother to fetus or newborn via passive transfer through placenta [153]. IgD responses were also elicited upon *M. penumoniae* infection however the role of IgD in not well understood [153, 158].

Consistent with mycoplasmas being predominantly mucosal pathogens, antibody responses at mucosal sites are primarily dominated by IgA and IgE antibody isotypes. Mycoplasma specific IgA was found in response to both genital and respiratory tract infections [159-162]. Resistance to *M. pneumoniae* infection in fact correlates better with a mucosal IgA response compared to serum IgG or IgM response [153]. IgE has also been found during respiratory mycoplasmosis and as IgE is associated with allergic response, it is believed that a *M. pneumoniae* mediated IgE response leads to

exacerbation of asthma [163, 164]. However a recent study identified association of anti *M. pneumoniae* IgM, but not IgG or IgE in asthmatic children [165]. Therefore it seems from majority of studies that local antibody production may be more important than circulating antibodies in control of mycoplasmas.

Taken together, it is safe to say that mycoplasmas are able to precipitate both innate and adaptive components of the host immune responses. Whereas macrophages are significant components aiding in phagocytosis and clearance of mycoplasma, PMNs may in fact aid in dissemination of mycoplasmas to other tissues [138]. Both B and T cells play critical roles in clearance and prevention of dissemination of mycoplasma but may also contribute to the significant immunopathology associated with the disease. Hence induction of innate and adaptive immune response to mycoplasma whereas beneficial, promotes lesion severity and can be considered a double-edged sword. Therefore, induction of local neutralizing antibodies without promoting severe immuno-pathology is to be considered during production of mycoplasma vaccines.

1.5.3 Role of chemokines and cytokines during mycoplasma infection

Chemokines and cytokines also play a major contributory role in manifestation of disease during mycoplasma infection. During epithelial colonization of mucosal pathogens, chemokines and cytokines produced by airway epithelial cells are known to recruit leukocytes to the submucosa [166]. Even though there is scarcity of data on response of epithelial cells to mycoplasma infections, a handful of studies support the notion that these molecules play an important role in the host response, both protective and maladaptive. During *Mycoplasma genitalium* infection, human cultured endocervical epithelial cells were found to produce IL-6, IL-7, IL-8, MCP-1, GM-CSF during acute

stages of infection while IL-8, MIP-1β and MCP-1 predominated during chronic stages of infection [112, 167, 168]. *M. pneumoniae* infection triggered the release of IL-8 and RANTES from human nasal epithelial cells and expression of IL-1β, IL-8 and TNF-α from A549 cells [169]. These chemokines and cytokines are well known for their role in recruitment and activation of leukocytes and therefore may play a significant role in the immunopathology observed during mycoplasma infection.

Macrophages and monocytes have also been found to produce TNF-α, IL-1β, and MIP-1β upon infection to live mycoplasma or mycoplasma lipid associated membrane proteins (LAMPs) [86, 100, 108, 120, 170, 171]. Cytokines produced by lymphocytes are often major factors in determining the outcome of immune responses and the complex interplay between cells and cytokines often determine disease outcomes. Various studies on mycoplasmas identified differential cytokine patterns during the acute and chronic stages of the disease. The presence of IL-4, IL-5, IL-6, IFN-γ, TNF-α and several other cytokines was found to correspond with the presence of gross inflammatory lesions [153, 172, 173]. Increased levels of IL-4 in broncho-alveolar lavage fluid was found in patients with *M. pneumoniae* infection suggesting a Th2 type response [174]. However a recent study implicated vaccine-mediated exacerbation of disease upon *M. pneumoniae* infection that was associated with increased IL-17 level and corresponding eosinophilia [175] and mycoplasma specific IFN-γ production by lymphocytes were observed in *M. pneumoniae* patients. *M. pulmonis* infected mice were also found to generate both Th1 and Th2 type cytokine response in lung by day 14 which included IFN-γ, IL-2 as well as IL-4, IL-6 and IL-13 [156]. Mycoplasma infections have been shown to mediate a shift from Th2 to a mixed Th1/Th2 response [153]. Even though a Th1 response is associated

with the exacerbation of disease, cytokines like IFN-γ can activate macrophages, allowing them to kill mycoplasmas but generation of a Th2 response may dampen macrophage function and promote development of chronic inflammatory disease [143]. Therefore the balance between Th1 and Th2 cytokine responses may ultimately determine the fate of the disease.

Often a pro-inflammatory cytokine response can have a concomitant anti-inflammatory response as has been observed during mycoplasma infections. *M. hyopneumoniae* infection lead to a significant increase in IL-10 production in broncho-alveolar lavage fluids and IL-10 mRNA levels were found to increase in *M. gallisepticum* and murine mycoplasma diseases [176, 177]. Thus it remains unknown which cytokines are protective and which promote disease; hence, further studies are required to fully elucidate the chemokine and cytokine responses and identify their specific role during mycoplasma infections.

1.5.4 Non-specific stimulation of immune cells by mycoplasma

Several mycoplasma species including *M. fermentans*, *M. pneumoniae*, *M. hyorhinis*, *M. argini*, *M. penetrans* and *M. pulmonis* have been found to non-specifically stimulate B and T cells [178, 179]. Most of these early studies showed lymphocyte activating capabilities of mycoplasmas, without involvement of any accessory cells [179]. MAM or *M. arthritidis* mitogen triggers proliferation of T-cell clones and induces cytotoxic T cell development as well as non-specifically stimulates B cells. However unlike other mitogens, stimulation through MAM requires antigen presentation by MHC-II bearing cells but does not require processing of the antigen [1, 180]. *M. fermentans* also possesses mitogenic potential to human B lymphocytes and was found to polyclonally

activate B cells leading to proliferation and subsequent differentiation into plasma cells producing non-specific antibodies [178, 181, 182]. Polyclonal activation of B cells by mycoplasmas also has been observed *in vivo*. However characterization of the *M. fermentans* B-cell mitogen only led to partial success and was identified as a membrane protein [178]. In addition to mitogenic potential of various mycoplasmas, membrane protein preparations of *M. pulmonis* were also able to chemoattract resting B cells *in vitro* [183]. Taken together it is assumed that mycoplasmas possess significant mitogenic activities on host lymphocytes may exacerbate disease severity.

1.5.5 Surface variable expression of mycoplasma lipoproteins and immune evasion

There are several mechanisms by which pathogens evade host innate and adaptive immune responses, which play a significant role in disease causation. Some of these mechanisms include molecular mimicry including ligand and receptor mimics, complement inhibitors and capsules. Pathogens also have the ability to hide from immune surveillance by staying latent or hiding in phagosomes and preventing phagolysosome fusion. They also possess antigenic hypervariability thereby escaping from antibody and T cell recognition. Some pathogens are able to kill immune cells and phagocytes, block adaptive immunity and cytokine production, interfere with TLRs and also modulate apoptosis and autophagy [184]. Mycoplasmas possess many mechanisms for antigenic hypervariability and are capable of surface variable expression of the major antigenic proteins which are essentially components of the lipoprotein family. They possess several mechanisms that drive the high frequency of surface variation including spontaneous mutation by nucleotide insertion or deletion in short repeat sequences, DNA

rearrangements and gene conversion or reciprocal recombination. But only the first two mechanisms affect the variable expression of surface lipoproteins aiding in immune evasion [185-193]

A genetic system of 3-8 elements in the chromosome encoding surface lipoproteins namely Vlps, were first identified in *M. hyorhinis*. Each of these *vlp* genes represents one transcriptional element containing a poly-A tract in the promoter region between -35 and -10 and are only transcribed when the poly-A tract is 17 bp in length. Therefore insertion or deletion of single nucleotide can completely abolish their expression making it a very simple ON/OFF switch [185, 192, 194, 195]. *M. gallisepticum* possesses a similar mechanism regulating expression of the *vlhA* gene family. The importance of these molecules is reflected by the percentage of their genome devoted to lipoproteins (*vlhA*, 103kb or 10.4% of total genome), which includes 38 genes with signature *vlhA* gene features and 5 pseudogenes possessing *vlhA* sequence homology. Phase variable expression of *vlhA* occurs in the presence of 12 GAA trinucleotide repeats located at the 5' end of the promoter upstream of -35 box. [103, 196-200]. Other types of molecular switching associated with phase variation exist including a DNA recombination mechanism to alternate silent genes behind a functional promoter in *M. bovis*, *M. pulmonis* and *M. agalactiae*. A third type of mechanism involves unidirectional or reciprocal gene recombination for generating antigenic variation in *M. synoviae* and *M. genitalium*.

Although there are a significant number of studies describing mechanisms of phase variation in various mycoplasmas, their role in pathogenesis is still poorly understood. Different mycoplasmas possess an inherent ability to undergo phase and antigenic

variation and it is believed that antigenic variations resulting from phase variations are mechanisms of immune evasion helping mycoplasmas to establish chronic infection in the host despite the vigorous inflammatory and immune response [56]. Studies report that phase variable *vlhA* expression is observed in *M. gallisepticum* within a few days of infection, possibly due to selective pressure of the adaptive immune system, when the dominant *vlhA* gene is no longer expressed by most of the re-isolates. Over the ensuing weeks they express other members of the gene family suggesting a dual phase response during infection [56, 199]. Similar observations have also been made *in vitro* in the presence of antibodies to phase variable lipoproteins or sera from infected animals. It has been observed that the presence of antibody or antisera inhibits expression of dominant Vsp lipoprotein in *M. bovis* and upon removal of antibody it regains expression of that specific lipoprotein. *M. gallisepticum* was also found to express alternative VlhA variants in the presence of specific antibodies [56, 196, 197, 201]. While it is believed that phase and antigenic variation is a mechanism of immune evasion, the role of size variation in surface lipoproteins of mycoplasmas however is still poorly understood. Size variable expression of mycoplasma lipoproteins have been observed in *M. hyorhinis* as it is able to increase the length of Vlp antigens, which helps in protection from host serum antibodies. Nevertheless these sophisticated mechanisms of phase and antigenic variation and devotion of a significant proportion of their genome to these mechanisms indicate that adaptation to host immune responses is a priority for all pathogenic mycoplasmas.

1.5.6 Vaccination against mycoplasma diseases

Mycoplasma diseases are often associated with significant morbidity and sometimes mortality in animals and humans. They can cause respiratory and reproductive diseases in

hosts as well as conjunctivitis and arthritis depending on the species of mycoplasma. Despite the seriousness of the diseases, very few safe and effective vaccines have been developed to combat these pathogens. Most mycoplasma vaccines are either bacterins (a suspension of killed mycoplasma) or live attenuated strains. Some of these vaccines are mildly virulent providing only partial or transient immunity and are often associated with morbidity and may even revert to a virulent phenotype. Some vaccines may even lead to exacerbation of disease upon challenge or exposure to virulent strains due to their immuno-pathological nature [175, 202]. Therefore, due to these challenges and ineffectiveness of antibiotics in controlling mycoplasma infections, the need for safe and effective vaccines is urgently required.

One of the first mycoplasma vaccines developed was for *Mycoplasma mycoides* subsp. *mycoides* small colony type (MmmSC), the first identified mycoplasma. In fact preventive inoculations with inactivated organisms were given even before the organism was identified. Later a partially attenuated CBPP (contagious bovine pleuropneumonia) vaccine T_1/44 was created by 44 serial passages in eggs and has been used for last 60 years. However, T_1/44 only provides short-term immunity and causes adverse effects in cattle. CBPP outbreaks were also observed in closed vaccinated herds suggesting that T1/44 vaccine retained virulence [203]. Two other vaccines were also created, one a recombinant subunit vaccine using immunogenic lipoprotein LppQ [204], which did not show protective efficacy despite of two vaccinations at an interval of 6 weeks, but exacerbated the disease. Another subunit vaccine was created based on MmmSC capsular polysachharide. Antibody response was generated when this vaccine was conjugated with ovalbumin, but did not show significant reduction in mycoplasmaemia in blood

suggesting inefficient protection [202, 205]. A quadrivalent vaccine for *Mycoplasma bovis* along with respiratory syncytial virus, parainfluenza type 3 and *Mycoplasma dispar* was developed 20 years ago, and showed some protection. Another divalent vaccine for *M. bovis* with *Mannheimia hemolytica* was also found to be partially protective. More recently generated inactivated vaccine containing saponin killed cells was found to be more safe and effective against *M. bovis* [157, 202, 206].

Mycoplasma agalactiae, Mycoplasma capricolum subsp *capricolum* (Mcc) and *Mycoplasma mycoides* subsp *mycoides* large colony type (MmmLC) are pathogens of sheeps and goats. Live attenuated vaccines (LAV) were generated for *M. agalactiae* and MmmLC and have been used in Turkey however are known to cause transient infection and shedding of mycoplasmas. In Europe, inactivated laboratory strains with added adjuvants were created as a trivalent vaccine for *M. agalactiae*, MmmLC and MCC, and are currently marketed. Other vaccines for these pathogens were also created and some has shown partial protective efficacy [202].

Adjuvanated LAVs to control *Mycoplasma hyopneumoniae*, has been successful and led to significant improvement in porcine health worldwide. A recombinant vaccine using fusion protein of *M. hyopneumoniae* subunit R2 of riboneuclotide reductase NrdF and B-galactosidase was also proven effective in pigs [207]. DNA vaccines based on heat shock protein P42 were able to inhibit growth of *M. hyopneumoniae in vitro*, and also elicited both Th1 and Th2 response in mice. *M. hyopneumoniae* mucosal vaccines using bacterial toxin adjuvants, containing P97 adhesin and *E-Coli* enterotoxin LTB were also developed but their efficacy were not tested in any animal models[207].

Vaccines for rodent pathogen *Mycoplasma pulmonis* that causes respiratory disease in mice and rats had also been developed. A plasmid based expression library vaccine and DNA vaccine coding two *M. pulmonis* antigens were proven to be partially successful. Also a subunit vaccine and monoclonal antibodies against the same proteins inhibited growth of this pathogen and showed protection [202].

Two significant avian pathogens are *Mycoplasma gallisepticum* and *Mycoplasma synoviae*. A temperature sensitive mutant (MS-H) of *M. synoviae*, produced by chemical mutagenesis was developed, tested and found to be partially protective. This vaccine is currently in use worldwide and its protective efficacy lasts for about 40 weeks [202, 208]. The significant economic impact of *M. gallisepticum* infection in poultry birds led to employment of several control measures. Administration of antibiotics and maintaining single aged *M. gallisepticum* free flocks has been used to avoid vertical spread. However vaccination is the preferred mean of controlling infections.

Both killed whole cell and live attenuated vaccines have been developed to control *M. gallisepicum* infection and have been used commercially. *M. gallisepticum* bacterins with oil and aqueous emulsion or adjuvanated with aluminum hydroxide were found to be effective in preventing severe inflammatory responses and reductions in egg production, however could not could not stop mycoplasma from colonizing the respiratory tract and neither conferred complete protection [209] . Later use of bacterins was supplanted by more effective LAVs. The three most well-known live attenuated *M. gallisepticum* are the F-strain, ts-11 and 6/85 and are commercially produced. The F strain is a field-isolate occurring naturally and ts-11 is a temperature sensitive mutant created by chemical mutagenesis. The 6/85 strain is also an attenuated strain, however the nature of

attenuation is a trade secret [202, 210, 211]. Vaccination of F strain was shown to be effective for inhibiting reduction in egg production, but show low to moderate virulence in broiler chickens and turkeys[212-214]. The 6/85 strain is avirulent in both chickens and turkeys and shows significant protection [215, 216]. The ts-11 strain that can replicate in the upper respiratory tract was found to elicit protective immune responses without causing disease [217, 218]. Later a ts-11 mutant containing *M. gallisepticum* cytadhesion protein GapA was created and showed a marked increase in protective immunity [219, 220].

Papazisi *et al*. also developed another attenuated strain, GT5, which is based on non-virulent strain R_{high} strain complemented with the primary cytadhesin GapA [221]. This strain is also devoid of cytadhesion capabilities but is protective in chickens. It showed high serum IgG and comparatively lower mucosal IgA, with a corresponding reduction in lesion severity and colonization [221]. Protection involved reduced $CD4^+$ and $CD8^+$ T cells and B cells compared to control birds infected with *M. gallisepticum* strain R_{low} alone. However higher number of mycoplasma specific IgG and IgA secreting plasma cells within the trachea of vaccinated birds by day 4 post inoculation suggest significant role of antibodies in protective immunity conferred by this vaccine [162]. Another metabolically attenuated vaccine strain Mg-7 was also created in 2006 by Hudson *et al* with a mutation in the di-hydrolipoamide (lpd) gene [222] that also showed protective efficacy by inducing high *M. gallisepticum* specific IgG response and lower lesion scores [223].

Two important human mycoplasmas are *Mycoplasma pneumoniae* that cause atypical pneumonia in children and adolescents, and *Mycoplasma genitalium* that causes non-

gonococcal urethritis in women. Use of inactivated vaccines for *M. pneumoniae* still remains unsuccessful. Some heat killed vaccines showed partial protective immunity upon oral inoculation in hamsters, however led to disease exacerbation upon parenteral vaccination. Hemolytic attenuated mutants in certain cases showed only 10-50% protective efficacies but temperature sensitive mutants retained significant virulence and therefore has not been used in humans [202, 224-227]. A recent study that tested a cytadhesion deficient mutant of *M. pneumoniae* identified its capability to exacerbate disease upon challenge in mice [175].

1.6 *Mycoplasma gallisepticum*

Mycoplasma gallisepticum is a significant poultry pathogen involved in severe economic losses of the poultry industry due to a reduction in egg production, hatchability and downgrading of carcasses. Both horizontal and vertical disease transmission leads to rapid spreading of this pathogen in flocks. This respiratory and reproductive tract pathogen can cause severe chronic respiratory disease (CRD) when present in concert with other poultry pathogens including Newcastle disease virus, Infectious bronchitis virus and *E. coli* [38-40]. *M. gallisepticum* infection in chickens is characterized by coughing, nasal discharge, respiratory rales, mucous production leading to blockade of tracheal lumen, difficulty breathing and sometimes conjunctivitis [228]. More recently *M. gallisepticum* was found to cause ocular infection in house finches leading to significant decline in house finch populations [229, 230].

1.6.1 Virulence factors and pathogenesis

The critical event for *M. gallisepticum* pathogenesis is attachment and colonization of host respiratory epithelium [44, 45], leading to further pathological

changes in the host [82]. *M. gallisepticum* attachment is mediated by their primary cytadhesion molecule GapA, whereas another molecule CrmA facilitates attachment. The primary cytadhesin is an orthologoue of *M. pneumonia* cytadhesion protein P1, and CrmA shows sequence homology with ORF6 of *M. pneumonia*e [53, 231, 232]. Upon attachment they can cause release of mucus from goblet cells leading to obstruction of tracheal lumen, and rounding and exfoliation of epithelial cells as well as ciliostasis, squamous metaplasia and sometimes lysis of epithelial cells [233, 234]. Although it is known that *M. gallisepticum* can invade a variety of cell types as previously described, invasion of the tracheal epithelium is not yet documented. An unpublished observation from our laboratory using *M. gallisepticum* 16S riboprobe, did not identify invading mycoplasmas in the tracheal submucosa even at day 4 post infection (unpublished, see appendix). Another ultrastructural study of *M. gallisepticum* infection of chicken trachea, also was not able to identify invasive capabilities of this pathogen within epithelial cells [235]. Yet they are able to cause severe immunopathology associated with infiltration of macrophages, heterophils and lymphocytes.

Evaluation of *in vivo* pathogenicity of *M. gallisepticum*, performed by determining severity of tracheal and air sac lesions and feasibility of re-isolation of mycoplasma from these organs determined that a low passage strain R_{low} was virulent whereas other strains like A5969 and R_{high} was avirulent and F and S-6 only cause intermediate lesions [53, 236, 237]. Comparative analyses of these strains revealed that R_{high}, or the high passage avirulent strain do not possess the cytadhesion genes *gapA* and *crmA*, as well as fibronectin binding genes *hlp3* and *plpA* and a high affinity transporter gene *hatA*. Not only that, but this strain has 64 other point mutations compared to R_{low}

[57, 237, 238]. A virulence associated gene *mslA* was also differentially transcribed in R_{low} strain at levels 6-fold higher than in F strain. Another gene *MGA1107* was also found to be associated with virulence [238].

Variable expression of *vlhA's* or variable lipoprotein hemagglutinins in *M. gallisepticum* is known to be associated with evasion and subversion of host immune responses. A distinct VlhA antigen is believed to be predominantly expressed in a population of bacterial cells. When a host humoral response in mounted against it, another bacterial population expression a different VlhA proliferate, thereby evading the host immune response and establishing a chronic infection. These VlhA's are also known to be involved in cytadhesion to tracheal explants and RBCs and may help in mycoplasma dissemination [238, 239].

1.6.2 Immune/Inflammatory response

M. gallisepticum infection is associated with infiltration of heterophils, macrophages and lymphocytes to the tracheal submucosa, and involve both innate and adaptive responses [39, 162, 234]. Early studies investigating immune response to this pathogen identified that bursectomy and thymectomy of chickens significantly increased their susceptibility to infection suggesting the important role of B and T cells. The importance of adaptive immune response was also evident through successful vaccination using LAVs [210, 240]. It was observed that B cells play a more significant role in *M. gallisepticum* infection as maternal antibodies (IgY) were able to interfere with day 1 vaccination of chicks, and transfer of *M. gallisepticum* antisera conferred protection against air-sacculitis [241]. Protection was also correlated with local antibody response in respiratory tract that inhibited attachment of *M. gallisepticum* to ex-vivo

cultured tracheal rings [242]. Antibody mediated protection in vaccinated chickens were also observed in a study previously published from our laboratory. Vaccinated chickens were found to have higher serum IgG titers, and significantly higher numbers of *M. gallisepticum* specific IgG- and IgA-secreting plasma cells in the trachea compared to unvaccinated chickens [162]. However the cellular nature of the inflammatory responses to *M. gallisepticum* infection in the tracheal mucosa still remains controversial. A study published from our laboratory in 2005 reported B cells to be the pre-dominant cell types in tracheal submucosa of chicken after *M. gallisepticum* infection as early as 1 day post infection. By day 4, B cells were found to be surrounded by clusters of $CD4^+$ T cells in vaccinated chickens and unvaccinated chickens had an increased number of B cells clustered or scattered throughout the lamina propria with interspersed $CD4^+$T cells. $CD8^+$T cells were also found scattered throughout the lamina propria [162]. Gaunson *et al*. on the other hand, reported initial response dominated by $CD8^+$T cells and gradually replaced by $CD4^+$T cells and subsequently predominated by B cells. However their experiments were done using a different strain of *M. gallisepticum* and might explain the observed differences [243]. Even though presence of various phenotypes of T lymphocytes are known to infiltrate tracheal tissue during *M. gallisepticum* infection in chickens, very little evidence remains on the role of T cells, especially cytotoxic T cells [39].

Chemokines and cytokines have also been found to play a significant role during *M. gallisepticum* infection [115]. Production of pro-inflammatory chemokines and cytokines were found to non-specifically stimulate B and T cells [1]. Lam *et al* showed that *in vitro* infection of monocytes and macrophages caused induction of specific chemokines and

chemokines [170]. However, how epithelial cells, the primary site of *M. gallisepticum* attachment, influence the induction of inflammatory responses is not yet known.

1.7 Specific Aims

Although several advances have been made in our understanding of mycoplasmal disease pathogenesis and host pathogen interactions, the dynamics of the interaction of *M. gallisepticum* with its host remains incomplete. The research described in this dissertation aims to characterize the cellular and molecular events of host-pathogen interactions during *M. gallisepticum* disease pathogenesis in more relevant situations.

The first study was aimed at identifying how *M. gallisepticum* lipid associated membrane proteins or LAMPs interact with chicken tracheal epithelial cells; and how these interactions may be important for mycoplasma disease pathogenesis. The hypothesis of this study was that *M. gallisepticum* LAMPs ligate to TLR-2 on chicken tracheal epithelial cells and up-regulate inflammatory genes via activation of NF-κB. The specific aims for this research study were

1. To characterize differential gene expression in primary tracheal epithelial cells cultured *in vitro* upon exposure to LAMPs from a virulent (R_{low}) and a non-virulent (R_{high}) *M. gallisepticum* strain as well as both the live strains using microarray and RT-qPCR analysis
2. To characterize expression of inflammatory genes in chicken tracheal epithelial cells upon *ex vivo* exposure of trachea to LAMPs and live mycoplasma using RT-qPCR

3. To identify if TLR-2 ligation of LAMPs are involved and leads to downstream activation of NF-κB to initiate the above mentioned phenomenon from chicken tracheal epithelial cells using specific inhibitors.

Whilst the first study was aimed at understanding the role of *M. gallisepticum* and associated LAMP mediated activation of tracheal epithelial cells, what happens next remained unknown. Mycoplasma infections as previously described are associated with severe leukocyte infiltration into the submucosa and macrophages are one of the very important cell type involved in this response. Whereas macrophages play significant role in mycoplasma clearance, they are also known for the production of cytokines associated with inflammatory lesions, and chemokines and cytokines associated with macrophage activation were observed to be differentially regulated in chicken trachea upon experimental infection with *M. gallispeticum* [115, 132, 170, 172, 173]. Therefore a second study was designed to identify how mycoplasma and host epithelial cell interaction might be responsible for the inflammatory responses observed during *M. gallisepticum* infection, in context of macrophages. In this study we also tried to identify why a virulent strain of mycoplasma is able to elicit a more robust immunopathology when compared to a non-virulent strain. Specific aims of this study involved are as follows

1. Chemotaxis assay for HD-11 cells (chicken macrophage cell line) towards the conditioned medium obtained from tracheal epithelial cells exposed to live *M. gallisepticum* strain R_{low} or R_{high}.

2. Co-culture assay of chicken macrophage cell line HD-11 with tracheal epithelial cells exposed to live *M. gallisepticum* strain R_{low} or R_{high} to identify chemokine and cytokine gene expression in HD-11 cells in response to epithelial cell derived mediators
3. Chemokine and cytokine profile of HD-11 cells upon exposure to various multiplicity of infection of live *M. gallisepticum* strain R_{low} or R_{high} as positive controls.

These studies contribute to a body of knowledge regarding the mechanisms by which *M. gallisepticum,* through its interaction with the host respiratory epithelial, influences the regulation of inflammatory genes and secondary events including macrophage chemotaxis and activation. This work bring us one step closer to understanding how this primarily extracellular pathogen mediates an over-exuberant inflammatory response by cytadhering to the respiratory epithelium, and how this can be mediated without mycoplasmal invasion of the epithelial cells or the underlying tissue. Our results also explain why virulent strains are always more efficient at initiating and sustaining inflammatory responses and advance our knowledge of how the pathogen manipulates host immune responses to establish a chronic infection.

1.8 References

1. Razin, S., D. Yogev, and Y. Naot, *Molecular biology and pathogenicity of mycoplasmas.* Microbiol Mol Biol Rev, 1998. **62**(4): p. 1094-156.
2. Mushegian, A.R. and E.V. Koonin, *A minimal gene set for cellular life derived by comparison of complete bacterial genomes.* Proc Natl Acad Sci U S A, 1996. **93**(19): p. 10268-73.
3. Gibson, D.G., *et al*., *Creation of a bacterial cell controlled by a chemically synthesized genome.* Science, 2010. **329**(5987): p. 52-6.
4. Dybvig, K. and L.L. Voelker, *Molecular biology of mycoplasmas.* Annu Rev Microbiol, 1996. **50**: p. 25-57.
5. Leigh, M.W. and W.A. Clyde, Jr., *Chlamydial and mycoplasmal pneumonias.* Semin Respir Infect, 1987. **2**(3): p. 152-8.
6. Gass, R., *et al*., *Donor-to-host transmission of Mycoplasma hominis in lung allograft recipients.* Clin Infect Dis, 1996. **22**(3): p. 567-8.
7. Meyer, R.D. and W. Clough, *Extragenital Mycoplasma hominis infections in adults: emphasis on immunosuppression.* Clin Infect Dis, 1993. **17 Suppl 1**: p. S243-9.
8. Razin, S., *Structure and function in mycoplasma.* Annu Rev Microbiol, 1969. **23**: p. 317-56.
9. *Mycoplasmas : molecular biology and pathogenesis*. 1992: American Society for Microbiology.
10. Edward, D.G. and E.A. Freundt, *The classification and nomenclature of organisms of the pleuropneumonia group.* J Gen Microbiol, 1956. **14**(1): p. 197-207.
11. Atkinson, T.P., M.F. Balish, and K.B. Waites, *Epidemiology, clinical manifestations, pathogenesis and laboratory detection of Mycoplasma pneumoniae infections.* Fems Microbiology Reviews, 2008. **32**(6): p. 956-973.
12. Waites, K.B. and D.F. Talkington, *Mycoplasma pneumoniae and its role as a human pathogen.* Clin Microbiol Rev, 2004. **17**(4): p. 697-728, table of contents.
13. Chu, H.W., *et al*., *Effects of respiratory Mycoplasma pneumoniae infection on allergen-induced bronchial hyperresponsiveness and lung inflammation in mice.* Infect Immun, 2003. **71**(3): p. 1520-6.
14. Sutherland, E.R. and R.J. Martin, *Asthma and atypical bacterial infection.* Chest, 2007. **132**(6): p. 1962-6.
15. Sethi, S., *et al*., *Mycoplasma genitalium: an emerging sexually transmitted pathogen.* Indian J Med Res, 2012. **136**(6): p. 942-55.
16. Short, V.L., *et al*., *Clinical presentation of Mycoplasma genitalium Infection versus Neisseria gonorrhoeae infection among women with pelvic inflammatory disease.* Clin Infect Dis, 2009. **48**(1): p. 41-7.
17. Uuskula, A. and P.K. Kohl, *Genital mycoplasmas, including Mycoplasma genitalium, as sexually transmitted agents.* Int J STD AIDS, 2002. **13**(2): p. 79-85.
18. Napierala Mavedzenge, S. and H.A. Weiss, *Association of Mycoplasma genitalium and HIV infection: a systematic review and meta-analysis.* AIDS, 2009. **23**(5): p. 611-20.

19. Lo, S.C., *et al*., *Adhesion onto and invasion into mammalian cells by mycoplasma penetrans: a newly isolated mycoplasma from patients with AIDS.* Mod Pathol, 1993. **6**(3): p. 276-80.
20. Berman, S.M., *et al*., *Low birth weight, prematurity, and postpartum endometritis. Association with prenatal cervical Mycoplasma hominis and Chlamydia trachomatis infections.* JAMA, 1987. **257**(9): p. 1189-94.
21. Pilo, P., J. Frey, and E.M. Vilei, *Molecular mechanisms of pathogenicity of Mycoplasma mycoides subsp. mycoides SC.* Vet J, 2007. **174**(3): p. 513-21.
22. Hale, H.H., *et al*., *Bovine mastitis caused by a Mycoplasma species.* Cornell Vet, 1962. **52**: p. 582-91.
23. Caswell, J.L. and M. Archambault, *Mycoplasma bovis pneumonia in cattle.* Anim Health Res Rev, 2007. **8**(2): p. 161-86.
24. Thiaucourt, F. and G. Bolske, *Contagious caprine pleuropneumonia and other pulmonary mycoplasmoses of sheep and goats.* Rev Sci Tech, 1996. **15**(4): p. 1397-414.
25. Bergonier, D., X. Berthelot, and F. Poumarat, *Contagious agalactia of small ruminants: current knowledge concerning epidemiology, diagnosis and control.* Rev Sci Tech, 1997. **16**(3): p. 848-73.
26. Thacker, E.L., *Immunology of the porcine respiratory disease complex.* Vet Clin North Am Food Anim Pract, 2001. **17**(3): p. 551-65.
27. Kobisch, M. and N.F. Friis, *Swine mycoplasmoses.* Rev Sci Tech, 1996. **15**(4): p. 1569-605.
28. Cole, B.C., L. Golightly, and J.R. Ward, *Characterization of mycoplasma strains from cats.* J Bacteriol, 1967. **94**(5): p. 1451-8.
29. Johnsrude, J.D., *et al*., *Isolation of Mycoplasma felis from a serval (Felis serval) with severe respiratory disease.* J Wildl Dis, 1996. **32**(4): p. 691-4.
30. Ogilvie, T.H., *et al*., *Mycoplasma felis as a cause of pleuritis in horses.* J Am Vet Med Assoc, 1983. **182**(12): p. 1374-6.
31. Cole, B.C., *The immunobiology of Mycoplasma arthritidis and its superantigen MAM.* Curr Top Microbiol Immunol, 1991. **174**: p. 107-19.
32. Brown, D.R., L.A. Zacher, and W.G. Farmerie, *Spreading factors of Mycoplasma alligatoris, a flesh-eating mycoplasma.* J Bacteriol, 2004. **186**(12): p. 3922-7.
33. Brown, D.R., *et al*., *Mycoplasma alligatoris sp. nov., from American alligators.* Int J Syst Evol Microbiol, 2001. **51**(Pt 2): p. 419-24.
34. Brown, D.R., *et al*., *Pathology of experimental mycoplasmosis in American alligators.* J Wildl Dis, 2001. **37**(4): p. 671-9.
35. Kirchhoff, H. and R. Rosengarten, *Isolation of a motile mycoplasma from fish.* J Gen Microbiol, 1984. **130**(9): p. 2439-45.
36. Stipkovits, L. and I. Kempf, *Mycoplasmoses in poultry.* Rev Sci Tech, 1996. **15**(4): p. 1495-525.
37. Jordan, F.T., *Mycoplasmosis in poultry.* Isr J Med Sci, 1981. **17**(7): p. 540-7.
38. Sato, S., *et al*., *Mixed infection with Mycoplasma gallisepticum and the B1 strain of Newcastle disease virus in chickens.* Natl Inst Anim Health Q (Tokyo), 1970. **10**(2): p. 58-65.

39. Gaunson, J.E., *et al*., *Lymphocytic infiltration in the chicken trachea in response to Mycoplasma gallisepticum infection.* Microbiology, 2000. **146 (Pt 5)**: p. 1223-9.
40. Stipkovits, L., *et al*., *Effect of low-pathogenicity influenza virus H3N8 infection on Mycoplasma gallisepticum infection of chickens.* Avian Pathol, 2012. **41**(1): p. 51-7.
41. Fischer, J.R., *et al*., *Mycoplasmal conjunctivitis in wild songbirds: the spread of a new contagious disease in a mobile host population.* Emerg Infect Dis, 1997. **3**(1): p. 69-72.
42. Luttrell, M.P., *et al*., *Mycoplasma gallisepticum in house finches (Carpodacus mexicanus) and other wild birds associated with poultry production facilities.* Avian Dis, 2001. **45**(2): p. 321-9.
43. Ley, D.H., J.E. Berkhoff, and J.M. McLaren, *Mycoplasma gallisepticum isolated from house finches (Carpodacus mexicanus) with conjunctivitis.* Avian Dis, 1996. **40**(2): p. 480-3.
44. Razin, S. and E. Jacobs, *Mycoplasma adhesion.* J Gen Microbiol, 1992. **138**(3): p. 407-22.
45. Razin, S., *Adherence of pathogenic mycoplasmas to host cells.* Biosci Rep, 1999. **19**(5): p. 367-72.
46. Rottem, S., *Interaction of mycoplasmas with host cells.* Physiol Rev, 2003. **83**(2): p. 417-32.
47. Krause, D.C., *Mycoplasma pneumoniae cytadherence: unravelling the tie that binds.* Mol Microbiol, 1996. **20**(2): p. 247-53.
48. Dallo, S.F., *et al*., *Biofunctional domains of the Mycoplasma pneumoniae P30 adhesin.* Infect Immun, 1996. **64**(7): p. 2595-601.
49. Ogle, K.F., K.K. Lee, and D.C. Krause, *Nucleotide sequence analysis reveals novel features of the phase-variable cytadherence accessory protein HMW3 of Mycoplasma pneumoniae.* Infect Immun, 1992. **60**(4): p. 1633-41.
50. Layh-Schmitt, G. and M. Harkenthal, *The 40- and 90-kDa membrane proteins (ORF6 gene product) of Mycoplasma pneumoniae are responsible for the tip structure formation and P1 (adhesin) association with the Triton shell.* FEMS Microbiol Lett, 1999. **174**(1): p. 143-9.
51. Krause, D.C., *Mycoplasma pneumoniae cytadherence: organization and assembly of the attachment organelle.* Trends Microbiol, 1998. **6**(1): p. 15-8.
52. Himmelreich, R., *et al*., *Complete sequence analysis of the genome of the bacterium Mycoplasma pneumoniae.* Nucleic Acids Res, 1996. **24**(22): p. 4420-49.
53. Papazisi, L., *et al*., *Analysis of cytadherence-deficient, GapA-negative Mycoplasma gallisepticum strain R.* Infect Immun, 2000. **68**(12): p. 6643-9.
54. Cole, B.C., R.A. Daynes, and J.R. Ward, *Stimulation of mouse lymphocytes by a mitogen derived from Mycoplasma arthritidis. I. Transformation is associated with an H-2-linked gene that maps to the I-E/I-C subregion.* J Immunol, 1981. **127**(5): p. 1931-6.
55. Moritz, T., *et al*., *Lymphoproliferative responses of spleen cells of inbred rat strains to Mycoplasma arthritidis mitogen.* Scand J Immunol, 1984. **20**(4): p. 365-9.

56. Marshall, L.J., *et al*., *IL-8 released constitutively by primary bronchial epithelial cells in culture forms an inactive complex with secretory component.* J Immunol, 2001. **167**(5): p. 2816-23.
57. Papazisi, L., *et al*., *GapA and CrmA coexpression is essential for Mycoplasma gallisepticum cytadherence and virulence.* Infect Immun, 2002. **70**(12): p. 6839-45.
58. Shimizu, T., Y. Kida, and K. Kuwano, *Cytoadherence-dependent induction of inflammatory responses by Mycoplasma pneumoniae.* Immunology. **133**(1): p. 51-61.
59. Shimizu, T., *et al*., *Cytadherence of Mycoplasma pneumoniae Induces Inflammatory Responses through Autophagy and Toll-like Receptor 4.* Infect Immun, 2014.
60. Shimizu, T. and M. Miyata, *Electron microscopic studies of three gliding Mycoplasmas, Mycoplasma mobile, M. pneumoniae, and M. gallisepticum, by using the freeze-substitution technique.* Curr Microbiol, 2002. **44**(6): p. 431-4.
61. Wolgemuth, C.W., O. Igoshin, and G. Oster, *The motility of mollicutes.* Biophys J, 2003. **85**(2): p. 828-42.
62. Bose, S.R., M.F. Balish, and D.C. Krause, *Mycoplasma pneumoniae cytoskeletal protein HMW2 and the architecture of the terminal organelle.* J Bacteriol, 2009. **191**(21): p. 6741-8.
63. Indikova, I., M. Vronka, and M.P. Szostak, *First identification of proteins involved in motility of Mycoplasma gallisepticum.* Vet Res, 2014. **45**(1): p. 99.
64. Lo, S.C., *et al*., *Mycoplasma penetrans sp. nov., from the urogenital tract of patients with AIDS.* Int J Syst Bacteriol, 1992. **42**(3): p. 357-64.
65. Taylor-Robinson, D., *et al*., *Intracellular location of mycoplasmas in cultured cells demonstrated by immunocytochemistry and electron microscopy.* Int J Exp Pathol, 1991. **72**(6): p. 705-14.
66. Jensen, J.S., J. Blom, and K. Lind, *Intracellular location of Mycoplasma genitalium in cultured Vero cells as demonstrated by electron microscopy.* Int J Exp Pathol, 1994. **75**(2): p. 91-8.
67. Rosengarten, R., *et al*., *Host-pathogen interactions in mycoplasma pathogenesis: virulence and survival strategies of minimalist prokaryotes.* Int J Med Microbiol, 2000. **290**(1): p. 15-25.
68. Winner, F., R. Rosengarten, and C. Citti, *In vitro cell invasion of Mycoplasma gallisepticum.* Infect Immun, 2000. **68**(7): p. 4238-44.
69. Meseguer, M.A., *et al*., *Mycoplasma pneumoniae: a reduced-genome intracellular bacterial pathogen.* Infect Genet Evol, 2003. **3**(1): p. 47-55.
70. Diaz-Garcia, F.J., *et al*., *Mycoplasma hominis attaches to and locates intracellularly in human spermatozoa.* Hum Reprod, 2006. **21**(6): p. 1591-8.
71. Much, P., *et al*., *Mycoplasma gallisepticum: Influence of cell invasiveness on the outcome of experimental infection in chickens.* FEMS Immunol Med Microbiol, 2002. **34**(3): p. 181-6.
72. Groebel, K., *et al*., *Mycoplasma suis invades porcine erythrocytes.* Infect Immun, 2009. **77**(2): p. 576-84.
73. Dusanic, D., *et al*., *Mycoplasma synoviae invades non-phagocytic chicken cells in vitro.* Vet Microbiol, 2009. **138**(1-2): p. 114-9.

74. van der Merwe, J., T. Prysliak, and J. Perez-Casal, *Invasion of bovine peripheral blood mononuclear cells and erythrocytes by Mycoplasma bovis.* Infect Immun, 2010. **78**(11): p. 4570-8.
75. Kornspan, J.D., M. Tarshis, and S. Rottem, *Invasion of melanoma cells by Mycoplasma hyorhinis: enhancement by protease treatment.* Infect Immun, 2010. **78**(2): p. 611-7.
76. Vogl, G., *et al*., *Mycoplasma gallisepticum invades chicken erythrocytes during infection.* Infect Immun, 2008. **76**(1): p. 71-7.
77. Tarshis, M., *et al*., *Intracellular location and survival of Mycoplasma penetrans within HeLa cells.* Curr Microbiol, 2004. **49**(2): p. 136-40.
78. Yavlovich, A., *et al*., *Mycoplasma fermentans binds to and invades HeLa cells: involvement of plasminogen and urokinase.* Infect Immun, 2004. **72**(9): p. 5004-11.
79. Furnkranz, U., *et al*., *Factors influencing the cell adhesion and invasion capacity of Mycoplasma gallisepticum.* Acta Vet Scand, 2013. **55**: p. 63.
80. Indikova, I., *et al*., *Role of the GapA and CrmA cytadhesins of Mycoplasma gallisepticum in promoting virulence and host colonization.* Infect Immun, 2013.
81. Yavlovich, A., M. Tarshis, and S. Rottem, *Internalization and intracellular survival of Mycoplasma pneumoniae by non-phagocytic cells.* FEMS Microbiol Lett, 2004. **233**(2): p. 241-6.
82. Tajima, M., T. Nunoya, and T. Yagihashi, *An ultrastructural study on the interaction of Mycoplasma gallisepticum with the chicken tracheal epithelium.* Am J Vet Res, 1979. **40**(7): p. 1009-14.
83. Krivan, H.C., *et al*., *Adhesion of Mycoplasma pneumoniae to sulfated glycolipids and inhibition by dextran sulfate.* J Biol Chem, 1989. **264**(16): p. 9283-8.
84. Roberts, D.D., *et al*., *Sialic acid-dependent adhesion of Mycoplasma pneumoniae to purified glycoproteins.* J Biol Chem, 1989. **264**(16): p. 9289-93.
85. Geary, S.J., *et al*., *Identification of Mycoplasma binding proteins utilizing a 100 kilodalton lung fibroblast receptor.* J Recept Res, 1989. **9**(6): p. 465-78.
86. Shimizu, T., Y. Kida, and K. Kuwano, *A dipalmitoylated lipoprotein from Mycoplasma pneumoniae activates NF-kappa B through TLR1, TLR2, and TLR6.* J Immunol, 2005. **175**(7): p. 4641-6.
87. Zhang, Q., T.F. Young, and R.F. Ross, *Glycolipid receptors for attachment of Mycoplasma hyopneumoniae to porcine respiratory ciliated cells.* Infect Immun, 1994. **62**(10): p. 4367-73.
88. Hsu, T., S. Artiushin, and F.C. Minion, *Cloning and functional analysis of the P97 swine cilium adhesin gene of Mycoplasma hyopneumoniae.* J Bacteriol, 1997. **179**(4): p. 1317-23.
89. Raymond, B.B., *et al*., *P159 from Mycoplasma hyopneumoniae binds porcine cilia and heparin and is cleaved in a manner akin to ectodomain shedding.* J Proteome Res, 2013. **12**(12): p. 5891-903.
90. Deutscher, A.T., *et al*., *Mycoplasma hyopneumoniae Surface proteins Mhp385 and Mhp384 bind host cilia and glycosaminoglycans and are endoproteolytically processed by proteases that recognize different cleavage motifs.* J Proteome Res, 2012. **11**(3): p. 1924-36.

91. Prince, O.A., T.M. Krunkosky, and D.C. Krause, *In vitro spatial and temporal analysis of Mycoplasma pneumoniae colonization of human airway epithelium.* Infect Immun, 2014. **82**(2): p. 579-86.
92. Mall, M.A., *Role of cilia, mucus, and airway surface liquid in mucociliary dysfunction: lessons from mouse models.* J Aerosol Med Pulm Drug Deliv, 2008. **21**(1): p. 13-24.
93. DeBey, M.C. and R.F. Ross, *Ciliostasis and loss of cilia induced by Mycoplasma hyopneumoniae in porcine tracheal organ cultures.* Infect Immun, 1994. **62**(12): p. 5312-8.
94. Engelhardt, J.A. and M.G. Gabridge, *Effect of squamous metaplasia on infection of hamster trachea organ cultures with Mycoplasma pneumoniae.* Infect Immun, 1977. **15**(2): p. 647-55.
95. Wann, A.K. and M.M. Knight, *Primary cilia elongation in response to interleukin-1 mediates the inflammatory response.* Cell Mol Life Sci, 2012. **69**(17): p. 2967-77.
96. Kabelitz, D. and R. Medzhitov, *Innate immunity--cross-talk with adaptive immunity through pattern recognition receptors and cytokines.* Curr Opin Immunol, 2007. **19**(1): p. 1-3.
97. Browning, G.F. and C. Citti, *Mollicutes: Molecular Biology and Pathogenesis*. 2014: Caister Academic Press.
98. Kumar, A. and L.K. Yerneni, *Semi-automated relative quantification of cell culture contamination with mycoplasma by Photoshop-based image analysis on immunofluorescence preparations.* Biologicals, 2009. **37**(1): p. 55-60.
99. Zuo, L.L., Y.M. Wu, and X.X. You, *Mycoplasma lipoproteins and Toll-like receptors.* J Zhejiang Univ Sci B, 2009. **10**(1): p. 67-76.
100. Shimizu, T., Y. Kida, and K. Kuwano, *A triacylated lipoprotein from Mycoplasma genitalium activates NF-kappaB through Toll-like receptor 1 (TLR1) and TLR2.* Infect Immun, 2008. **76**(8): p. 3672-8.
101. Chambaud, I., H. Wroblewski, and A. Blanchard, *Interactions between mycoplasma lipoproteins and the host immune system.* Trends Microbiol, 1999. **7**(12): p. 493-9.
102. You, X.X., Y.H. Zeng, and Y.M. Wu, *Interactions between mycoplasma lipid-associated membrane proteins and the host cells.* J Zhejiang Univ Sci B, 2006. **7**(5): p. 342-50.
103. Papazisi, L., *et al*., *The complete genome sequence of the avian pathogen Mycoplasma gallisepticum strain R(low).* Microbiology, 2003. **149**(Pt 9): p. 2307-16.
104. Muhlradt, P.F., *et al*., *Isolation, structure elucidation, and synthesis of a macrophage stimulatory lipopeptide from Mycoplasma fermentans acting at picomolar concentration.* J Exp Med, 1997. **185**(11): p. 1951-8.
105. Shibata, K., *et al*., *The N-terminal lipopeptide of a 44-kDa membrane-bound lipoprotein of Mycoplasma salivarium is responsible for the expression of intercellular adhesion molecule-1 on the cell surface of normal human gingival fibroblasts.* J Immunol, 2000. **165**(11): p. 6538-44.
106. Deiters, U. and P.F. Muhlradt, *Mycoplasmal lipopeptide MALP-2 induces the chemoattractant proteins macrophage inflammatory protein 1alpha (MIP-*

1alpha), monocyte chemoattractant protein 1, and MIP-2 and promotes leukocyte infiltration in mice. Infect Immun, 1999. **67**(7): p. 3390-8.
107. Seya, T. and M. Matsumoto, *A lipoprotein family from Mycoplasma fermentans confers host immune activation through Toll-like receptor 2.* Int J Biochem Cell Biol, 2002. **34**(8): p. 901-6.
108. Shimizu, T., Y. Kida, and K. Kuwano, *Triacylated lipoproteins derived from Mycoplasma pneumoniae activate nuclear factor-kappaB through toll-like receptors 1 and 2.* Immunology, 2007. **121**(4): p. 473-83.
109. He, J., *et al.*, *Mycoplasma genitalium-derived lipid-associated membrane proteins activate NF-kappaB through toll-like receptors 1, 2, and 6 and CD14 in a MyD88-dependent pathway.* Clin Vaccine Immunol, 2009. **16**(12): p. 1750-7.
110. He, J., *et al.*, *Binding of CD14 to Mycoplasma genitalium-Derived Lipid-Associated Membrane Proteins Upregulates TNF-alpha.* Inflammation, 2014. **37**(2): p. 322-30.
111. Liu, Y.C., *et al.*, *Proteomics characterization of cytoplasmic and lipid-associated membrane proteins of human pathogen Mycoplasma fermentans M64.* PLoS One, 2012. **7**(4): p. e35304.
112. McGowin, C.L., *et al.*, *Mycoplasma genitalium-encoded MG309 activates NF-kappaB via Toll-like receptors 2 and 6 to elicit proinflammatory cytokine secretion from human genital epithelial cells.* Infect Immun, 2009. **77**(3): p. 1175-81.
113. Takeuchi, O., *et al.*, *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 Immunol, 2000. **164**(2): p. 554-7.
114. Kaufmann, A., *et al.*, *Induction of cytokines and chemokines in human monocytes by Mycoplasma fermentans-derived lipoprotein MALP-2.* Infect Immun, 1999. **67**(12): p. 6303-8.
115. Mohammed, J., *et al.*, *Chemokine and cytokine gene expression profiles in chickens inoculated with Mycoplasma gallisepticum strains Rlow or GT5.* Vaccine, 2007. **25**(51): p. 8611-21.
116. Markham, P.F., *et al.*, *Homologue of macrophage-activating lipoprotein in Mycoplasma gallisepticum is not essential for growth and pathogenicity in tracheal organ cultures.* J Bacteriol, 2003. **185**(8): p. 2538-47.
117. Bercic, R.L., *et al.*, *Identification of major immunogenic proteins of Mycoplasma synoviae isolates.* Vet Microbiol, 2008. **127**(1-2): p. 147-54.
118. Lavric, M., *et al.*, *Mycoplasma synoviae lipoprotein MSPB, the N-terminal part of VlhA haemagglutinin, induces secretion of nitric oxide, IL-6 and IL-1beta in chicken macrophages.* Vet Microbiol, 2007. **121**(3-4): p. 278-87.
119. Romero, F., *et al.*, *In vivo immunomodulation by Mycoplasma fermentans membrane lipoprotein.* Curr Microbiol, 2004. **48**(3): p. 237-9.
120. Shimizu, T., Y. Kida, and K. Kuwano, *Mycoplasma pneumoniae-derived lipopeptides induce acute inflammatory responses in the lungs of mice.* Infect Immun, 2008. **76**(1): p. 270-7.

121. Love, W., *et al*., *Toll-like receptor 2 (TLR2) plays a major role in innate resistance in the lung against murine Mycoplasma.* PLoS One, 2010. **5**(5): p. e10739.
122. Into, T., *et al*., *Extracellular ATP regulates cell death of lymphocytes and monocytes induced by membrane-bound lipoproteins of Mycoplasma fermentans and Mycoplasma salivarium.* Microbiol Immunol, 2002. **46**(10): p. 667-75.
123. Into, T., *et al*., *Mycoplasmal lipoproteins induce toll-like receptor 2- and caspases-mediated cell death in lymphocytes and monocytes.* Microbiol Immunol, 2002. **46**(4): p. 265-76.
124. Into, T., *et al*., *Stimulation of human Toll-like receptor (TLR) 2 and TLR6 with membrane lipoproteins of Mycoplasma fermentans induces apoptotic cell death after NF-kappa B activation.* Cell Microbiol, 2004. **6**(2): p. 187-99.
125. Wu, Y., *et al*., *Mycoplasma genitalium lipoproteins induce human monocytic cell expression of proinflammatory cytokines and apoptosis by activating nuclear factor kappaB.* Mediators Inflamm, 2008. **2008**: p. 195427.
126. Zeng, Y., *et al*., *Apoptosis induced by lipid-associated membrane proteins from Mycoplasma penetrans is mediated by nuclear factor kappaB activation in mouse macrophage.* Can J Microbiol, 2008. **54**(2): p. 150-8.
127. Gerlic, M., *et al*., *The inhibitory effect of Mycoplasma fermentans on tumour necrosis factor (TNF)-alpha-induced apoptosis resides in the membrane lipoproteins.* Cell Microbiol, 2007. **9**(1): p. 142-53.
128. Hopfe, M. and B. Henrich, *OppA, the ecto-ATPase of Mycoplasma hominis induces ATP release and cell death in HeLa cells.* BMC Microbiol, 2008. **8**: p. 55.
129. Jordan, J.L., *et al*., *Protein P200 is dispensable for Mycoplasma pneumoniae hemadsorption but not gliding motility or colonization of differentiated bronchial epithelium.* Infect Immun, 2007. **75**(1): p. 518-22.
130. Oven, I., *et al*., *Diacylated lipopeptide from Mycoplasma synoviae mediates TLR15 induced innate immune responses.* Vet Res, 2013. **44**: p. 99.
131. Marshall, A.J., R.J. Miles, and L. Richards, *The phagocytosis of mycoplasmas.* J Med Microbiol, 1995. **43**(4): p. 239-50.
132. Hickman-Davis, J.M., *Role of innate immunity in respiratory mycoplasma infection.* Front Biosci, 2002. **7**: p. d1347-55.
133. Rubartelli, A., *DAMP-Mediated Activation of NLRP3-Inflammasome in Brain Sterile Inflammation: The Fine Line between Healing and Neurodegeneration.* Front Immunol, 2014. **5**: p. 99.
134. Khare, S., *et al*., *An NLRP7-containing inflammasome mediates recognition of microbial lipopeptides in human macrophages.* Immunity, 2012. **36**(3): p. 464-76.
135. Into, T., *et al*., *Synergic effects of mycoplasmal lipopeptides and extracellular ATP on activation of macrophages.* Infect Immun, 2002. **70**(7): p. 3586-91.
136. Xu, Y., *et al*., *Mycoplasma hyorhinis activates the NLRP3 inflammasome and promotes migration and invasion of gastric cancer cells.* PLoS One, 2013. **8**(11): p. e77955.
137. Howard, C.J. and G. Taylor, *Variation in the virulence of strains of Mycoplasma pulmonis related to susceptibility to killing by macrophages in vivo.* J Gen Microbiol, 1979. **114**(2): p. 289-94.

138. Webster, A.D., *et al*., *Critical dependence on antibody for defence against mycoplasmas.* Clin Exp Immunol, 1988. **71**(3): p. 383-7.
139. Ueno, N. and M.E. Wilson, *Receptor-mediated phagocytosis of Leishmania: implications for intracellular survival.* Trends Parasitol, 2012. **28**(8): p. 335-44.
140. Howard, C.J., *Variation in the susceptibility of bovine mycoplasmas to killing by the alternative complement pathway in bovine serum.* Immunology, 1980. **41**(3): p. 561-8.
141. Howard, C.J., R.N. Gourlay, and G. Taylor, *Immunity to Mycoplasma bovis infections of the respiratory tract of calves.* Res Vet Sci, 1980. **28**(2): p. 242-9.
142. Bronte, V. and P. Zanovello, *Regulation of immune responses by L-arginine metabolism.* Nat Rev Immunol, 2005. **5**(8): p. 641-54.
143. Hickman-Davis, J.M., *et al*., *Surfactant protein A mediates mycoplasmacidal activity of alveolar macrophages.* Am J Physiol, 1998. **274**(2 Pt 1): p. L270-7.
144. Hickman-Davis, J., *et al*., *Surfactant protein A mediates mycoplasmacidal activity of alveolar macrophages by production of peroxynitrite.* Proc Natl Acad Sci U S A, 1999. **96**(9): p. 4953-8.
145. Hickman-Davis, J. and S. Matalon, *Surfactant protein B deficiency worsens hyperoxic injury to the alveolar epithelium.* Am J Respir Cell Mol Biol, 1999. **21**(4): p. 449-50.
146. D'Orazio, J.A., B.C. Cole, and J. Stein-Streilein, *Mycoplasma arthritidis mitogen up-regulates human NK cell activity.* Infect Immun, 1996. **64**(2): p. 441-7.
147. Lai, W.C., *et al*., *Resistance to Mycoplasma pulmonis mediated by activated natural killer cells.* J Infect Dis, 1990. **161**(6): p. 1269-75.
148. Davis, J.K. and G.H. Cassell, *Murine respiratory mycoplasmosis in LEW and F344 rats: strain differences in lesion severity.* Vet Pathol, 1982. **19**(3): p. 280-93.
149. Davis, J.K., *et al*., *Murine respiratory mycoplasmosis in F344 and LEW rats: evolution of lesions and lung lymphoid cell populations.* Infect Immun, 1982. **36**(2): p. 720-9.
150. Rodriguez, F., *et al*., *Immunohistochemical characterization of lung lesions induced experimentally by Mycoplasma agalactiae and Mycoplasma bovis in goats.* J Comp Pathol, 2000. **123**(4): p. 285-93.
151. Cartner, S.C., *et al*., *Roles of innate and adaptive immunity in respiratory mycoplasmosis.* Infect Immun, 1998. **66**(8): p. 3485-91.
152. Evengard, B., *et al*., *Intranasal inoculation of Mycoplasma pulmonis in mice with severe combined immunodeficiency (SCID) causes a wasting disease with grave arthritis.* Clin Exp Immunol, 1994. **98**(3): p. 388-94.
153. Blanchard, A. and G. Browning, *Mycoplasmas: Molecular Biology Pathogenicity and Strategies for Control*. 2005: CRC Press. 600.
154. Foy, H.M., *Infections caused by Mycoplasma pneumoniae and possible carrier state in different populations of patients.* Clin Infect Dis, 1993. **17 Suppl 1**: p. S37-46.
155. Hayakawa, M., *et al*., *Animal model of Mycoplasma pneumoniae infection using germfree mice.* Clin Diagn Lab Immunol, 2002. **9**(3): p. 669-76.

156. Jones, H.P., *et al*., *Depletion of CD8+ T cells exacerbates CD4+ Th cell-associated inflammatory lesions during murine mycoplasma respiratory disease.* J Immunol, 2002. **168**(7): p. 3493-501.
157. Howard, C.J., *et al*., *Protection against respiratory disease in calves induced by vaccines containing respiratory syncytial virus, parainfluenza type 3 virus, Mycoplasma bovis and M dispar.* Vet Rec, 1987. **121**(16): p. 372-6.
158. Tamura, H., T. Fujinaga, and T. Kuroume, *IgD antibody to Mycoplasma pneumoniae in human sera as measured by the RAST.* Int Arch Allergy Appl Immunol, 1982. **69**(4): p. 335-9.
159. Avakian, A.P. and D.H. Ley, *Protective immune response to Mycoplasma gallisepticum demonstrated in respiratory-tract washings from M. gallisepticum-infected chickens.* Avian Dis, 1993. **37**(3): p. 697-705.
160. Brown, M.B., *et al*., *Measurement of antibody to Ureaplasma urealyticum by an enzyme-linked immunosorbent assay and detection of antibody responses in patients with nongonococcal urethritis.* J Clin Microbiol, 1983. **17**(2): p. 288-95.
161. Brown, M.B., *et al*., *Antigens of Mycoplasma hominis.* Sex Transm Dis, 1983. **10**(4 Suppl): p. 247-55.
162. Javed, M.A., *et al*., *Correlates of immune protection in chickens vaccinated with Mycoplasma gallisepticum strain GT5 following challenge with pathogenic M. gallisepticum strain R(low).* Infect Immun, 2005. **73**(9): p. 5410-9.
163. Seggev, J.S., *et al*., *Mycoplasma pneumoniae is a frequent cause of exacerbation of bronchial asthma in adults.* Ann Allergy, 1986. **57**(4): p. 263-5.
164. Yano, T., *et al*., *Association of Mycoplasma pneumoniae antigen with initial onset of bronchial asthma.* Am J Respir Crit Care Med, 1994. **149**(5): p. 1348-53.
165. Smith-Norowitz, T.A., *et al*., *Asthmatic children have increased specific anti-Mycoplasma pneumoniae IgM but not IgG or IgE-values independent of history of respiratory tract infection.* Pediatr Infect Dis J, 2013. **32**(6): p. 599-603.
166. Gomez, M.I. and A. Prince, *Airway epithelial cell signaling in response to bacterial pathogens.* Pediatr Pulmonol, 2008. **43**(1): p. 11-9.
167. McGowin, C.L., *et al*., *Persistent Mycoplasma genitalium infection of human endocervical epithelial cells elicits chronic inflammatory cytokine secretion.* Infect Immun, 2012. **80**(11): p. 3842-9.
168. McGowin, C.L., V.L. Popov, and R.B. Pyles, *Intracellular Mycoplasma genitalium infection of human vaginal and cervical epithelial cells elicits distinct patterns of inflammatory cytokine secretion and provides a possible survival niche against macrophage-mediated killing.* BMC Microbiol, 2009. **9**: p. 139.
169. Yang, J., *et al*., *Regulation of proinflammatory cytokines in human lung epithelial cells infected with Mycoplasma pneumoniae.* Infect Immun, 2002. **70**(7): p. 3649-55.
170. Lam, K.M., *The macrophage inflammatory protein-1beta in the supernatants of Mycoplasma gallisepticum-infected chicken leukocytes attracts the migration of chicken heterophils and lymphocytes.* Dev Comp Immunol, 2002. **26**(1): p. 85-93.
171. Shimizu, T., Y. Kida, 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): p. 121-9.

172. Faulkner, C.B., *et al*., *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.* Infect Immun, 1995. **63**(10): p. 4084-90.
173. Nishimoto, M., *et al*., *Gene expression of tumor necrosis factor alpha and interferon gamma in the lungs of Mycoplasma pulmonis-infected mice.* Microbiol Immunol, 1994. **38**(5): p. 345-52.
174. Koh, Y.Y., *et al*., *Levels of interleukin-2, interferon-gamma, and interleukin-4 in bronchoalveolar lavage fluid from patients with Mycoplasma pneumonia: implication of tendency toward increased immunoglobulin E production.* Pediatrics, 2001. **107**(3): p. E39.
175. Szczepanek, S.M., *et al*., *Vaccination of BALB/c mice with an avirulent Mycoplasma pneumoniae P30 mutant results in disease exacerbation upon challenge with a virulent strain.* Infect Immun, 2012. **80**(3): p. 1007-14.
176. Bodhankar, S., *et al*., *Interferon gamma and interleukin 4 have contrasting effects on immunopathology and the development of protective adaptive immunity against mycoplasma respiratory disease.* J Infect Dis, 2010. **202**(1): p. 39-51.
177. Lorenzo, H., *et al*., *Cytokine expression in porcine lungs experimentally infected with Mycoplasma hyopneumoniae.* Vet Immunol Immunopathol, 2006. **109**(3-4): p. 199-207.
178. Simecka, J.W., *et al*., *Interactions of mycoplasmas with B cells: antibody production and nonspecific effects.* Clin Infect Dis, 1993. **17 Suppl 1**: p. S176-82.
179. Ruuth, E. and F. Praz, *Interactions between mycoplasmas and the immune system.* Immunol Rev, 1989. **112**: p. 133-60.
180. Matthes, M., *et al*., *Clonal analysis of human T cell activation by the Mycoplasma arthritidis mitogen (MAS).* Eur J Immunol, 1988. **18**(11): p. 1733-7.
181. Cheek, R.F., *et al*., *In vitro detection of Mycoplasma fermentans binding to B-lymphocytes in fresh peripheral blood using flow cytometry.* Cytometry, 1997. **28**(1): p. 90-5.
182. Feng, S.H. and S.C. Lo, *Induced mouse spleen B-cell proliferation and secretion of immunoglobulin by lipid-associated membrane proteins of Mycoplasma fermentans incognitus and Mycoplasma penetrans.* Infect Immun, 1994. **62**(9): p. 3916-21.
183. Ross, S.E., *et al*., *Mycoplasma pulmonis possesses a novel chemoattractant for B lymphocytes.* Infect Immun, 1992. **60**(2): p. 669-74.
184. Finlay, B.B. and G. McFadden, *Anti-immunology: evasion of the host immune system by bacterial and viral pathogens.* Cell, 2006. **124**(4): p. 767-82.
185. Citti, C., L.X. Nouvel, and E. Baranowski, *Phase and antigenic variation in mycoplasmas.* Future Microbiol, 2010. **5**(7): p. 1073-85.
186. Rosengarten, R., *et al*., *Antigenic variation in Mycoplasma hyorhinis: increased repertoire of variable lipoproteins expanding surface diversity and structural complexity.* Infect Immun, 1993. **61**(5): p. 2224-8.
187. Wise, K.S., *Adaptive surface variation in mycoplasmas.* Trends Microbiol, 1993. **1**(2): p. 59-63.
188. Wise, K.S., *et al*., *A family of strain-variant surface lipoproteins of Mycoplasma fermentans.* Infect Immun, 1993. **61**(8): p. 3327-33.

189. Yogev, D., R. Rosengarten, and K.S. Wise, *Variation and genetic control of surface antigen expression in mycoplasmas: the Vlp system of Mycoplasma hyorhinis.* Zentralbl Bakteriol, 1993. **278**(2-3): p. 275-86.
190. Chopra-Dewasthaly, R., *et al.*, *Construction of the first shuttle vectors for gene cloning and homologous recombination in Mycoplasma agalactiae.* FEMS Microbiol Lett, 2005. **253**(1): p. 89-94.
191. Citti, C. and R. Rosengarten, *Mycoplasma genetic variation and its implication for pathogenesis.* Wien Klin Wochenschr, 1997. **109**(14-15): p. 562-8.
192. Citti, C., *et al.*, *Gene families encoding phase- and size-variable surface lipoproteins of Mycoplasma hyorhinis.* J Bacteriol, 2000. **182**(5): p. 1356-63.
193. Glew, M.D., *et al.*, *Characterization of a multigene family undergoing high-frequency DNA rearrangements and coding for abundant variable surface proteins in Mycoplasma agalactiae.* Infect Immun, 2000. **68**(8): p. 4539-48.
194. Cleavinger, C.M., *et al.*, *Identification of mycoplasma membrane proteins by systematic Tn phoA mutagenesis of a recombinant library.* Mol Microbiol, 1995. **18**(2): p. 283-93.
195. Levisohn, S., R. Rosengarten, and D. Yogev, *In vivo variation of Mycoplasma gallisepticum antigen expression in experimentally infected chickens.* Vet Microbiol, 1995. **45**(2-3): p. 219-31.
196. Glew, M.D., *et al.*, *Expression of the pMGA genes of Mycoplasma gallisepticum is controlled by variation in the GAA trinucleotide repeat lengths within the 5' noncoding regions.* Infect Immun, 1998. **66**(12): p. 5833-41.
197. Markham, P.F., *et al.*, *Expression of two members of the pMGA gene family of Mycoplasma gallisepticum oscillates and is influenced by pMGA-specific antibodies.* Infect Immun, 1998. **66**(6): p. 2845-53.
198. Markham, P.F., *et al.*, *The organisation of the multigene family which encodes the major cell surface protein, pMGA, of Mycoplasma gallisepticum.* FEBS Lett, 1994. **352**(3): p. 347-52.
199. Glew, M.D., *et al.*, *pMGA phenotypic variation in Mycoplasma gallisepticum occurs in vivo and is mediated by trinucleotide repeat length variation.* Infect Immun, 2000. **68**(10): p. 6027-33.
200. Liu, L., V.S. Panangala, and K. Dybvig, *Trinucleotide GAA repeats dictate pMGA gene expression in Mycoplasma gallisepticum by affecting spacing between flanking regions.* J Bacteriol, 2002. **184**(5): p. 1335-9.
201. Bai, F., *et al.*, *Mycoplasma hyopneumoniae-derived lipid-associated membrane proteins induce apoptosis in porcine alveolar macrophage via increasing nitric oxide production, oxidative stress, and caspase-3 activation.* Vet Immunol Immunopathol, 2013. **155**(3): p. 155-61.
202. Nicholas, R.A., R.D. Ayling, and L. McAuliffe, *Vaccines for Mycoplasma diseases in animals and man.* J Comp Pathol, 2009. **140**(2-3): p. 85-96.
203. Mbulu, R.S., *et al.*, *Contagious bovine pleuropneumonia (CBPP) caused by vaccine strain T1/44 of Mycoplasma mycoides subsp. mycoides SC.* Vet Microbiol, 2004. **98**(3-4): p. 229-34.
204. Abdo, E.M., J. Nicolet, and J. Frey, *Antigenic and genetic characterization of lipoprotein LppQ from Mycoplasma mycoides subsp. mycoides SC.* Clin Diagn Lab Immunol, 2000. **7**(4): p. 588-95.

205. Waite, E.R. and J.B. March, *Capsular polysaccharide conjugate vaccines against contagious bovine pleuropneumonia: Immune responses and protection in mice.* J Comp Pathol, 2002. **126**(2-3): p. 171-82.
206. Nicholas, R.A., R.D. Ayling, and L.P. Stipkovits, *An experimental vaccine for calf pneumonia caused by Mycoplasma bovis: clinical, cultural, serological and pathological findings.* Vaccine, 2002. **20**(29-30): p. 3569-75.
207. Fagan, P.K., *et al.*, *Molecular characterization of a ribonucleotide reductase (nrdF) gene fragment of Mycoplasma hyopneumoniae and assessment of the recombinant product as an experimental vaccine for enzootic pneumonia.* Infect Immun, 1996. **64**(3): p. 1060-4.
208. Jones, J.F., *et al.*, *Duration of immunity with Mycoplasma synoviae: comparison of the live attenuated vaccine MS-H (Vaxsafe MS) with its wild-type parent strain, 86079/7NS.* Avian Dis, 2006. **50**(2): p. 228-31.
209. Yagihashi, T., T. Nunoya, and M. Tajima, *Immunity induced with an aluminum hydroxide-adsorbed Mycoplasma gallisepticum bacterin in chickens.* Avian Dis, 1987. **31**(1): p. 149-55.
210. Whithear, K.G., *Control of avian mycoplasmoses by vaccination.* Rev Sci Tech, 1996. **15**(4): p. 1527-53.
211. Whithear, K.G., K.E. Harrigan, and S.H. Kleven, *Standardized method of aerosol challenge for testing the efficacy of Mycoplasma gallisepticum vaccines.* Avian Dis, 1996. **40**(3): p. 654-60.
212. Rodriguez, R. and S.H. Kleven, *Evaluation of a vaccine against Mycoplasma gallisepticum in commercial broilers.* Avian Dis, 1980. **24**(4): p. 879-89.
213. Rodriguez, R. and S.H. Kleven, *Pathogenicity of two strains of Mycoplasma gallisepticum in broilers.* Avian Dis, 1980. **24**(4): p. 800-7.
214. Lin, M.Y. and S.H. Kleven, *Cross-immunity and antigenic relationships among five strains of Mycoplasma gallisepticum in young Leghorn chickens.* Avian Dis, 1982. **26**(3): p. 496-507.
215. Evans, R.D. and Y.S. Hafez, *Evaluation of a Mycoplasma gallisepticum strain exhibiting reduced virulence for prevention and control of poultry mycoplasmosis.* Avian Dis, 1992. **36**(2): p. 197-201.
216. Evans, R.D., Y.S. Hafez, and F.W. Orthel, *Mycoplasma gallisepticum vaccination-challenge: an egg-production model.* Avian Dis, 1992. **36**(4): p. 956-63.
217. Whithear, K.G., *et al.*, *Immunogenicity of a temperature sensitive mutant Mycoplasma gallisepticum vaccine.* Aust Vet J, 1990. **67**(5): p. 168-74.
218. Whithear, K.G., *et al.*, *Safety of temperature sensitive mutant Mycoplasma gallisepticum vaccine.* Aust Vet J, 1990. **67**(5): p. 159-65.
219. Shil, P.K., *et al.*, *GapA+ Mycoplasma gallisepticum ts-11 has improved vaccine characteristics.* Microbiology, 2011. **157**(Pt 6): p. 1740-9.
220. Shil, P.K., *et al.*, *Development and immunogenicity of recombinant GapA(+) Mycoplasma gallisepticum vaccine strain ts-11 expressing infectious bronchitis virus-S1 glycoprotein and chicken interleukin-6.* Vaccine, 2011. **29**(17): p. 3197-205.
221. Papazisi, L., *et al.*, *A modified live Mycoplasma gallisepticum vaccine to protect chickens from respiratory disease.* Vaccine, 2002. **20**(31-32): p. 3709-19.

222. Hudson, P., *et al*., *Identification of a virulence-associated determinant, dihydrolipoamide dehydrogenase (lpd), in Mycoplasma gallisepticum through in vivo screening of transposon mutants.* Infect Immun, 2006. **74**(2): p. 931-9.
223. Gates, A.E., *et al*., *Comparative assessment of a metabolically attenuated Mycoplasma gallisepticum mutant as a live vaccine for the prevention of avian respiratory mycoplasmosis.* Vaccine, 2008. **26**(16): p. 2010-9.
224. Kottaridis, S.D., W.R. Dunlop, and S.C. Smith, *Tissue-culture-propagated mycoplasma for the control of chronic respiratory disease.* Avian Dis, 1967. **11**(4): p. 528-31.
225. Smith, C.B., W.T. Friedewald, and R.M. Chanock, *Inactivated Mycoplasma pneumoniae vaccine. Evaluation in volunteers.* JAMA, 1967. **199**(6): p. 353-8.
226. Lai, W.C., *et al*., *Vaccination of Lewis rats with temperature-sensitive mutants of Mycoplasma pulmonis: adoptive transfer of immunity by spleen cells but not by sera.* Infect Immun, 1991. **59**(1): p. 346-50.
227. Lai, W.C., *et al*., *Potential subunit vaccine against Mycoplasma pulmonis purified by a protective monoclonal antibody.* Vaccine, 1991. **9**(3): p. 177-84.
228. Nunoya, T., *et al*., *Natural case of salpingitis apparently caused by Mycoplasma gallisepticum in chickens.* Avian Pathol, 1997. **26**(2): p. 391-8.
229. Tulman, E.R., *et al*., *Extensive variation in surface lipoprotein gene content and genomic changes associated with virulence during evolution of a novel North American house finch epizootic strain of Mycoplasma gallisepticum.* Microbiology, 2012. **158**(Pt 8): p. 2073-88.
230. Stallknecht, D.E., *et al*., *Potential for transmission of the finch strain of Mycoplasma gallisepticum between house finches and chickens.* Avian Dis, 1998. **42**(2): p. 352-8.
231. Goh, M.S., *et al*., *Molecular and biochemical analysis of a 105 kDa Mycoplasma gallisepticum cytadhesin (GapA).* Microbiology, 1998. **144 (Pt 11)**: p. 2971-8.
232. Willby, M.J., *et al*., *HMW1 is required for stability and localization of HMW2 to the attachment organelle of Mycoplasma pneumoniae.* J Bacteriol, 2004. **186**(24): p. 8221-8.
233. Hod, I., *et al*., *Early detection of tracheal damage in chickens by scanning electron microscopy.* Avian Dis, 1982. **26**(2): p. 450-7.
234. Dykstra, M.J., *et al*., *Evaluation of cytopathologic changes induced in chicken tracheal epithelium by Mycoplasma gallisepticum in vivo and in vitro.* Am J Vet Res, 1985. **46**(1): p. 116-22.
235. Abu-Zahr, M.N. and M. Butler, *Ultrastructural features of Mycoplasma gallisepticum in tracheal explants under transmission and stereoscan electron microscopy.* Res Vet Sci, 1978. **24**(2): p. 248-53.
236. Levisohn, S., *et al*., *Comparison of in vivo and in vitro methods for pathogenicity evaluation for Mycoplasma gallisepticum in respiratory infection.* Avian Pathol, 1986. **15**(2): p. 233-46.
237. May, M., *et al*., *Identification of fibronectin-binding proteins in Mycoplasma gallisepticum strain R.* Infect Immun, 2006. **74**(3): p. 1777-85.
238. Szczepanek, S.M., *et al*., *Comparative genomic analyses of attenuated strains of Mycoplasma gallisepticum.* Infect Immun, 2010. **78**(4): p. 1760-71.

239. Forsyth, M.H., M.E. Tourtellotte, and S.J. Geary, *Localization of an immunodominant 64 kDa lipoprotein (LP 64) in the membrane of Mycoplasma gallisepticum and its role in cytadherence.* Mol Microbiol, 1992. **6**(15): p. 2099-106.
240. Levisohn, S. and S.H. Kleven, *Avian mycoplasmosis (Mycoplasma gallisepticum).* Rev Sci Tech, 2000. **19**(2): p. 425-42.
241. Lin, M.Y. and S.H. Kleven, *Transferred humoral immunity in chickens to Mycoplasma gallisepticum.* Avian Dis, 1984. **28**(1): p. 79-87.
242. Avakian, A.P. and D.H. Ley, *Inhibition of Mycoplasma gallisepticum growth and attachment to chick tracheal rings by antibodies to a 64-kilodalton membrane protein of M. gallisepticum.* Avian Dis, 1993. **37**(3): p. 706-14.
243. Gaunson, J.E., *et al*., *The cellular immune response in the tracheal mucosa to Mycoplasma gallisepticum in vaccinated and unvaccinated chickens in the acute and chronic stages of disease.* Vaccine, 2006. **24**(14): p. 2627-33.

Chapter II

Mycoplasma gallisepticum Lipid Associated Membrane Proteins Up-regulate Inflammatory Genes in Chicken Tracheal Epithelial Cells via TLR-2 Ligation Through an NF-κB Dependent Pathway

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Published: PLoS One, November 2014, 9(11): e112796

2.1 Abstract

Mycoplasma gallisepticum-mediated respiratory inflammation in chickens is associated with accumulation of leukocytes in the tracheal submucosa. However the molecular mechanisms underpinning these changes have not been well described. We hypothesized that the initial inflammatory events are initiated upon ligation of mycoplasma lipid associated membrane proteins (LAMP) to TLRs expressed on chicken tracheal epithelial cells (TEC). To test this hypothesis, live bacteria or LAMPs isolated from a virulent (R_{low}) or a non-virulent (R_{high}) strain were incubated with primary TECs or chicken tracheae *ex vivo*. Microarray analysis identified up-regulation of several inflammatory and chemokine genes in TECs as early as 1.5 hours post-exposure. Kinetic analysis using RT-qPCR identified the peak of expression for most genes to be at either 1.5 or 6 hours. *Ex-vivo* exposure also showed up-regulation of inflammatory genes in epithelial cells by 1.5 hours. Among the commonly up-regulated genes were IL-1β, IL-6, IL-8, IL-12p40, CCL-20, and NOS-2, all of which are important immune-modulators and/or chemo-attractants of leukocytes. While these inflammatory genes were up-regulated in all four treatment groups, R_{low} exposed epithelial cells both *in vitro* and *ex vivo* showed the most dramatic up-regulation, inducing over 100 unique genes by 5-fold or more in TECs. Upon addition of a TLR-2 inhibitor, LAMP-mediated gene expression of IL-1β and CCL-20 was reduced by almost 5-fold while expression of IL-12p40, IL-6, IL-8 and NOS-2 mRNA was reduced by about 2-3 fold. Conversely, an NF-κB inhibitor abrogated the response entirely for all six genes. miRNA-146a, a negative regulator of TLR-2 signaling, was up-regulated in TECs in response to either R_{low} or R_{high} exposure. Taken together we conclude that LAMPs isolated from both R_{high} and R_{low} induced rapid,

TLR-2 dependent but transient up-regulation of inflammatory genes in primary TECs through an NF-κB dependent pathway.

2.2 Introduction

Mycoplasma gallisepticum (*M. gallisepticum*) is an avian respiratory pathogen causing severe inflammation of the trachea, air sacs and lungs, especially in the presence of a co-infection [1-3]. This pathogen is known to invade, survive and multiply inside a variety of non-phagocytic cells such as chicken RBCs, HeLa cells, and chicken fibroblasts, [4-9]. In addition, *M. gallisepticum* is known to colonize many extra-pulmonary tissues including blood, heart, spleen, liver and brain [4, 5, 7, 8, 10]. Indikova et al. (2013) suggested that invasion may occur at the air sac, where the mucosal barrier is quite thin [7]. However, there is yet no clear evidence that *M. gallisepticum* invades tracheal epithelial cells *in vivo* [unpublished observations], as it predominantly colonizes the mucosal surface and only rarely is found inside phagocytic vacuoles [11]. Nonetheless, the organism orchestrates immuno-pathological changes in the tracheal mucosa marked by infiltration of heterophils, macrophages and lymphocytes [2, 12, 13] soon after attachment and colonization of the respiratory surface.

A previous study from our laboratory reported up-regulation of several chemokines including lymphotactin, CXCL-13, RANTES and MIP-1β in chicken trachea isolated from live birds within 24 hours of experimental *M. gallisepticum* infection [12]. These chemokines are primarily produced by macrophages, lymphocytes and NK cells; cell types not found in large numbers in the uninfected tracheal mucosa [14-19]. However, chemokines and cytokines that are produced by epithelial cells upon infection are known

for their ability to recruit phagocytic cells and lymphocytes into infected tissues [20]. Due to the protective layer of mucus, it is not clear if the initial interaction of mycoplasmas with the host epithelium is driven by viable organisms or microbial components such as lipoprotein-bearing membrane fragments or both, although substantial evidence supports the notion that the initial "pathogen perception" occurs upon interaction of various PAMPs with TLRs [20-24]. Previous studies conducted using other mycoplasma species suggest an important role for epithelial cells in inflammation. For example, A549 human lung epithelial cells increase their production of IL-8, TNF-α, IL-1β, and IL-6 following *Mycoplasma pneumoniae (M. pneumoniae)* exposure [25]. Similarly, cultured human endocervical epithelial cells exposed to *Mycoplasma genitalium (M. genitalium)* secreted several pro-inflammatory chemokines and cytokines including IL-6, IL-7, IL-8, MCP-1 and GM-CSF [26-28].

Due to the lack of a peptidoglycan cell wall or outer membrane, mycoplasmas do not possess lipopolysaccharides (LPS), lipotechoic acid or flagella. Even though certain mycoplasmas are known for production of exotoxins, like the *M. pneumoniae* CARDS toxin or *Mycoplasma arthritidis* mitogen MAM [29-32], the majority of mycoplasmas including *M. gallisepticum* are not known to produce or secrete any exotoxin. Their surface-exposed membranes are composed of a single lipid bi-layer with numerous embedded integral and peripheral proteins and membrane anchored lipoproteins [33-35]. Phase and antigenic variable expression of these membrane lipoproteins provides a mechanism of immune evasion [36-46], and the importance of these molecules is reflected by the percentage of the mycoplasma genome devoted to lipoproteins. For example, in *M. gallisepticum* about 10% of the genome is devoted to *vlhA*s (variable

lipoprotein hemagglutinins) which includes 38 genes with signature *vlhA* features and 5 pseudogenes possessing *vlhA* sequence homology [47].

Mycoplasma lipoproteins are known to partition into the Triton X-114 detergent phase during phase partitioning. This detergent phase fraction may also contain other hydrophobic proteins besides lipoproteins [48], and therefore has been termed “lipid associated membrane proteins" (LAMPs) [48-51]. In other mycoplasma species, the detergent phase fraction containing these LAMPs was found to activate NF-κB via TLR-1, 2, 6 as well as CD-14 via a MyD88 pathway, and induce expression of pro-inflammatory cytokines in monocytes and macrophages [43, 48, 50-53]. Recently, it was also found that mycoplasma LAMPs are capable of activating the NLRP3 inflammasome resulting in the induction of IL-1β [54]. Several other studies found that lipoproteins purified from the TX-114 fraction induce inflammatory responses via TLR-2 or TLR-1/2 andTLR-2/6 heterodimers [28, 34, 48-50, 55-59]. However, the vast majority of these *in-vitro* studies were performed using leukocytes even though the initial interactions between mycoplasma membrane components and host cells occur at the mucosal surface upon contact with epithelial cells [28, 60, 61]. Thus, we hypothesized that LAMPs of *M. gallisepticum* ligate TLR-2 on respiratory epithelial cells, resulting in the up-regulation of inflammatory chemokine and cytokine genes via an NF- κB dependent pathway. To test this hypothesis, *M. gallisepticum* LAMPs were incubated with primary chicken tracheal epithelial cells in culture (TEC) or tracheae *ex vivo* to examine differential gene expression and to determine if the response is mediated via TLR-2 ligation. Similar studies using viable organisms were conducted to assess the relative contribution of

LAMPs to early inflammatory events, using the low passage, adherent and virulent *M. gallisepticum* strain R_{low} or a high passage, non-adherent and non-virulent strain, $R_{high.}$

2.3 Materials and Methods

Bacterial Strains and Culture Conditions

M. gallisepticum low passage virulent strain R_{low} (passage 17) and high passage non-virulent strain R_{high} (passage 167) were cultured at 37^{o} C in modified Hayflicks medium supplemented with 10% horse serum and 5% yeast extract until mid-log phase as determined by acid-mediated shift of phenol red dye from red to orange, as previously described [2]. Cell density was measured and a mycoplasma concentration of 0.9 x 10^{8} to1.6 x 10^{8} cfu (colony forming unit) per milliliter, approximately mid-log phase, was used.

Isolation of TX-114 phase proteins

M. gallisepticum strains R_{low} and R_{high} were grown to mid-log phase as previously described and pelleted by centrifuging at 10,000 x g for 20 minutes. Pellets were washed twice with PBS and suspended in 750µl of TS buffer (10mM Tris, 150 mM NaCl, pH 7.5) containing 1mM PMSF and 1% TX-114 and rocked for 30 minutes at 4^{o}C, followed by centrifugation at 10,000 x g at 4^{o}C for 5 minutes. The soluble phase was transferred to a new tube and incubated at 37^{o}C with rocking for 10 minutes and then centrifuged for 5 minutes at room temperature to separate the aqueous and detergent phases. 500 µl of TS buffer containing 1mM PMSF and 1% TX114 was added to the detergent phase obtained in the previous step and thoroughly mixed and incubated for 15 min at 4^{o}C followed by 10 min at 37^{o}C with rocking followed by centrifugation for 5 minutes (at 10,000x g) at

room temperature. The detergent phase was collected and the previous step was repeated to obtain the final detergent phase. The LAMPs were precipitated overnight at -20^{o}C with 2 volumes of methanol and centrifuged at 15,000 xg for 20 minutes. Precipitated protein was suspended in PBS by sonication for 30 seconds at output 5 using Biosonik ultrasonic disintegrator (Bronwill Scientific; Rochester, NY). Protein concentration was determined by Quick Start^{TM} Bradford protein assay kit (Bio Rad; Hercules, CA) according to manufacturer's instructions.

Cell culture and exposure/stimulation

A primary chicken tracheal epithelial cell culture was established based upon two previously described protocols [62, 63]. Five-week-old female specific pathogen free chicken tracheae were obtained from SPAFAS (Charles River Laboratories; Mansfield, CT) in sterile PBS (phosphate-buffered saline) containing 1X penicillin-streptomycin to inhibit bacterial and fungal growth. The tracheae were rinsed twice in DMEM (Dulbecco's modified eagles medium) (Gibco®, Life technologies; Grand Island, NY) under sterile conditions. After removal of the surrounding adipose and muscular tissues, tracheae were cut into 2cm pieces and incised vertically. They were then twice rinsed for 5 minutes in PBS/DTT (Dithiothreitol) (Sigma Aldrich; St. Louis, Missouri) (0.0385 g DTT- in 50ml 1X PBS) to remove non-adherent mucus. The pieces were then rinsed twice with PBS and DMEM. To dissociate the epithelial cells from the underlying connective tissue, about 3-4 tracheal pieces were placed in a T25 flask containing 25ml dissociation solution [DMEM 50ml, protease type XIV 0.14g (Sigma Aldrich), DNAse 0.01g (Sigma Aldrich) and antibiotic/antimycotic 1X (Gibco®, Life technologies)]. The flasks were gently shaken for 15 minutes at 37°C to slightly loosen the cells. 10% FBS

(Gibco®, Life technologies) was added to block protease activity after incubation. The tracheae were washed twice with fresh DMEM. The luminal surface of tracheae was scraped gently with a sterile scalpel in a petri dish containing fresh DMEM to obtain the epithelial cells. Cells were centrifuged at 1,250 rpm for 5 minutes to remove media. The epithelial cells were placed in 10 ml dissociation solution for 10 minutes at 37°C and then pipetted up and down several times to dissociate clumped cells. 10% FBS was added to the dissociation solution to stop the reaction. Cells were centrifuged at 1,250 rpm for 5 min to remove residual enzymes and washed twice with PBS. Cells were suspended in DMEM supplemented with 10% FBS and plated in a T75 flask for 4 hours to allow adherent cells (primarily fibroblasts and macrophages) to attach. The unattached cells were collected and precipitated by centrifugation at 1,250 rpm for 5 minutes and suspended in ATE medium [DMEM F-12+ Glutamax, (Gibco®, Life technologies) 10% FBS (Gibco®, Life technologies), 10% chick embryo extract (US Biologicals; Salem, MA), 1X MEM non-essential amino acids (NEAA) (Gibco®, Life technologies), 1X antibiotic/antimycotic (Gibco®, Life technologies)], counted on a hemocytometer and plated on 5% matrigel (BD Biosciences; San Jose, CA) coated T12.5 flasks. Exposure studies were done at 96 hours post-plating when the flasks reached 70-80% confluence, at which time they were exposed to either $5x10^8$ cfu (roughly equal to 500 multiplicity of infection (MOI) live strain $R_{low,}$ R_{high} as previously described [64] or LAMPs isolated from each strain at a concentration of 5µg/mL. This concentration was based on preliminary studies where 5µg/mL and 50 µg/mL LAMPs showed comparable potency (based on changes in gene regulation) and roughly equivalent to 500 MOI ($5x10^8$ cfu)

live mycoplasma. All experiments were done with 6 replicates each for 1.5, 6, and 24 hours.

Whole tracheal exposure

Tracheae from 5-week-old female specific-pathogen free white leghorn chickens were obtained from SPAFAS (Charles River Laboratories; Mansfied, CT) in sterile PBS containing 1X penicillin-streptomycin to inhibit bacterial and fungal growth. Surrounding adipose tissues were removed and tracheae were cut into 0.5 inch pieces by vertical incision and rinsed 3 times with PBS and DMEM under sterile conditions. The tracheae were exposed to either 10^9 cfu *M. gallisepticum* strains R_{low} and R_{high} or 10 µg/mL LAMPs from either strain for 1.5 or 6 hours in ATE medium at 37^0C, 5% CO_2. After exposure, tracheae were digested in dissociation solution (described previously) for 15 minutes at 37^0C and immediately placed on ice. Epithelial cells from the luminal surface of tracheal pieces were lightly scraped using a sterile scalpel. Epithelial cells were then preserved in RNA later (Ambion, Life Technologies; Grand Island, NY) at 4^0C for future RNA isolation (detailed below). All exposures were done with 6 replicates each for 1.5, and 6 hours.

Immunocytostaining

Tracheal epithelial cells and DF-1(Chicken embryonic fibroblast) cells grown on coverslips were fixed with 10% formalin and permeablized with 0.25% Triton X-100. Immunocytochemistry was performed using primary monoclonal anti-vimentin antibody (Sigma-aldrich) and anti E-cadherin antibody (Millipore; Billerica, MA) at a concentration of 1:200. Fluorescence-tagged secondary antibodies used were goat anti

mouse IgG FITC (Sigma-aldrich) for visualization of vimentin and Alexa Fluor® 546 Goat Anti-Rabbit IgG (H+L) (Life Technologies; Grand Island, NY) for visualization of E-cadherin at a 1:250 dilution. Vectashied® HardSet mounting medium containing DAPI (4′, 6-Diamidino-2-phenylindole) (Vector Laboratories; Burlingame, CA) was used to mount cells on a slide for imaging. Images of immunostaining were captured using a Nikon A1R Spectral Confocal Microscope (Nikon Instruments Inc.; Melville, NY).

Signaling inhibitors

Tracheal epithelial cells were incubated with either 3 µg/mL R_{low} LAMPs or 5 µg/mL R_{high} LAMPs in the presence or absence of signaling inhibitors. TLR-2/4 signaling inhibitor OxPAPC (oxidated 1-palmitoyl-2-arachidonyl-sn- glycero-3-phosphorylcholine), TLR-4 inhibitor CLI-095, and NF-κB inhibitor Celastrol were purchased from Invivogen (Invivogen; San Diego, CA). Cells were co-incubated with either 10 µg/mL or 30 µg/mL OxPAPC and LAMPs for 6 hours, in accordance with the manufacturer's instruction. Cells were pre-incubated with 1 µg/mL CLI-095 for 6 hours before exposure to LAMPs and then further incubated for 6 hours after LAMP exposure. Pre-incubation of cells with 5 µM Celastrol for 30 minutes was done before LAMP exposure for 6 hours. All experiments were performed with 6 replicates each.

RNA isolation and cDNA synthesis

Total RNA was extracted from cells using TRIzol reagent (Life Technologies) and purified using RNeasy mini columns according to the manufacturer's instructions (Qiagen; Valencia, CA). On-column DNase digestion was done using RNase free DNase (Qiagen;). RNA quality and quantity was assessed using the Agilent 2100 Bioanalyzer

with the RNA pico kit (Agilent technologies; Mendon, MA). All RNA samples had a RNA integrity number of 8 or more. 500 ng of RNA from individual samples was reverse transcribed using iScript^{TM} reverse transcription supermix for RT-qPCR (Bio Rad; Hercules, CA) according to the manufacturer's recommendation, and 2µg RNA was reverse transcribed for PCR reaction to assess epithelial-specific gene expression.

Microarray

Agilent chicken gene expression microarray slides were utilized (Agilent technologies). Total RNA (50 ng) was utilized as starting material for microarray hybridization. Four biological replicates for each of five exposure conditions utilized were: R_{high}, R_{low}, R_{high} LAMPs, R_{low} LAMPs and media control. Dye swap technical replicates were created for each biological replicate totaling eight replicates for each exposure condition. The Agilent two-color microarray-based gene expression analysis protocol version 6.5 (http://www.genome.duke.edu/cores/microarray/services/agilent-microarrays/documents/LIQA_G4140-90050_GeneExpression_Two_Color_v6.5.pdf) was followed. All incubations were performed utilizing the Applied Biosystem® Geneamp PCR system 9700 (Life Technologies). The Agilent two-color low-input quick amp labeling kit (Agilent technologies) was utilized for cDNA synthesis, *in-vitro* amplification and labeling of nucleic acids. Purification of labeled cRNA was performed with the Qiagen RNeasy mini kit). Purified cRNA was fragmented using the Agilent gene expression hybridization kit (Agilent technologies). Microarray slides and hybridization gasket were assembled in a hybridization chamber (Agilent technologies). Samples were placed in a rotating hybridization oven set to 10 rpm at 65°C for 17 hours. Microarray slides were scanned using a GenePix 4000B laser scanner (Molecular Devices;

Sunnyvale, CA) following the instructions in the GenePixPro 7 user guide. Feature extraction was performed utilizing GenePixPro 7 software (Molecular Devices). Microarray images were visually inspected for quality control of features (spots on microarray slide that have spotted oligonucleotide probes). Background fluorescence of each feature was calculated as the mean of the five closest negative control features. Each channel's background-corrected median fluorescence value was used in an intensity-based analysis utilizing Agilent GeneSpring (v12.5) software (Agilent technologies). Quantile normalization was performed on all the background-corrected intensity values and samples were clustered according to their original exposure group. Features were removed if they were at saturating intensity or flagged "bad" in the aforementioned visual inspection. Genes were removed from downstream analysis if <80% of the features representing a gene were already excluded following preprocessing of data. Genes with duplicate or triplicate features on the microarray were grouped and their mean values were used for a gene-level analysis. Quantile normalization was then performed on the gene-level *in-silico* experiment. All microarray datasets have been deposited into Gene Expression Omnibus database repository, accession number GSE61520 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE61520).

Polymerase Chain Reaction

PCR reactions were performed using cDNA to identify expression of epithelial cell-specific genes in tracheal epithelial cell cultures using GoTaq® Green master mix (Promega; Madison, WI). Cycle conditions were as follows: 94°C for 3 minutes, 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 1 minute. Step 2 to 4 was repeated 30 times.

Final extension was performed at 72°C for 10 minutes. Primers for the epithelial cells specific genes are listed on **Table 2.1**.

Real time quantitative PCR (RT-qPCR)

Primers specific for chicken genes were designed using Primer 3 input version 0.4.0 (http://frodo.wi.mit.edu/) or as described in Mohammed et al [12] **(Table 2.2)**. RT-qPCR was performed using 1µl of cDNA from the reverse transcription reaction using iTaq™ universal SYBR® green supermix (Bio Rad). Amplification was performed using an Applied Biosystem® 7900 HT (Life Technologies) by incubating samples at 50°C for 2 minutes, 95 °C for 1 min, followed by 40 cycles of 95 °C for 15 seconds and 58–60 °C (depending on Tm values of specific primers) for 60 seconds. Melt curve analysis was performed to confirm that a single, product-specific amplification had occurred. A stepwise temperature gradient was used (65-95 °C) with 0.5 °C increments and 2 sec/step. Amplicon sizes were confirmed by agarose gel electrophoresis. The reference gene for all real time reactions was GAPDH. Absolute fold change compared to the media controls were calculated using the Ct values based upon the following equation:

$$Fold\ change\left(\frac{Test}{Control}\right) = 2^{\Delta\Delta}Ct = 2^{[(Ct\ gene\ of\ interest - Ct\ GAPDH)_{Control} - (Ct\ gene\ of\ interest - Ct\ GAPDH)_{Test}]}$$

Gel electrophoresis was performed using a 0.8% agarose gel to determine the product size for each gene.

SDS PAGE and proteomics

200 µg of R_{low} LAMPs was separated by SDS-PAGE. Briefly, laemelli buffer was added to the LAMPs to 1:1 ratio and incubated at 70°C for 10 minutes. The LAMPs were then ran in 4-20% mini TGX gel (Bio-Rad) with tris glycine buffer. Bands were then

stained using coomassie blue reagent (Bio-Rad) for 1 hour and destained overnight at 4^{o}C in distilled water. Total 13 bands were cut and homogenized in 1% SDS in a tissuelyzer and centrifuged to remove the polyacrylamide gel. Each fraction was precipitated using 5 volumes of acetone at -20^{o}C overnight. TECs in 24 well plates at 70-80% confluency were exposed to LAMPs from each band at a concentration of 1µg/mL.

For proteomic characterization of R_{low} LAMPS, each of the 13 gel bands were cut and prepared for LC-MS using in gel tryptic digestion kit (Thermo Scientific; Waltham, MA). All LC-MS based characterizations were done at Mass Spectrometry Facility of University of Connecticut, Department of Chemistry with help from Dr. Xudong Yao and Dr. Fu You-Jun.

Statistical analysis

All statistical analyses were performed using the Statistical Analysis Software (SAS) program Version 9.2 (SAS Institute; Inc Cary, NC). Multiple pairwise comparisons of mRNA fold differences were analyzed using the mixed design analysis of variance (ANOVA) with repeated measures, with mRNA fold difference being the dependent variable and treatments or time being the independent variables. Post hoc mean comparison analyses were performed using least square means. Results were denoted as fold change ± SEM. Significant differences were denoted as * = $P< 0.05$, **= $P <0.01$, *** = $P < 0.001$.

2.4 Results

Tracheal epithelial cell culture and immunocytostaining

Primary tracheal epithelial cell cultures were established based on published methods [62, 63]. Confirmation was performed using PCR amplification of epithelial cell specific genes and morphological examination based on E-cadherin staining patterns. A chicken embryonic fibroblast cell line (DF-1) was used as a negative control. E-cadherin staining was observed only at the contact points between cells, which is typical of epithelial cell morphology. Very few cells in the TEC culture stained for vimentin, suggesting an extremely low level of fibroblast contamination. As a positive control, DF-1 chicken fibroblast cells stained positively for vimentin and not for E-cadherin **(Figure 2.1A-D, Figure 2.2).**

Microarray analysis – global gene expression profile

Microarray analysis of chicken tracheal epithelial cells at 1.5 hours post-exposure identified a total of 166, 43, 55 and 38 genes differentially regulated ≥ 5-fold ($p \leq 0.05$) after exposure to either live R_{low} and R_{high}, or to 5µg/mL R_{low} or R_{high} LAMPs respectively (**Figure 2.3).** 23 genes were commonly up-regulated ≥ 5-fold in TECs in all four exposure groups, which included many inflammatory chemokine and cytokine genes **(Figure 2.3; indicated by the asterisk*).** Gene ontology analysis of commonly up-regulated genes (≥2-fold) identified categories such as immune system processes, signal transduction, regulation of apoptosis and stress response **(Table 2.3, Figure 2.4)**. Exposure to viable R_{low} resulted in differential expression of 110 unique genes by a factor of ≥ 5- fold ($p \leq 0.05$) **(Table 2.4)** whereas only six or fewer genes were unique in TECs

exposed to R_{high}, R_{low} LAMPs or R_{high} LAMPs **(Figure 2.3)**. Pathway analysis identified differential expression of genes in the TLR signaling pathway, with 12 out of 70 genes commonly up-regulated in all four exposure groups. Other pathways of note included the TNF-α/NF-κB signaling, apoptosis, and type II interferon pathways **(Table 2.5 and Figure 2.5).**

Differential gene expression in R_{low}, R_{low} LAMP, R_{high} and R_{high} LAMP exposed TECs

A concentration of 5 μg/mL LAMPs optimally induced the expression of IL-1β and CCL-20, which was roughly comparable to the up-regulation induced by 500 MOI ($5x10^8$ cfu) live mycoplasma (data not shown). mRNA transcripts of six immune response associated genes up-regulated in TECs upon exposure to each of the four treatments were validated by RT-qPCR at 1.5, 6 and 24 hours. TECs' expression of IL-12p40, IL-1β and IL-6 peaked at 1.5 hours post-exposures in all four exposure groups and waned significantly thereafter in contrast to CCL-20 and nitric oxide synthase-2 (NOS-2) which peaked at 6 hours and returned to near baseline levels within 24 hours. IL-8 expression peaked at 6 hours in R_{low} exposed TECs, however R_{high} and R_{low} LAMP exposed TECs exhibited the highest level of IL-8 gene expression at 1.5 hours ($p \leq 0.01$ and $p \leq 0.001$) **(Figure 2.6A-D)**. Five out of six genes (IL-6, IL-8, IL-12p40, CCL-20 and NOS-2) were expressed at significantly higher levels in R_{low} exposed TECs when compared to TECs exposed to R_{high} or LAMPs isolated from either strain, at one or more time point **(Figure 2.7A-E)**. IL-1β gene expression in TECs did not significantly differ between any of the four exposures at any time point **(Figure 2.7F)**. We also observed that R_{low} LAMP exposed TECs had a significantly higher level of the IL-12p40 gene expression when

compared to TECs exposed to R_{high} LAMP at 1.5 hours ($p \leq 0.05$) **(Figure 2.7A)** and CCL-20 when compared to TECs exposed to R_{high} or R_{high} LAMP at 1.5 and 6 hours ($p \leq 0.01$ and $p \leq 0.001$) **(Figure 2.7D)**.

Differential gene expression in TECs by mycoplasma LAMPs in the presence of signaling inhibitors

OxPAPC, a TLR-2 and 4 signaling inhibitor, reduced expression of all 6 genes in a concentration dependent manner **(Figure 2.8A-F)**. IL-12p40 gene expression was reduced by more than 2-fold in TECs exposed to LAMPs when incubated with OxPAPC at 30 µg/mL when compared to both R_{low} ($P < 0.05$) and R_{high} LAMP ($P<0.01$) exposed TECs **(Figure 2.8A)**. IL-1β and CCL-20 gene expression were reduced by more than 5-fold compared to TECs exposed to R_{low} ($P < 0.001$) or R_{high} ($P < 0.001$) LAMPs in the absence of the inhibitor **(Figure 2.8B, E).** IL-8 ($P < 0.001$), IL-6 ($P < 0.01$) and NOS-2 ($P < 0.001$) gene expression was also significantly reduced by approximately 2-3 fold **(Figure 2.8C, D, and F).** No difference was observed upon exposure to CLI-095, a selective TLR-4 inhibitor, for any of the genes analyzed in this study, supporting the hypothesis that *M. gallisepticum* LAMPs signal via TLR-2 and not TLR-4 **(Figure 2.8A-F).** In the presence of Celastrol, an NF-κB inhibitor, cell signaling was abolished entirely (comparable to control levels) in TECs exposed to any of the four treatments ($P<0.001$ for all six genes analyzed) **(Figure 2.8A-F)**.

Comparison of gene regulation in R_{low}, R_{low} LAMP, R_{high} and R_{high} LAMP exposed tracheal epithelial cells *ex vivo*

mRNA transcripts of all six genes were also up-regulated in tracheal epithelial cells isolated from whole tracheal tissues exposed *ex vivo* to live R_{low}, R_{high} or the LAMPs isolated therefrom. Most genes followed a similar pattern of expression as seen in the *in vitro* experiments. IL-12p40 was expressed at a significantly higher level in R_{low} exposed trachea at both time points compared to others, and CCL-20 was significantly higher in R_{low} exposed trachea than all others at 1.5 hours ($p \leq 0.001$) **(Figure 2.9A, 5B).** IL-8 expression was higher in both R_{low} and R_{high} exposed trachea when compared to the LAMP exposed tracheae at both time points but IL-6 was found to be higher in the LAMP-exposed tissues ($p \leq 0.001$) at 1.5 hours and lower at 6 hours ($p \leq 0.01$, $p \leq 0.05$) when compared to tracheae exposed to live R_{low} or R_{high}. Although IL-1β expression did not differ between any exposure groups at 1.5 hours, at 6 hours tracheae exposed to the live mycoplasma were found to express IL-1β at a higher level than either LAMP-exposed tracheae ($p \leq 0.001$ for R_{low} , $p \leq 0.05$ for R_{high}). NOS-2 expression did not differ between any exposure groups at any time point **(Figure 2.9A, 5B).**

Micro RNA and IL-10 gene expression

Expression analysis of four miRNA genes including mir-21, mir-146a, mir-146b, and mir-146c1, as well as IL-10 was assessed in TECs exposed to either LAMPs or live organisms. IL-10 gene expression in all TECs peaked at 1.5 hours, concomitant with the pro-inflammatory genes **(Figure 2.10A).** Expression analysis of miRNAs showed that miRNA146a increased over time in TECs exposed to live mycoplasmas; however in TECs exposed to the LAMPs from both strains, miRNA146a expression peaked at 6

hours **(Figure 2.10B).** Expression of miRNA21, miRNA146b and miRNA146c1 did not differ from media controls at any time point in any exposure group (data not shown).

IL-1β expression by TECs upon exposure to SDS-PAGE separated LAMPs

LAMPs from R_{low} were separated using 1D SDS-PAGE and 13 distinct band of various molecular weight were obtained **(Figure 2.11A)**. TECs at 70-80% confluency on 24 well plates were exposed to 1µg/mL of LAMPs from each of the 13 fractions obtained from SDS-PAGE separation. IL-1β expression were up-regulated significantly in TECs upon exposure to LAMPs from 10 out of 13 bands (2 fold or more). 5 or more fold up-regulation of IL-1β were observed during exposure to LAMPs from bands of 110kd, 100kd, 64kd, 52kd, 23kd and 20kd **(Figure 2.11B).** When proteomic characterization of each of the 13 bands was done, several *M. gallisepticum* lipoproteins were identified as well as several hypothetical proteins **(Table 2.6).** The 6 bands that showed highest activity contained many lipoproteins, including VlhA 3.03, the primary *vlhA* expressed by *M. gallisepticum* strain R_{low} as well as virulence associated lipoprotein MslA, being the two most common lipoproteins in these bands **(Table 2.6).**

2.5 Discussion

Bacterial cell envelope components such as LPS, lipotechoic acid, peptidoglycan, flagella and lipoproteins are well characterized PAMPs that interact with host cell pattern recognition receptors such as TLRs, thereby contributing in part to the inflammation that ensues post-infection [20, 43, 65]. With the exception of lipoproteins, *M. gallisepticum* is devoid of these PAMPs, yet is able to initiate a robust inflammatory response marked by infiltration of leukocytes to the submucosa, often in absence of tissue invasion [2, 12, 13,

66, 67]. Mycoplasma lipoproteins are well known for their pro-inflammatory properties, initiated upon TLR ligation and NF-κB activation [34, 48-51, 55, 56, 68]. However, the vast majority of studies examining these effects have focused on monocytes/macrophages maintained in culture rather than epithelial cells, the primary site of mycoplasma attachment and colonization [11, 69].

Previous studies from our laboratory in which live birds were exposed to *M. gallisepticum* intra-tracheally reported alterations in inflammatory gene expression in whole tracheal tissue [12]. The current study is novel in that it reports interaction of *M. gallisepticum* lipid associated membrane proteins, a mixture of lipoproteins, specifically with host airway epithelial cells.

A study by Walter et al (2001) reported that IL-12p40 was expressed by airway epithelial cells during viral tracheobronchitis [70, 71]. We also observed IL-12p40 mRNA to be significantly up-regulated both in TECs and epithelial cells from tracheae upon exposure to live mycoplasma or LAMPs within 1.5 hours of exposure. But this signal waned rapidly thereafter, suggesting that IL-12p40 acts as an early, but not sustained, inflammatory event. IL-1β and IL-6 mRNA expression were also up-regulated and followed similar kinetics to that observed with IL-12p40. mRNA expression of two important chemokines, CCL-20 and IL-8 were also significantly up-regulated in epithelial cells *in-vitro* and *ex-vivo* after exposure to LAMPs or live organisms, as was NOS-2. These molecules are known for their ability to chemo-attract and activate leukocytes at the site of infection [72-77]. Several other inflammation associated genes, including TLR-15, were up-regulated to a lesser degree in TECs in all four exposure groups **(Table 2.3,** RT-qPCR data not shown**)**. This cytokine and chemokine expression

profile is in keeping with earlier studies that reported *M. pneumoniae* and *M. genitalium* mediated production of IL-8, TNF-α, IL-1β, IL-6 IL-7, MCP-1 and GM-CSF from epithelial cells [25-28].

A previously published study from our laboratory however, reported down-regulation of IL-12p40, IL-8, IL-1β, and CCL-20 mRNA in tracheal tissues at day-1 post-infection, whereas chemokines like MIP-1β, CXCL-13, RANTES and lymphotactin were found to be up-regulated [12]. As this later set of chemokines are known to be produced primarily by macrophages, lymphocytes and NK cells [14-19], types of cells not found in large numbers in normal tracheal mucosa, we believe transient but robust expression of chemokines and cytokines like IL-12p40, IL-8, IL-6, IL-1β, and CCL-20 by tracheal epithelial cells may be responsible for initially attracting the inflammatory cells into the tracheal submucosa. However these signals appear to be transient and subsequent signaling events appear to involve a unique set of inflammatory genes not observed in epithelial cells.

In the current study we also observed that R_{low} was by far the most potent stimulus for initiating differential gene expression by epithelial cells when compared to live R_{high} or LAMPs from either strain. TECs exposed to R_{low} not only up-regulated inflammatory genes to a significantly higher extent, but up-regulated more than a hundred additional unique genes by ≥5 fold. The R_{high} strain is non-virulent as it lacks several virulence determinant proteins of R_{low}, *especially* GapA and CrmA [7, 78] two significant cytadhesion-associated proteins homologous to P1 and P40/P90 of *M. pneumoniae* [78-84]. The significantly diminished cytadhesion capability of R_{high} [7, 82, 85, 86], may explain the reduced differential gene expression induced by this strain. Subtle differences

in gene expression observed between the LAMPs prepared from R_{low} and R_{high} may also be explained by the differences identified by Szczepanek et al. involving 29 mutations in the variable GAA repeat region associated with phase variable expression of vlhA genes between these two strains [86].

When TECs were exposed to LAMPs in the presence of OxPAPC, a competitive inhibitor of lipoprotein and LPS mediated signaling via TLR-2 and TLR-4 respectively [87], expression of all six genes was significantly reduced. However in the presence of CLI-095, a selective TLR-4 inhibitor, no changes in gene expression were observed. When TECs were exposed to Celastrol, an NF-κB inhibitor [88], LAMP-mediated gene expression was completely abrogated. This observation is in keeping with previous studies from other mycoplasma species in which LAMP-induced inflammation was mediated upon TLR-2 ligation and activation of NF-κB [28, 34, 49-51, 53, 55, 56]. Moreover, these observations apply to early time-points post-exposure, in a highly relevant cell population.

The kinetics of inflammatory gene expression in the current study was found to be rapid, peaking at either 1.5 hours or 6 hours, and then waning by 24 hours. As modulation of host responses is often accompanied by concurrent pro- and anti-inflammatory mechanisms [89], we hypothesized that a compensatory, homeostatic mechanism may be working in concert with the inflammatory response. Upon NF-κB activation, certain anti-inflammatory genes and micro RNA's are expressed that participate in the homeostatic regulation of inflammatory responses. For example, miRNA-146a, which is induced by LPS via NF-κB activation, down-regulates IRAK-1 and TRAF-6 and in turn suppresses further activation of NF-κB [90]. miRNA146a also negatively regulate TLR-2 signaling

[91]. miRNA-21, on the other hand can promote IL-10 production by regulating PDCD4 (programmed cell death 4), an inhibitor of IL-10 production [90]. We observed miRNA-146a expression to be increasingly up-regulated until 24 hours in TECs exposed to either R_{low} or R_{high} in contrast to the pattern observed upon exposure to LAMPs, which peaked at 6 hours and waned thereafter, likely due to the lack of LAMP re-stimulation [91]. No difference in expression was observed for miRNA146b, miRNA146c1 or miRNA21. Conversely, the anti-inflammatory cytokine IL-10 showed no reciprocal relationship to pro-inflammatory gene expression, but was significantly up-regulated by the epithelial cells initially upon exposure to live R_{low} and the LAMPs (but not R_{high}). IL-10 is known to selectively inhibit nuclear localization of NF-κB by blocking IκB kinase activity and inducing nuclear translocation and DNA-binding of the repressive p50-p50 homodimer [92, 93]. Therefore, miRNA-146a and in part IL-10 may play a role in regulating the over-exuberant pro-inflammatory response observed during *M.gallisepticum* infection.

When proteomic characterization of R_{low} LAMPs were done 10 out of 13 bands up-regulated IL-1β significantly with 6 of them up-regulating IL-1β by more than 5 fold. MslA, a significant virulence associated lipoprotein [30] was found in 4 of the 6 bands and 3 other bands showing significant activity. The primary VlhA of *M. gallisepticum* strain R_{low}, VlhA 3.03 a 64 kd protein was also found in 3 out of 6 bands and was found to be the most abundant lipoprotein in the LAMPs. These results suggest that MslA and VlhA 3.03 are possibly two major immunogenic lipoproteins of *M. gallisepticum*, however more sophisticated studies are required to confirm these preliminary findings.

Taken together our data suggest that *M. gallisepticum* LAMPs have potent inflammatory properties and can mediate changes in gene expression in chicken tracheal

epithelial cells almost immediately upon exposure. However, the response appears to be transient in nature likely due to host compensatory mechanisms. Thus, continuous LAMP-mediated stimulation by adherent, replicating mycoplasma may be necessary to sustain the response. Studies using chemical inhibitors of specific signaling pathways indicated that mycoplasma LAMPs ligate TLR2 on TECs and activate NF-κB resulting in downstream expression of several pro-inflammatory chemokines and cytokines. Among the up-regulated genes are chemokines and cytokines known for leukocyte chemo-attraction and activation, consistent with the immunopathology associated with infection. Our data also support the notion that the virulent R_{low} strain possesses additional mechanisms of initiating inflammatory responses in tracheal epithelial cells beyond that mediated by LAMPs alone.

2.6 Acknowledgements

We acknowledge Dr. Steven J. Geary for providing us with the *Mycoplasma gallisepticum* strains and for his critical review of the manuscript; Dr. Steven M. Szczepanek for his helpful inputs during the early stages of project development and critical review of the manuscript. We acknowledge Dr. Tania B. Huedo-Medina for her assistance with the statistical analyses. We also thank Ms. Xiaofen Liao for her help during isolation of mycoplasma LAMPs and Ms. Debra Rood for her maintenance of the laboratory. This project was supported, in part, by the USDA National Institute of Food and Agriculture, Hatch project number CONS00930.

2.7 References

1. Sato, S., et al., *Mixed infection with Mycoplasma gallisepticum and the B1 strain of Newcastle disease virus in chickens.* Natl Inst Anim Health Q (Tokyo), 1970. **10**(2): p. 58-65.
2. Gaunson, J.E., et al., *Lymphocytic infiltration in the chicken trachea in response to Mycoplasma gallisepticum infection.* Microbiology, 2000. **146 (Pt 5)**: p. 1223-9.
3. Stipkovits, L., et al., *Effect of low-pathogenicity influenza virus H3N8 infection on Mycoplasma gallisepticum infection of chickens.* Avian Pathol, 2012. **41**(1): p. 51-7.
4. Winner, F., R. Rosengarten, and C. Citti, *In vitro cell invasion of Mycoplasma gallisepticum.* Infect Immun, 2000. **68**(7): p. 4238-44.
5. Vogl, G., et al., *Mycoplasma gallisepticum invades chicken erythrocytes during infection.* Infect Immun, 2008. **76**(1): p. 71-7.
6. Furnkranz, U., et al., *Factors influencing the cell adhesion and invasion capacity of Mycoplasma gallisepticum.* Acta Vet Scand, 2013. **55**: p. 63.
7. Indikova, I., et al., *Role of the GapA and CrmA cytadhesins of Mycoplasma gallisepticum in promoting virulence and host colonization.* Infect Immun, 2013.
8. Much, P., et al., *Mycoplasma gallisepticum: Influence of cell invasiveness on the outcome of experimental infection in chickens.* FEMS Immunol Med Microbiol, 2002. **34**(3): p. 181-6.
9. Jenkins, C., et al., *The Mycoplasma gallisepticum OsmC-like protein MG1142 resides on the cell surface and binds heparin.* Microbiology, 2007. **153**(Pt 5): p. 1455-63.
10. Grodio, J.L., et al., *Detection and quantification of Mycoplasma gallisepticum genome load in conjunctival samples of experimentally infected house finches (Carpodacus mexicanus) using real-time polymerase chain reaction.* Avian Pathol, 2008. **37**(4): p. 385-91.
11. Tajima, M., T. Nunoya, and T. Yagihashi, *An ultrastructural study on the interaction of Mycoplasma gallisepticum with the chicken tracheal epithelium.* Am J Vet Res, 1979. **40**(7): p. 1009-14.
12. Mohammed, J., et al., *Chemokine and cytokine gene expression profiles in chickens inoculated with Mycoplasma gallisepticum strains Rlow or GT5.* Vaccine, 2007. **25**(51): p. 8611-21.
13. Gaunson, J.E., et al., *The cellular immune response in the tracheal mucosa to Mycoplasma gallisepticum in vaccinated and unvaccinated chickens in the acute and chronic stages of disease.* Vaccine, 2006. **24**(14): p. 2627-33.
14. Bystry, R.S., et al., *B cells and professional APCs recruit regulatory T cells via CCL4.* Nat Immunol, 2001. **2**(12): p. 1126-32.
15. Sherry, B., et al., *Resolution of the two components of macrophage inflammatory protein 1, and cloning and characterization of one of those components, macrophage inflammatory protein 1 beta.* J Exp Med, 1988. **168**(6): p. 2251-9.
16. Legler, D.F., et al., *B cell-attracting chemokine 1, a human CXC chemokine expressed in lymphoid tissues, selectively attracts B lymphocytes via BLR1/CXCR5.* J Exp Med, 1998. **187**(4): p. 655-60.

17. Gunn, M.D., et al., *A B-cell-homing chemokine made in lymphoid follicles activates Burkitt's lymphoma receptor-1.* Nature, 1998. **391**(6669): p. 799-803.
18. Kelner, G.S., et al., *Lymphotactin: a cytokine that represents a new class of chemokine.* Science, 1994. **266**(5189): p. 1395-9.
19. Schall, T.J., et al., *A human T cell-specific molecule is a member of a new gene family.* J Immunol, 1988. **141**(3): p. 1018-25.
20. Gomez, M.I. and A. Prince, *Airway epithelial cell signaling in response to bacterial pathogens.* Pediatr Pulmonol, 2008. **43**(1): p. 11-9.
21. Fournier, B. and D.J. Philpott, *Recognition of Staphylococcus aureus by the innate immune system.* Clin Microbiol Rev, 2005. **18**(3): p. 521-40.
22. Prince, A., *Flagellar activation of epithelial signaling.* Am J Respir Cell Mol Biol, 2006. **34**(5): p. 548-51.
23. Liu, X., et al., *Human airway epithelial cell responses to Neisseria lactamica and purified porin via Toll-like receptor 2-dependent signaling.* Infect Immun, 2010. **78**(12): p. 5314-23.
24. Roussel, L., et al., *P. aeruginosa drives CXCL8 synthesis via redundant toll-like receptors and NADPH oxidase in CFTRF508 airway epithelial cells.* J Cyst Fibros, 2011. **10**(2): p. 107-13.
25. Yang, J., et al., *Regulation of proinflammatory cytokines in human lung epithelial cells infected with Mycoplasma pneumoniae.* Infect Immun, 2002. **70**(7): p. 3649-55.
26. McGowin, C.L., et al., *Persistent Mycoplasma genitalium infection of human endocervical epithelial cells elicits chronic inflammatory cytokine secretion.* Infect Immun, 2012. **80**(11): p. 3842-9.
27. McGowin, C.L., V.L. Popov, and R.B. Pyles, *Intracellular Mycoplasma genitalium infection of human vaginal and cervical epithelial cells elicits distinct patterns of inflammatory cytokine secretion and provides a possible survival niche against macrophage-mediated killing.* BMC Microbiol, 2009. **9**: p. 139.
28. McGowin, C.L., et al., *Mycoplasma genitalium-encoded MG309 activates NF-kappaB via Toll-like receptors 2 and 6 to elicit proinflammatory cytokine secretion from human genital epithelial cells.* Infect Immun, 2009. **77**(3): p. 1175-81.
29. Krishnan, M., T.R. Kannan, and J.B. Baseman, *Mycoplasma pneumoniae CARDS toxin is internalized via clathrin-mediated endocytosis.* PLoS One, 2013. **8**(5): p. e62706.
30. Medina, J.L., et al., *Mycoplasma pneumoniae CARDS toxin induces pulmonary eosinophilic and lymphocytic inflammation.* Am J Respir Cell Mol Biol, 2012. **46**(6): p. 815-22.
31. Cole, B.C., *The immunobiology of Mycoplasma arthritidis and its superantigen MAM.* Curr Top Microbiol Immunol, 1991. **174**: p. 107-19.
32. Cole, B.C., K.E. Aldridge, and J.R. Ward, *Mycoplasma-dependent activation of normal lymphocytes: mitogenic potential of mycoplasmas for mouse lymphocytes.* Infect Immun, 1977. **18**(2): p. 393-9.
33. Zuo, L.L., Y.M. Wu, and X.X. You, *Mycoplasma lipoproteins and Toll-like receptors.* J Zhejiang Univ Sci B, 2009. **10**(1): p. 67-76.

34. Shimizu, T., Y. Kida, and K. Kuwano, *A triacylated lipoprotein from Mycoplasma genitalium activates NF-kappaB through Toll-like receptor 1 (TLR1) and TLR2.* Infect Immun, 2008. **76**(8): p. 3672-8.
35. Chambaud, I., H. Wroblewski, and A. Blanchard, *Interactions between mycoplasma lipoproteins and the host immune system.* Trends Microbiol, 1999. **7**(12): p. 493-9.
36. Tulman, E.R., et al., *Extensive variation in surface lipoprotein gene content and genomic changes associated with virulence during evolution of a novel North American house finch epizootic strain of Mycoplasma gallisepticum.* Microbiology, 2012. **158**(Pt 8): p. 2073-88.
37. Xu, C.G., et al., *Molecular cloning and immune response analysis of putative variable lipoproteins from Mycoplasma mycoides subsp capri.* Genet Mol Res, 2014. **13**(1): p. 1527-39.
38. Noormohammadi, A.H., et al., *A novel mechanism for control of antigenic variation in the haemagglutinin gene family of mycoplasma synoviae.* Mol Microbiol, 2000. **35**(4): p. 911-23.
39. Yogev, D., et al., *A surface epitope undergoing high-frequency phase variation is shared by Mycoplasma gallisepticum and Mycoplasma bovis.* Infect Immun, 1994. **62**(11): p. 4962-8.
40. Chopra-Dewasthaly, R., et al., *Role of Vpma phase variation in Mycoplasma agalactiae pathogenesis.* FEMS Immunol Med Microbiol, 2012. **66**(3): p. 307-22.
41. Wu, H.N., et al., *"Mycoplasmal antigen modulation," a novel surface variation suggested for a lipoprotein specifically localized on Mycoplasma mobile.* Curr Microbiol, 2012. **64**(5): p. 433-40.
42. Nouvel, L.X., et al., *Occurrence, plasticity, and evolution of the vpma gene family, a genetic system devoted to high-frequency surface variation in Mycoplasma agalactiae.* J Bacteriol, 2009. **191**(13): p. 4111-21.
43. You, X.X., Y.H. Zeng, and Y.M. Wu, *Interactions between mycoplasma lipid-associated membrane proteins and the host cells.* J Zhejiang Univ Sci B, 2006. **7**(5): p. 342-50.
44. Behrens, A., et al., *A family of phase- and size-variant membrane surface lipoprotein antigens (Vsps) of Mycoplasma bovis.* Infect Immun, 1994. **62**(11): p. 5075-84.
45. Calcutt, M.J., et al., *Differential posttranslational processing confers intraspecies variation of a major surface lipoprotein and a macrophage-activating lipopeptide of Mycoplasma fermentans.* Infect Immun, 1999. **67**(2): p. 760-71.
46. Citti, C. and K.S. Wise, *Mycoplasma hyorhinis vlp gene transcription: critical role in phase variation and expression of surface lipoproteins.* Mol Microbiol, 1995. **18**(4): p. 649-60.
47. Papazisi, L., et al., *The complete genome sequence of the avian pathogen Mycoplasma gallisepticum strain R(low).* Microbiology, 2003. **149**(Pt 9): p. 2307-16.
48. Liu, Y.C., et al., *Proteomics characterization of cytoplasmic and lipid-associated membrane proteins of human pathogen Mycoplasma fermentans M64.* PLoS One, 2012. **7**(4): p. e35304.

49. He, J., et al., *Binding of CD14 to Mycoplasma genitalium-Derived Lipid-Associated Membrane Proteins Upregulates TNF-alpha.* Inflammation, 2014. **37**(2): p. 322-30.
50. He, J., et al., *Mycoplasma genitalium-derived lipid-associated membrane proteins activate NF-kappaB through toll-like receptors 1, 2, and 6 and CD14 in a MyD88-dependent pathway.* Clin Vaccine Immunol, 2009. **16**(12): p. 1750-7.
51. Logunov, D., et al., *[Lipid-associated membrane lipopeptides of M. arginini activate NF-kB by interacting with TLR2/1, TLR2/6, and TLR2/CD14]*. Mol Gen Mikrobiol Virusol, 2009(2): p. 25-8.
52. You, X., et al., *Mycoplasma genitalium-derived lipid-associated membrane proteins induce activation of MAPKs, NF-kappaB and AP-1 in THP-1 cells.* FEMS Immunol Med Microbiol, 2008. **52**(2): p. 228-36.
53. Qiu, H., et al., *[Mycoplasma genitalium lipid-associated membrane proteins induce human monocytic cell express proinflammatory cytokines and apoptosis by activating nuclear factor kappaB]*. Wei Sheng Wu Xue Bao, 2007. **47**(5): p. 899-904.
54. Xu, Y., et al., *Mycoplasma hyorhinis activates the NLRP3 inflammasome and promotes migration and invasion of gastric cancer cells.* PLoS One, 2013. **8**(11): p. e77955.
55. Shimizu, T., Y. Kida, and K. Kuwano, *A dipalmitoylated lipoprotein from Mycoplasma pneumoniae activates NF-kappa B through TLR1, TLR2, and TLR6.* J Immunol, 2005. **175**(7): p. 4641-6.
56. Shimizu, T., Y. Kida, and K. Kuwano, *Triacylated lipoproteins derived from Mycoplasma pneumoniae activate nuclear factor-kappaB through toll-like receptors 1 and 2.* Immunology, 2007. **121**(4): p. 473-83.
57. Seya, T. and M. Matsumoto, *A lipoprotein family from Mycoplasma fermentans confers host immune activation through Toll-like receptor 2.* Int J Biochem Cell Biol, 2002. **34**(8): p. 901-6.
58. Deiters, U. and P.F. Muhlradt, *Mycoplasmal lipopeptide MALP-2 induces the chemoattractant proteins macrophage inflammatory protein 1alpha (MIP-1alpha), monocyte chemoattractant protein 1, and MIP-2 and promotes leukocyte infiltration in mice.* Infect Immun, 1999. **67**(7): p. 3390-8.
59. Into, T., et al., *Stimulation of human Toll-like receptor (TLR) 2 and TLR6 with membrane lipoproteins of Mycoplasma fermentans induces apoptotic cell death after NF-kappa B activation.* Cell Microbiol, 2004. **6**(2): p. 187-99.
60. Fichorova, R.N., et al., *Response to Neisseria gonorrhoeae by cervicovaginal epithelial cells occurs in the absence of toll-like receptor 4-mediated signaling.* J Immunol, 2002. **168**(5): p. 2424-32.
61. Choi, S.Y., et al., *Reactive oxygen species mediate Jak2/Stat3 activation and IL-8 expression in pulmonary epithelial cells stimulated with lipid-associated membrane proteins from Mycoplasma pneumoniae.* Inflammation Research, 2012. **61**(5): p. 493-501.
62. Zaffuto, K.M., C.N. Estevez, and C.L. Afonso, *Primary chicken tracheal cell culture system for the study of infection with avian respiratory viruses.* Avian Pathol, 2008. **37**(1): p. 25-31.

63. Shen, C.I., et al., *The infection of primary avian tracheal epithelial cells with infectious bronchitis virus.* Vet Res. **41**(1): p. 6.
64. Bischof, D.F., et al., *Cytotoxicity of Mycoplasma mycoides subsp. mycoides small colony type to bovine epithelial cells.* Infect Immun, 2008. **76**(1): p. 263-9.
65. Love, W., et al., *Toll-like receptor 2 (TLR2) plays a major role in innate resistance in the lung against murine Mycoplasma.* PLoS One, 2010. **5**(5): p. e10739.
66. Razin, S., D. Yogev, and Y. Naot, *Molecular biology and pathogenicity of mycoplasmas.* Microbiol Mol Biol Rev, 1998. **62**(4): p. 1094-156.
67. Javed, M.A., et al., *Correlates of immune protection in chickens vaccinated with Mycoplasma gallisepticum strain GT5 following challenge with pathogenic M. gallisepticum strain R(low).* Infect Immun, 2005. **73**(9): p. 5410-9.
68. Rawadi, G., *Mycoplasma fermentans interaction with monocytes/macrophages: molecular basis.* Microbes Infect, 2000. **2**(8): p. 955-64.
69. Rottem, S., *Interaction of mycoplasmas with host cells.* Physiol Rev, 2003. **83**(2): p. 417-32.
70. Walter, M.J., et al., *Interleukin 12 p40 production by barrier epithelial cells during airway inflammation.* J Exp Med, 2001. **193**(3): p. 339-51.
71. Oppmann, B., et al., *Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12.* Immunity, 2000. **13**(5): p. 715-25.
72. Hieshima, K., et al., *Molecular cloning of a novel human CC chemokine liver and activation-regulated chemokine (LARC) expressed in liver. Chemotactic activity for lymphocytes and gene localization on chromosome 2.* J Biol Chem, 1997. **272**(9): p. 5846-53.
73. Baggiolini, M. and I. Clark-Lewis, *Interleukin-8, a chemotactic and inflammatory cytokine.* FEBS Lett, 1992. **307**(1): p. 97-101.
74. Zamora, R., Y. Vodovotz, and T.R. Billiar, *Inducible nitric oxide synthase and inflammatory diseases.* Mol Med, 2000. **6**(5): p. 347-73.
75. Medina, S., et al., *Nitric oxide released by accessory cells mediates the gastrin-releasing peptide effect on murine lymphocyte chemotaxis.* Regul Pept, 2005. **131**(1-3): p. 46-53.
76. Hsieh, C.S., et al., *Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages.* Science, 1993. **260**(5107): p. 547-9.
77. Xing, Z., A. Zganiacz, and M. Santosuosso, *Role of IL-12 in macrophage activation during intracellular infection: IL-12 and mycobacteria synergistically release TNF-alpha and nitric oxide from macrophages via IFN-gamma induction.* J Leukoc Biol, 2000. **68**(6): p. 897-902.
78. Papazisi, L., et al., *GapA and CrmA coexpression is essential for Mycoplasma gallisepticum cytadherence and virulence.* Infect Immun, 2002. **70**(12): p. 6839-45.
79. Dallo, S.F., A. Chavoya, and J.B. Baseman, *Characterization of the gene for a 30-kilodalton adhesion-related protein of Mycoplasma pneumoniae.* Infect Immun, 1990. **58**(12): p. 4163-5.

80. Krause, D.C. and M.F. Balish, *Structure, function, and assembly of the terminal organelle of Mycoplasma pneumoniae.* FEMS Microbiol Lett, 2001. **198**(1): p. 1-7.
81. Kahane, I., et al., *Detection of the major adhesin P1 in triton shells of virulent Mycoplasma pneumoniae.* Infect Immun, 1985. **50**(3): p. 944-6.
82. Papazisi, L., et al., *Analysis of cytadherence-deficient, GapA-negative Mycoplasma gallisepticum strain R.* Infect Immun, 2000. **68**(12): p. 6643-9.
83. Krause, D.C. and M.F. Balish, *Cellular engineering in a minimal microbe: structure and assembly of the terminal organelle of Mycoplasma pneumoniae.* Mol Microbiol, 2004. **51**(4): p. 917-24.
84. Krause, D.C., *Mycoplasma pneumoniae cytadherence: unravelling the tie that binds.* Mol Microbiol, 1996. **20**(2): p. 247-53.
85. May, M., et al., *Identification of fibronectin-binding proteins in Mycoplasma gallisepticum strain R.* Infect Immun, 2006. **74**(3): p. 1777-85.
86. Szczepanek, S.M., et al., *Comparative genomic analyses of attenuated strains of Mycoplasma gallisepticum.* Infect Immun, 2010. **78**(4): p. 1760-71.
87. Erridge, C., et al., *Oxidized phospholipid inhibition of toll-like receptor (TLR) signaling is restricted to TLR2 and TLR4: roles for CD14, LPS-binding protein, and MD2 as targets for specificity of inhibition.* J Biol Chem, 2008. **283**(36): p. 24748-59.
88. Sethi, G., et al., *Celastrol, a novel triterpene, potentiates TNF-induced apoptosis and suppresses invasion of tumor cells by inhibiting NF-kappaB-regulated gene products and TAK1-mediated NF-kappaB activation.* Blood, 2007. **109**(7): p. 2727-35.
89. Griesinger, G., et al., *Production of pro- and anti-inflammatory cytokines of human placental trophoblasts in response to pathogenic bacteria.* J Soc Gynecol Investig, 2001. **8**(6): p. 334-40.
90. Alam, M.M. and L.A. O'Neill, *MicroRNAs and the resolution phase of inflammation in macrophages.* Eur J Immunol, 2011. **41**(9): p. 2482-5.
91. Quinn, E.M., et al., *MicroRNA-146a is upregulated by and negatively regulates TLR2 signaling.* PLoS One, 2013. **8**(4): p. e62232.
92. Wang, P., et al., *Interleukin (IL)-10 inhibits nuclear factor kappa B (NF kappa B) activation in human monocytes. IL-10 and IL-4 suppress cytokine synthesis by different mechanisms.* J Biol Chem, 1995. **270**(16): p. 9558-63.
93. Driessler, F., et al., *Molecular mechanisms of interleukin-10-mediated inhibition of NF-kappaB activity: a role for p50.* Clin Exp Immunol, 2004. **135**(1): p. 64-73.

2.8 Figure Legends:

Figure 2.1: Primary chicken tracheal epithelial cell culture (TEC)

Primary chicken tracheal epithelial cells were isolated and cultured as described in the Methods section. **1A:** Primary chicken tracheal epithelial cells at 100X magnification. **1B:** Confirmation of tracheal epithelial cell identity both *in vitro* and freshly isolated (*ex vivo*) from tracheae after *ex-vivo* exposure: PCR amplified epithelial cell specific genes from cDNA in agarose gel, compared to chicken embryonic fibroblast (DF-1) cells. **1C:** Tracheal epithelial cells stained for E-cadherin and Vimentin at (400X magnification). Left panel shows TECs at different filter setting Blue (DAPI) for nuclear staining, Green (FITC) for Vimentin and Red (AlexaFluor® 546) for E-cadherin, right panel shows merged picture for all filters. **1D:** DF-1 fibroblast cells stained for E-cadherin and Vimentin at 400X magnification. Left panel shows DF-1 cells at different filter setting; Blue (DAPI) for nuclear staining, Green (FITC) for Vimentin and Red (AlexaFluor® 546) for E-cadherin; right panel shows merged picture for all filters.

Figure 2.2: Original agarose gels photos of amplified products of epithelial cell specific genes

1A: Confirmation of tracheal epithelial cells in culture *in vitro* using amplification of epithelial cell specific genes compared to chicken embryonic fibroblast (DF-1) cells. **1B:** Confirmation of epithelial cell specific genes in freshly isolated epithelial cells from tracheae after *ex-vivo* exposure.

Figure 2.3: Distribution of differentially regulated genes in TECs (>5 fold)

Differentially regulated genes (≥ 5 fold) in tracheal epithelial cell after exposure to live R_{low} , R_{high} or LAMPs isolated from either strain 1.5 hours after exposure. The star (*) in the Figure 2.represent commonly up-regulated genes upon all four exposures, from which six follow up genes were chosen. n=8 (4 biological replicates x2 dye swap technical replicates) for all microarray experiments.

Figure 2.4: Distribution of differentially regulated genes in TECs (>2 fold)

Differentially regulated genes (≥ 2 fold) in tracheal epithelial cells after exposure to live R_{low} , R_{high} or LAMPs isolated from either strain for 1.5 hours. n=8 (4 biological replicates x2 dye swap technical replicates) for all microarray experiments.

Figure 2.5: **TLR- Signaling pathway**

Toll like receptor signaling network: Common genes up-regulated in TECs exposed to R_{low}, R_{high}, R_{low} LAMP or R_{high} LAMP are depicted in yellow boxes.

Figure 2.6: Kinetic analysis of differentially regulated genes encoding inflammatory chemokines and cytokines

Differential gene expression in TECs exposed to R_{low}, R_{low} LAMP, R_{high} or R_{high} LAMP at 1.5, 6 and 24 hours respectively. Samples normalized to housekeeping gene GAPDH and un-exposed TECs as control. n=6 for all experiments. Results are denoted as fold change ± SEM with all control values set at 1. Significant differences denoted as * = $P< 0.05$, **= $P <0.01$, *** = $P < 0.001$. **A:** mRNA fold difference in R_{low} exposed cells

B: mRNA fold difference in R_{high} exposed cells. **C:** mRNA fold difference in R_{low} LAMP exposed cells. **D:** mRNA fold difference in R_{high} LAMP exposed cells.

Figure 2.7: **Differential gene expression in TECs post-exposure**

mRNA fold difference in TECs exposed to R_{low}, R_{low} LAMP, R_{high} or R_{high} LAMP at 1.5, 6 and 24 hours respectively. Samples normalized to housekeeping gene GAPDH and un-exposed TECs as control. n=6 for all experiments. Results are denoted as fold change ± SEM with all control values set at 1. Significant differences denoted as * = P< 0.05, **= P <0.01, *** = P < 0.001. **A:** IL-12p40 mRNA **B:** IL-8 mRNA **C** IL-6 mRNA **D:** CCL-20 mRNA **E.** NOS-2 mRNA. **F:** IL-1β mRNA.

Figure 2.8: Differential gene expression in TECs exposed to LAMPs in the presence of signaling inhibitors.

Epithelial cells were exposed to LAMPs isolated from R_{low} or R_{high} in the presence or absence of signaling inhibitors for 6 hours. Samples were normalized to the housekeeping gene GAPDH and un-exposed TECs served as control. n=6 for all experiments. Results are denoted as fold change ± SEM with all control values set at 1.Significant differences denoted as * = P< 0.05, **= P <0.01, *** = P < 0.001. **A**. IL-12p40. **B**. IL-1β. **C**. IL-8. **D**. IL-6. **E.** CCL-20. **F**. NOS-2.

Figure 2.9: **Differential gene expression in tracheal epithelial cells after *ex-vivo* exposure to LAMPs**

Comparison of mRNA fold difference in tracheal epithelial cells from tracheal explant exposed to R_{low}, R_{low} LAMP, R_{high} or R_{high} LAMP at 1.5 and 6 hours respectively. Samples normalized to housekeeping gene GAPDH and un-exposed tracheae as control.

n=6 for all experiments. Results are denoted as fold change ± SEM with all control values set at 1. Significant differences denoted as * = P< 0.05, **= P <0.01, *** = P < 0.001. **A:** mRNA fold difference of all genes at 1.5 hours **B:** mRNA fold difference of all genes at 6 hours

Figure 2.10: RT-qPCR analysis of miRNA and IL-10 differential expression in TECs

Epithelial cells were exposed to R_{low}, R_{low} LAMP, R_{high} or R_{high} LAMP at 1.5, 6 and 24 hours respectively. Samples were normalized to housekeeping gene GAPDH and un-exposed TECs as control. n=6 for all experiments. Results are denoted as fold change ± SEM with all control values set at 1. Significant differences denoted as * = P< 0.05, **= P <0.01, *** = P < 0.001. **A:** mRNA fold difference of IL-10 in TECs at all three time points post exposure. **B:** mRNA fold difference of miRNA-146a in TECs at all three time points post exposure.

Figure 2.11: SDS-PAGE fractionation of R_{low} LAMPs and IL-1β expression by TECs in presence of various SDS-PAGE fractions of R_{low} LAMPs

A: SDS-PAGE separation of R_{ow} LAMPs identified 13 distinct bands. **B:** mRNA fold difference of IL-1β in TECs upon exposure to 1μg/mL LAMPs from each band obtained during SDS-PAGE separation.

2.9 Tables:

Table 2.1: Epithelial gene specific primers

Gene ID	Primer Name	Sequence 5'-3'
395209	Retinoic acid responder Forward Retinoic acid responder Reverse	ACA TCA ACT CCC ACG AGG CGT CC ACT GCT GCC AAC AAT GGC CAA GC
408039	Keratin 14 Forward Keratin 14 Reverse	CAC TGC CAG CCC GCT GTG CT ACC TTG TCC AGG TAG GCG GCC
407779	Keratin 5 Forward Keratin 5 Reverse	TGC TGC TTT CCT GCT GCT CAG C ACG GTC ACT TCA TGG ATG CCA CC
414833	Cytochrome P-450 2C45 Forward Cytochrome P-450 2C45 Reverse	CCA CGT GGG AGA TGT TGC TCC TG TGG CAG CAA ACT CAT CCG CAC G

Table 2.2: Gene specific primers for RT-qPCR

Primer Name	Sequence 5'-3'
GAPDH Forward	ATT CTA CAC ACG GAC ACT TCA
GAPDH Reverse	CAC CAG TGG ACT CCA CAA CAT A
IL-12p40 Forward	TGAAGGAGTTCCCAGATGC
IL-12p40 Reverse	CGTCTTGCTTGGCTCTTTATA
IL-1β Forward	GCT GGA ACT GGG CAG AT
IL-1β Reverse	GGT AGA AGA TGA AGC GGG TC
IL-8 Forward	GTG CAT TAG CAC TCA TTC TAA GTT
IL-8 Reverse	GGC CAT AAG TGC CTT TAC G
IL-6 Forward	CCT GTT CGC CTT TCA GAC CTA
IL-6 Reverse	AGT CTG GGA TGA CCA CTT C
IL-10 Forward	AGAGATGCTGCGCTTCTACA
IL-10 Reverse	GCTTGATGGCTTTGCTCC
CCL-20 Forward	GCC AGA AGC TCA AGA GGA TG
CCL-20 Reverse	TCC AGA AGT TCA ACG GTT CC
NOS-2 Forward	TGA TCT TTG CTG CCA AAC AG
NOS-2 Reverse	TCC TCT GAG GGA AAA TGG TG
miRNA-146a Forward	GAGAACTGAATTCCATGGGTTG
miRNA-146a Reverse	TCCAAGCTGAAGAACTGAGC

Table 2.3: Differentially expressed genes (≥2-fold, p-value ≤0.05) common in TECs exposed to R_{low}, R_{high}, R_{low} lipoprotein and R_{high} lipoprotein: analyzed for gene ontology hierarchal clustering.

Entrez Gene ID	Gene Name	Fold Change in TECs exposed to			
		R_{low}	R_{high}	R_{low} lipoprotein	R_{high} lipoprotein
Immune system processes					
768950	CD80 molecule	2.96	4.77	2.44	4.02
395082	Chemokine (C-C motif) ligand 20	55.34	11.67	43.92	12.98
417465	Chemokine (C-C motif) ligand 5	2.63	2.65	2.38	2.65
396330	Interferon regulatory factor 7	6.57	5.21	5.44	4.22
395196	Interleukin 1, beta	9.89	7.86	6.75	5.65
404671	Interleukin 12B	207.80	71.80	194.67	78.21
395337	Interleukin 6	42.33	30.57	27.31	20.95
396495	Interleukin 8	22.92	20.72	17.87	19.44
396093	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	5.00	4.15	5.00	3.62
417247	Similar to TL1A; tumor necrosis factor (ligand) superfamily, member 15	4.22	14.47	4.08	10.33
421219	Toll-like receptor 15	2.75	2.53	3.69	2.11
770778	Gallus gallus interleukin 28B	14.34	11.44	10.28	11.08
Regulation of apoptosis					
395673	BCL2-related protein A1	4.92	4.47	4.53	3.40
423471	TNF receptor-associated factor 3	3.68	5.35	4.39	5.46
374012	Baculoviral IAP repeat-containing 2	5.42	4.07	5.45	3.48
395196	Interleukin 1, beta	9.89	7.86	6.75	5.65
404671	Interleukin 12B	207.80	71.80	194.67	78.21
417247	Similar to TL1A; tumor necrosis factor (ligand) superfamily, member 15	4.22	14.47	4.08	10.33
396033	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	2.55	2.08	2.58	2.02
396093	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	5.00	4.15	5.00	3.62
417319	Phosphoinositide-3-kinase, regulatory subunit 5, p101	6.34	3.27	5.17	3.01

Entrez gene ID	Gene name	Fold change in tecs exposed to			
		R_{low}	R_{high}	R_{low} lipoprotein	R_{high} lipoprotein
Signal transduction					
419844	Ras association (ralgds/af-6) domain family 5	5.03	3.80	4.24	3.98
415790	Ras-related associated with diabetes	4.88	3.03	4.27	3.25
423471	Tnf receptor-associated factor 3	3.68	5.35	4.39	5.46
422884	Tnfaip3 interacting protein 2	10.95	4.77	7.09	7.01
769087	Angiopoietin-like 4 angptl4	7.27	2.66	5.90	2.17
408036	Epiregulin	4.01	2.02	2.80	2.66
395196	Interleukin 1, beta	9.89	7.86	6.75	5.65
396033	Nuclear factor of kappa light polypeptide gene enhancer in b-cells 1	2.55	2.08	2.58	2.02
396093	Nuclear factor of kappa light polypeptide gene enhancer in b-cells inhibitor, alpha	5.00	4.15	5.00	3.62
423290	Similar to ras guanyl releasing protein 1	2.25	3.89	3.58	3.50
417247	Similar to tl1a; tumor necrosis factor (ligand) superfamily, member 15	4.22	14.47	4.08	10.33
416630	Suppressor of cytokine signaling 1	8.37	3.76	5.41	3.22
421219	Toll-like receptor 15	2.75	2.53	3.69	2.11
378803	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	2.59	2.09	2.40	3.32
Response to stress					
395654	Heparin-binding egf-like growth factor	6.66	4.39	5.22	3.61
395196	Interleukin 1, beta	9.89	7.86	6.75	5.65
395807	Nitric oxide synthase 2, inducible	10.53	3.68	8.41	3.94
418404	Nuclear factor of kappa light polypeptide gene enhancer in b-cells inhibitor, zeta	10.24	17.96	12.14	15.32
396424	Plasminogen activator, urokinase	7.15	16.65	7.95	14.86
396451	Prostaglandin-endoperoxide synthase 2	12.98	10.65	13.13	9.76
421219	Toll-like receptor 15	2.75	2.53	3.69	2.11
408036	Epiregulin	4.01	2.02	2.80	2.66
404671	Interleukin 12b	207.80	71.80	194.67	78.21
396093	Nuclear factor of kappa light polypeptide gene enhancer in b-cells inhibitor, alpha	5.00	4.15	5.00	3.62

Entrez gene ID	Gene name	Fold change in tecs exposed to			
		R_{low}	R_{high}	R_{low} lipoprotein	R_{high} lipoprotein
Cell communication					
395820	Delta-like 1 (drosophila); similar to c-delta-1	3.19	2.76	2.18	2.37
408036	Epiregulin	4.01	2.02	2.80	2.66
395337	Interleukin 6	42.33	30.57	27.31	20.95
420628	Sorting nexin 10	3.71	2.02	3.31	2.25
374168	Wingless-type mmtv integration site family, member 7a	3.27	4.42	2.79	4.47
Cell migration/locomotion					
395654	Heparin-binding egf-like growth factor	6.66	4.39	5.22	3.61
395196	Interleukin 1, beta	9.89	7.86	6.75	5.65
404671	Interleukin 12b	207.80	71.80	194.67	78.21
396424	Plasminogen activator, urokinase	7.15	16.65	7.95	14.86
417465	Chemokine (c-c motif) ligand 5	2.63	2.65	2.38	2.65
396495	Interleukin 8	22.92	20.72	17.87	19.44
Regulation of cell proliferation					
768950	Cd80 molecule	2.96	4.77	2.44	4.02
408036	Epiregulin	4.01	2.02	2.80	2.66
395654	Heparin-binding egf-like growth factor	6.66	4.39	5.22	3.61
395196	Interleukin 1, beta	9.89	7.86	6.75	5.65
404671	Interleukin 12b	207.80	71.80	194.67	78.21
395337	Interleukin 6	42.33	30.57	27.31	20.95
395807	Nitric oxide synthase 2, inducible	10.53	3.68	8.41	3.94
396093	Nuclear factor of kappa light polypeptide gene enhancer in b-cells inhibitor, alpha	5.00	4.15	5.00	3.62
396424	Plasminogen activator, urokinase	7.15	16.65	7.95	14.86
396451	Prostaglandin-endoperoxide synthase 2	12.98	10.65	13.13	9.76
417247	Similar to tl1a; tumor necrosis factor (ligand) superfamily, member 15	4.22	14.47	4.08	10.33

Entrez gene ID	Gene name	Fold change in tecs exposed to			
		R_{low}	R_{high}	R_{low} lipoprotein	R_{high} lipoprotein
Response to stimulus					
395082	Chemokine (c-c motif) ligand 20	55.34	11.67	43.92	12.98
417465	Chemokine (c-c motif) ligand 5	2.63	2.65	2.38	2.65
395654	Heparin-binding egf-like growth factor	6.66	4.39	5.22	3.61
418812	Immunoresponsive 1 homolog (mouse) irg1	47.48	28.24	43.40	29.42
396330	Interferon regulatory factor 7	6.57	5.21	5.44	4.22
395196	Interleukin 1, beta	9.89	7.86	6.75	5.65
404671	Interleukin 12b	207.80	71.80	194.67	78.21
395337	Interleukin 6	42.33	30.57	27.31	20.95
396495	Interleukin 8	22.92	20.72	17.87	19.44
395807	Nitric oxide synthase 2, inducible	10.53	3.68	8.41	3.94
396093	Nuclear factor of kappa light polypeptide gene enhancer in b-cells inhibitor, alpha	5.00	4.15	5.00	3.62
418404	Nuclear factor of kappa light polypeptide gene enhancer in b-cells inhibitor, zeta	10.24	17.96	12.14	15.32
396424	Plasminogen activator, urokinase	7.15	16.65	7.95	14.86
396451	Prostaglandin-endoperoxide synthase 2	12.98	10.65	13.13	9.76
417247	Similar to tl1a; tumor necrosis factor (ligand) superfamily, member 15	4.22	14.47	4.08	10.33
421219	Toll-like receptor 15	2.75	2.53	3.69	2.11
Receptor binding					
769087	Angiopoietin-like 4 angptl4	7.27	2.66	5.90	2.17
395082	Chemokine (c-c motif) ligand 20	55.34	11.67	43.92	12.98
417465	Chemokine (c-c motif) ligand 5	2.63	2.65	2.38	2.65
395820	Delta-like 1 (drosophila); similar to c-delta-1	3.19	2.76	2.18	2.37
408036	Epiregulin	4.01	2.02	2.80	2.66
395654	Heparin-binding egf-like growth factor	6.66	4.39	5.22	3.61
395196	Interleukin 1, beta	9.89	7.86	6.75	5.65
404671	Interleukin 12b	207.80	71.80	194.67	78.21
395337	Interleukin 6 (interferon, beta 2)	42.33	30.57	27.31	20.95
396495	Interleukin 8	22.92	20.72	17.87	19.44
417247	Similar to tl1a; tnf (ligand) superfamily, member15	4.22	14.47	4.08	10.33

Entrez gene ID	Gene name	Fold change in tecs exposed to			
		R_{low}	R_{high}	R_{low} lipoprotein	R_{high} lipoprotein
Protein binding					
417247	Similar to tl1a; tumor necrosis factor (ligand) superfamily, member 15	4.22	14.47	4.08	10.33
423163	Similar to traf6; tnf receptor-associated factor 6	3.35	3.01	3.29	2.24
420628	Sorting nexin 10	3.71	2.02	3.31	2.25
416630	Suppressor of cytokine signaling 1	8.37	3.76	5.41	3.22
421219	Toll-like receptor 15	2.75	2.53	3.69	2.11
419088	Tsukushin	4.24	2.68	3.76	3.02
378803	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	2.59	2.09	2.40	3.32
396250	V-ets erythroblastosis virus e26 oncogene homolog 2 (avian)	2.53	2.75	2.13	2.43
374168	Wingless-type mmtv integration site family, member 7a	3.27	4.42	2.79	4.47
Other genes					
420838	Cd83 molecule	5.98	4.53	5.63	3.75
428492	F-box and leucine-rich repeat protein 7	3.30	4.23	3.29	4.07
420343	F-box protein 32	-2.95	-3.37	-3.59	-3.77
422061	G protein-coupled receptor 116	3.57	3.99	3.68	3.21
416546	Nadph oxidase organizer 1	31.41	11.36	27.79	11.96
395138	St3 beta-galactoside alpha-2,3-sialyltransferase 6	3.33	4.85	3.07	4.08
771693	Tnfaip3 interacting protein 3	5.04	2.69	5.87	2.81
427985	Udp-gal:betaglcnac beta 1,3-galactosyltransferase, polypeptide 5	2.21	3.42	2.56	3.78
418422	Chromosome 3 open reading frame 52	2.45	2.39	2.22	2.10
417515	Deltex homolog 2	3.72	2.34	2.99	2.57
769474	Family with sequence similarity 83, member c	2.77	3.92	2.42	3.78
421747	Fyn-related kinase	3.17	3.69	2.62	3.57
417968	Guanylate cyclase 2c	2.10	2.46	2.01	2.16
422840	Heparan sulfate (glucosamine) 3-o-sulfotransferase 1	3.50	2.94	3.22	2.72

Entrez gene ID	Gene name	Fold change in tecs exposed to			
		R_{low}	R_{high}	R_{low} lipoprotein	R_{high} lipoprotein
Other genes					
423723	Peptidylprolyl isomerase f	4.28	2.33	2.44	2.11
423227	Phospholipase a2, group ive	2.45	2.65	3.18	2.44
419262	Regulator of telomere elongation helicase 1	2.78	3.65	2.27	2.95
420628	Sorting nexin 10	3.71	2.02	3.31	2.25
423477	Tumor necrosis factor, alpha-induced protein 2	9.29	4.90	8.42	4.39
421684	Tumor necrosis factor, alpha-induced protein 3	4.98	4.25	4.28	3.21
420942	Uridine phosphorylase 1	2.64	2.37	2.27	2.90
419618	Zinc finger ccch-type containing 12a	4.16	3.33	4.22	3.16
396384	Interferon regulatory factor 1 (irf1)	5.66	5.48	4.73	5.07
416325	Granulocyte-macrophage colony-stimulating factor	5.16	8.70	5.20	9.57
395827	Cytochrome p450, family 24, subfamily a, polypeptide 1 (cyp24a1)	3.06	3.17	3.30	3.17

Table 2.4: Differentially expressed unique genes in R_{low} exposed TECs (≥5-fold, p-value ≤0.05) (Excludes unknown genes listed as finished cDNA clones)

Entrez Gene ID	Gene Name	Fold change in R_{low} exposed TECs
395888	NK2 transcription factor related, locus 3	87.83
428065	Protocadherin 8	65.12
	Partial mRNA for immunoglobulin heavy chain variable region, clone U11B3.	33.71
418236	Parvin gamma	29.20
429104	Cyclin-dependent kinase inhibitor 2C	28.81
427105	POLR3G polymerase (RNA) III (DNA directed) polypeptide G (32kD)	19.60
374049	Tumor protein D52-like 1	19.52
378791	Reticulon 1	19.15
414343	Gal 9	15.95
374004	Myogenin (myogenic factor 4)	13.33
416953	Member RAS oncogene family similar to small GTP-binding protein Rab36	12.48
424940	Cholinergic receptor, nicotinic, delta	11.74
424171	G6PC2 glucose-6-phosphatase, catalytic, 2	9.32
428714	Probable tRNA threonylcarbamoyladenosine biosynthesis protein YwlC-like	7.55
408183	Cytochrome P450, family 26, subfamily A, polypeptide 1	7.25
427503	FSD2 fibronectin type III and SPRY domain containing 2	7.10
428247	HTR3A 5-hydroxytryptamine (serotonin) receptor 3A, ionotropic	7.04
373928	Peroxisome proliferator-activated receptor gamma	6.88
429283	Distal-less homeobox 1	6.36
426186	Transmembrane protein 106C	6.26
768668	Arylacetamide deacetylase-like 4-like	6.13
396260	Mature avidin	6.07
396114	Pyrimidinergic receptor P2Y, G-protein coupled, 6	5.95
396300	Kainate binding protein	5.87
427718	Leukemia inhibitory factor	5.78
395725	Deoxyribonuclease I	5.77
422307	Immune-responsive gene 1 protein-like	5.69
415820	Hepatic nuclear factor 4beta	5.63
416996	Inositol polyphosphate-5-phosphatase J	5.61
422219	Interleukin 13 receptor, alpha 2	5.39
417505	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 7	5.34
768877	SAG S-antigen; retina and pineal gland (arrestin)	5.19
428191	Ciliary rootlet coiled-coil, rootletin	5.18
427028	Olfactory receptor 14J1 like	5.13
429337	Apelin receptor	5.10
419844	Ras association (RalGDS/AF-6) domain family 5	5.03
430670	Chymotrypsin C	5.03
419751	Glutamate receptor, ionotropic, kainate 4	5.01
421686	Interleukin 20 receptor, alpha	5.01

Table 2.5: Pathway analysis of differentially regulated genes

Analysis performed using GeneSpring (v12.5), within genes that are differentially expressed ≥2-fold (p-value ≤0.05).

Pathway	No. of genes from different pathways up-regulated in TEC's exposed to:				Total no. of genes in pathway
	R_{low}	R_{high}	R_{low} LAMP	R_{high} LAMP	
Toll-like receptor signaling pathway	17	15	13	13	70
TNF-alpha NF-kB Signaling Pathway	10	9	11	10	157
Adipogenesis	14	7	4	4	100
Senescence and Autophagy	10	8	7	7	73
EGFR1 Signaling Pathway	8	4	5	2	148
MAPK signaling pathway	5	7	4	5	123
Apoptosis	5	6	5	6	65
Type II interferon signaling pathway	6	4	4	4	22

Table 2.6: Proteomic characterization of R_{low} LAMPs separated by SDS-PAGE

Sample	Peptide matches	Sequence coverage	Description
Sample 1 (175 kd)	69	35%	Hypothetical protein mga_0241 [*Mycoplasma gallisepticum* str. R(low)]
	50	24%	Hypothetical protein [*Mycoplasma gallisepticum* str. F]
	6	8%	Pts system glucose-specific transporter subunit iiabc [*Mycoplasma gallisepticum* str. R(low)]
	6	8%	Ptsg_1 gene product [*Mycoplasma gallisepticum* str. F]
	4	6%	M9 protein [*Mycoplasma gallisepticum*
Sample 2 (110 kd)	47	37%	Hypothetical protein mga_0319 [*Mycoplasma gallisepticum* str. R(low)]
	18	31%	Vlha.3.03 variable lipoprotein family protein [*Mycoplasma gallisepticum* str. R(low)]
	10	15%	Mycoplasma specific lipoprotein a [*Mycoplasma gallisepticum* str. R(low)]
	18	17%	Hypothetical protein mga_0321 [*Mycoplasma gallisepticum* str. R(low)]
	9	12%	Pts system glucose-specific transporter subunit iiabc [*Mycoplasma gallisepticum* str. R(low)]
	8	8%	Putative oligopeptide abc transporter solute binding protein oppa [*Mycoplasma gallisepticum* str. R(low)]
	9	10%	Hypothetical protein mga_0226 [*Mycoplasma gallisepticum* str. R(low)]
	5	14%	Putative multidrug abc transporter permease component [*Mycoplasma gallisepticum* str. R(low)]
	4	4%	120kda membrane protein [*Mycoplasma gallisepticum* str. R]
Sample 3 (100 kd)	49	43%	Putative oligopeptide abc transporter solute binding protein oppa [*Mycoplasma gallisepticum* str. R(low)]
	33	42%	Hypothetical protein mga_0226 [*Mycoplasma gallisepticum* str. R(low)]
	33	38%	120kda membrane protein [*Mycoplasma gallisepticum* str. R]
	28	22%	Hypothetical protein mga_0321 [*Mycoplasma gallisepticum* str. R(low)]
	27	23%	Hypothetical protein mga_0319 [*Mycoplasma gallisepticum* str. R(low)]
	5	6%	Pts system glucose-specific transporter subunit iiabc [*Mycoplasma gallisepticum* str. R(low)]
	7	7%	Cytadhesin protein gapa [*Mycoplasma gallisepticum* str. R(low)]
	5	7%	M9 protein [*Mycoplasma gallisepticum*

Sample	Peptide matches	Sequence coverage	Description
Sample 4 (75 kd)	13	16%	Pts system glucose-specific transporter subunit iiabc [*Mycoplasma gallisepticum* str. R(low)]
	12	17%	Mycoplasma specific lipoprotein a [*Mycoplasma gallisepticum* str. R(low)]
	9	12%	Vlha.5.03 variable lipoprotein family protein [*Mycoplasma gallisepticum* str. R(low)]
	8	13%	Hypothetical protein mga_0267 [*Mycoplasma gallisepticum* str. R(low)]
	10	16%	M9 protein [*Mycoplasma gallisepticum*
	7	11%	Vlha protein [*Mycoplasma gallisepticum*
	7	12%	Unknown [*Mycoplasma gallisepticum*
	6	6%	Hypothetical protein mga_0648 [*Mycoplasma gallisepticum* str. R(low)]
	3	5%	Vlha protein [*Mycoplasma gallisepticum*
	5	6%	Vlha.1.07 variable lipoprotein family protein [*Mycoplasma gallisepticum* str. R(low)]
Sample 5 (70 kd)	29	44%	Vlha.4.07 variable lipoprotein family protein [*Mycoplasma gallisepticum* str. R(low)]
	32	49%	Vlha.5.08 variable lipoprotein family protein [*Mycoplasma gallisepticum* str. R(low)]
	26	44%	Vlha.3.03 variable lipoprotein family protein [*Mycoplasma gallisepticum* str. R(low)]
	23	32%	Vlha.3.07 variable lipoprotein family protein [*Mycoplasma gallisepticum* str. R(low)]
	10	16%	Vlha.4.07.1 variable lipoprotein family protein [*Mycoplasma gallisepticum* str. R(low)]
	7	11%	Vlha.4.05 variable lipoprotein family protein [*Mycoplasma gallisepticum* str. R(low)]
	7	12%	Vlha.4.01 variable lipoprotein family protein [*Mycoplasma gallisepticum* str. R(low)]
	4	8%	Vlha.1.04 variable lipoprotein family protein [*Mycoplasma gallisepticum* str. R(low)]
	3	4%	Vlha.1.03 variable lipoprotein family protein [*Mycoplasma gallisepticum* str. R(low)]
	3	4%	Vlha.5.03 variable lipoprotein family protein [*Mycoplasma gallisepticum* str. R(low)]
	14	20%	Mycoplasma specific lipoprotein a [*Mycoplasma gallisepticum* str. R(low)]
	10	14%	Pts system glucose-specific transporter subunit iiabc [*Mycoplasma gallisepticum* str. R(low)]
	10	21%	Hypothetical protein mga_0495 [*Mycoplasma gallisepticum* str. R(low)]

Sample	Peptide matches	Sequence coverage	Description
	5	6%	Cell division protease ftsh-like protein [*Mycoplasma gallisepticum* str. R(low)]
	3	2%	Hypothetical protein mga_0319 [*Mycoplasma gallisepticum* str. R(low)]
	4	7%	Hypothetical protein mga_0267 [*Mycoplasma gallisepticum* str. R(low)]
	3	3%	Hypothetical protein mga_0849 [*Mycoplasma gallisepticum* str. R(low)]
	3	3%	Hypothetical protein mga_0045 [*Mycoplasma gallisepticum* str. R(low)]
Sample 6 (64 kd)	53	66%	Vlha.3.03 variable lipoprotein family protein [*Mycoplasma gallisepticum* str. R(low)]
	38	42%	Haemagglutinin precursor [*Mycoplasma gallisepticum* s6]
	40	47%	Variably expressed lipoprotein and hemagglutinin (vlha) family protein [*Mycoplasma gallisepticum* str. F]
	34	45%	Haemagglutinin homologue [*Mycoplasma gallisepticum* s6]
	34	45%	Pmga 1.2 [*Mycoplasma gallisepticum*
	33	45%	Pmga1.2 a homologue of pmga [*Mycoplasma gallisepticum*
	30	34%	Vlha protein [*Mycoplasma gallisepticum*
	30	38%	Vlha.5.05 variable lipoprotein family protein [*Mycoplasma gallisepticum* str. R(low)]
	20	39%	Hypothetical protein mga_0993 [*Mycoplasma gallisepticum* str. R(low)]
	17	35%	Vlha.4.01 variable lipoprotein family protein [*Mycoplasma gallisepticum* str. R(low)]
	16	32%	Hypothetical protein [*Mycoplasma gallisepticum* str. F]
	12	25%	Vlha.3.02_1 gene product [*Mycoplasma gallisepticum* str. R(high)]
	12	17%	Vlha.3.02 variable lipoprotein family protein [*Mycoplasma gallisepticum* str. R(low)]
	6	11%	Hypothetical protein mga_0079 [*Mycoplasma gallisepticum* str. R(low)]
	5	8%	Vlha.2.01 variable lipoprotein family protein [*Mycoplasma gallisepticum* str. R(low)]
	4	6%	Vlha.5.02 variable lipoprotein family protein [*Mycoplasma gallisepticum* str. R(low)]
	4	7%	Mycoplasma specific lipoprotein a [*Mycoplasma gallisepticum* str. R(low)]
	4	5%	Vlha.4.07 variable lipoprotein family protein [*Mycoplasma gallisepticum* str. R(low)]
	4	5%	Vlha.4.07.6 variable lipoprotein family protein [*Mycoplasma gallisepticum* str. R(low)]

Sample	Peptide matches	Sequence coverage	Description
Sample 7 (52 kd)	85	55%	Mycoplasma specific lipoprotein a [*Mycoplasma gallisepticum* str. R(low)]
	85	46%	Msla gene product [*Mycoplasma gallisepticum* str. F]
	22	38%	Vlha.2.01 variable lipoprotein family protein [*Mycoplasma gallisepticum* str. R(low)]
	5	10%	Vlha.5.13 variable lipoprotein family protein [*Mycoplasma gallisepticum* str. R(low)]
	8	16%	Hypothetical protein mga_0267 [*Mycoplasma gallisepticum* str. R(low)]
	8	13%	Hypothetical protein mga_1073 [*Mycoplasma gallisepticum* str. R(low)]
	7	11%	Hypothetical protein mga_0416 [*Mycoplasma gallisepticum* str. R(low)]
	3	6%	Phosphoenolpyruvate-protein kinase [*Mycoplasma gallisepticum* str. R(low)]
	6	9%	M9 protein [*Mycoplasma gallisepticum*
	4	9%	Pyruvate kinase [*Mycoplasma gallisepticum* str. R(low)]
	3	6%	Adenosinetriphosphatase [*Mycoplasma gallisepticum*
Sample 8 (45 kd)	44	38%	Macrophage-activating lipoprotein-like protein [*Mycoplasma gallisepticum* str. R(low)]
	19	46%	Dipeptide/oligopeptide/nickel abc transporter atp-binding protein dppd/oppd [*Mycoplasma gallisepticum* str. R(low)]
	19	23%	Vlha.5.03 variable lipoprotein family protein [*Mycoplasma gallisepticum* str. R(low)]
	9	14%	Vlha.4.05 variable lipoprotein family protein [*Mycoplasma gallisepticum* str. R(low)]
	7	12%	Vlha.4.06 variable lipoprotein family protein [*Mycoplasma gallisepticum* str. R(low)]
	23	35%	Hypothetical protein mga_0267 [*Mycoplasma gallisepticum* str. R(low)]
	19	30%	Mycoplasma specific lipoprotein a [*Mycoplasma gallisepticum* str. R(low)]
	11	38%	Translation longation factor tu (ef-tu) [*Mycoplasma gallisepticum* str. R(low)]
	10	17%	Vlha.1.01 variable lipoprotein family protein [*Mycoplasma gallisepticum* str. R(low)]
	6	9%	Vlha.1.04 variable lipoprotein family protein [*Mycoplasma gallisepticum* str. R(low)]
	9	15%	Vlha.3.03 variable lipoprotein family protein [*Mycoplasma gallisepticum* str. R(low)]
	5	8%	Vlha.4.07.6 variable lipoprotein family protein [*Mycoplasma gallisepticum* str. R(low)]
	13	30%	Hypothetical protein mga_0878 [*Mycoplasma gallisepticum* str. R(low)]

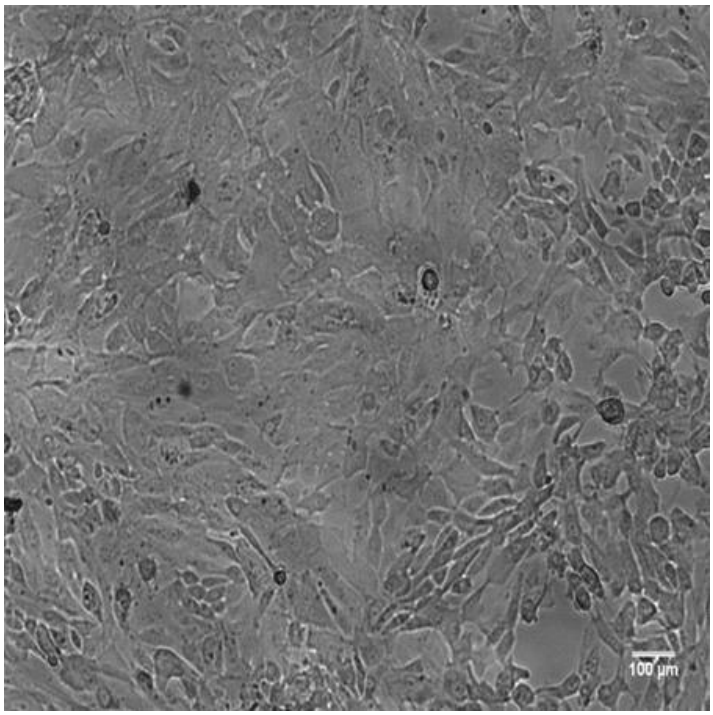
Sample	Peptide matches	Sequence coverage	Description
	16	46%	Glyceraldehyde-3-phosphate dehydrogenase [*Mycoplasma gallisepticum* str. R(low)]
	8	18%	Hypothetical protein mga_0335 [*Mycoplasma gallisepticum* str. R(low)]
	4	12%	Putative multidrug abc transporter permease component [*Mycoplasma gallisepticum* str. R(low)]
	8	22%	Dnaj-like molecular chaperone [*Mycoplasma gallisepticum* str. R(low)]
	6	8%	Cell division protease ftsh-like protein [*Mycoplasma gallisepticum* str. R(low)]
	3	7%	Cysteinyl-trna synthetase [*Mycoplasma gallisepticum* str. R(low)]
	3	3%	Pts system glucose-specific transporter subunit iiabc [*Mycoplasma gallisepticum* str. R(low)]
	5	4%	Hypothetical protein mga_0319 [*Mycoplasma gallisepticum* str. R(low)]
	3	7%	F0f1 atp synthase subunit beta [*Mycoplasma gallisepticum* str. R(low)]
	3	3%	Vlha.1.05 variable lipoprotein family protein [*Mycoplasma gallisepticum* str. R(low)]
	15	34%	L-lactate dehydrogenase [*Mycoplasma gallisepticum* str. R(low)]
	11	36%	High affinity abc transporter protein hata [*Mycoplasma gallisepticum* str. R(low)]
	11	36%	Hata gene product [*Mycoplasma gallisepticum* str. F]
	12	19%	Vlha.3.03 variable lipoprotein family protein [*Mycoplasma gallisepticum* str. R(low)]
	8	16%	Macrophage-activating lipoprotein-like protein [*Mycoplasma gallisepticum* str. R(low)]
	8	16%	Macrophage-activating lipoprotein-like protein [*Mycoplasma gallisepticum* str. F]
	6	10%	Vlha.5.03 variable lipoprotein family protein [*Mycoplasma gallisepticum* str. R(low)]
	7	22%	Glyceraldehyde-3-phosphate dehydrogenase [*Mycoplasma gallisepticum* str. R(low)]
	7	22%	Gapd gene product [*Mycoplasma gallisepticum* str. F]
	4	6%	Pts system glucose-specific transporter subunit iiabc [*Mycoplasma gallisepticum* str. R(low)]
	10	31%	Multidrug-like abc transporter atp-binding protein [*Mycoplasma gallisepticum* str. R(low)]
	7	14%	Macrophage-activating lipoprotein-like protein [*Mycoplasma gallisepticum*
	7	18%	Hypothetical protein mga_0878 [*Mycoplasma gallisepticum* str. R(low)]

Sample	Peptide matches	Sequence coverage	Description
	7	19%	Phosphate abc transporter periplasmic phosphate binding protein [*Mycoplasma gallisepticum* str. R(low)]
	7	9%	Pmga1.2 a homologue of pmga [*Mycoplasma gallisepticum*
	6	6%	Vlha.5.05 variable lipoprotein family protein [*Mycoplasma gallisepticum* str. R(low)]
	4	7%	Hypothetical protein mga_0267 [*Mycoplasma gallisepticum* str. R(low)]
	3	9%	Recname: full=dna-directed rna polymerase subunit alpha; short=rnap subunit alpha; altname: full=rna polymerase subunit alpha; altname: full=transcrip
	3	9%	Dna-directed rna polymerase subunit alpha [*Mycoplasma gallisepticum* str. R(low)]
Sample 9 (30 kd)	10	19%	Putative multidrug abc transporter permease component [*Mycoplasma gallisepticum* str. R(low)]
	10	14%	Dipeptide/oligopeptide/nickel abc transporter permease dppb/oppb [*Mycoplasma gallisepticum* str. R(low)]
	8	28%	Hypothetical protein mga_0244 [*Mycoplasma gallisepticum* str. R(low)]
	10	15%	M9 protein [*Mycoplasma gallisepticum*
	8	28%	Hypothetical protein [*Mycoplasma gallisepticum* str. F]
	9	23%	Nuclease-like lipoprotein [*Mycoplasma gallisepticum* str. R(low)]
	8	13%	Haemagglutinin precursor [*Mycoplasma gallisepticum* s6]
	7	12%	Vlha.3.08 variable lipoprotein family protein [*Mycoplasma gallisepticum* str. R(low)]
	9	20%	Hypothetical protein mga_0811 [*Mycoplasma gallisepticum* str. R(low)]
	8	20%	Hypothetical protein [*Mycoplasma gallisepticum* str. F]
	6	15%	Dipeptide/oligopeptide/nickel abc transporter permease dppc/oppc [*Mycoplasma gallisepticum* str. R(low)]
	7	13%	Pmga1.2 a homologue of pmga [*Mycoplasma gallisepticum*
	7	13%	Haemagglutinin homologue [*Mycoplasma gallisepticum* s6]
	7	13%	Pmga 1.2 [*Mycoplasma gallisepticum*
	4	18%	Adhesin pmga1.1 [*Mycoplasma gallisepticum*
	4	21%	Pmga 1.1 [*Mycoplasma gallisepticum*
	5	8%	Vlha.3.02 variable lipoprotein family protein [*Mycoplasma gallisepticum* str. R(low)]
	5	12%	Vlha.3.02_1 gene product [*Mycoplasma gallisepticum* str. R(high)]
	3	11%	Lema-family protein [*Mycoplasma gallisepticum* str. R(low)]

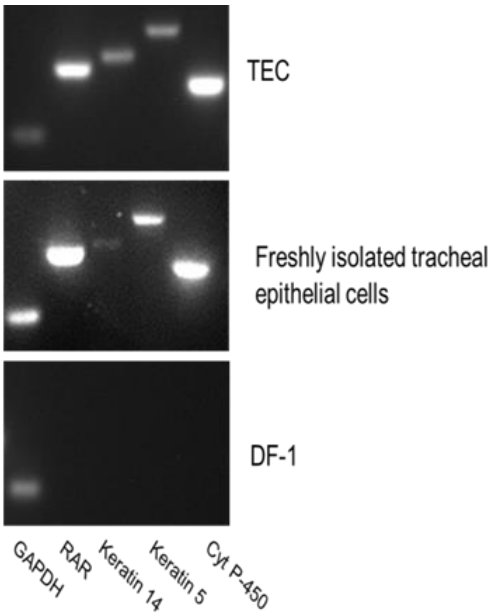
Sample	Peptide matches	Sequence coverage	Description
	3	11%	Lema-family protein [*Mycoplasma gallisepticum* str. F]
	4	7%	Cobalt abc transporter permease [*Mycoplasma gallisepticum* str. R(low)]
	4	7%	Cbiq gene product [*Mycoplasma gallisepticum* str. F]
Sample 10 (23 kd)	33	20%	Mycoplasma specific lipoprotein a [*Mycoplasma gallisepticum* str. R(low)]
	31	20%	Msla gene product [*Mycoplasma gallisepticum* str. F]
	10	17%	Putative multidrug abc transporter permease component [*Mycoplasma gallisepticum* str. R(low)]
	8	33%	Lema-family protein [*Mycoplasma gallisepticum* str. R(low)]
	6	20%	Lema-family protein [*Mycoplasma gallisepticum* str. F]
Sample 11 (20 kd)	12	29%	Hypothetical protein mga_0913 [*Mycoplasma gallisepticum* str. R(low)]
	4	20%	Ferritin-like protein [*Mycoplasma gallisepticum* str. R(low)]
	3	16%	50s ribosomal protein l5 [*Mycoplasma gallisepticum* str. R(low)]
	2	3%	Hypothetical protein mga_0267 [*Mycoplasma gallisepticum* str. R(low)]
	2	13%	Hypothetical protein mga_0957 [*Mycoplasma gallisepticum* str. R(low)]
	3	10%	Hypothetical protein mga_0119 [*Mycoplasma gallisepticum* str. R(low)]
Sample 12 (16 kd)	5	10%	Macrophage-activating lipoprotein-like protein [*Mycoplasma gallisepticum* str. R(low)]
Sample 13 (13 kd)	5	10%	Macrophage-activating lipoprotein-like protein [*Mycoplasma gallisepticum* str. F]
	2	13%	Putative pts system glucose-specific transporter subunit iib ptsg [*Mycoplasma gallisepticum* str. R(low)]
	4	20%	Organic hydroperoxide resistance protein [*Mycoplasma gallisepticum* str. R(low)]
	2	24%	Hypothetical protein mga_0340 [*Mycoplasma gallisepticum* str. R(low)]

FIGURE 2.1

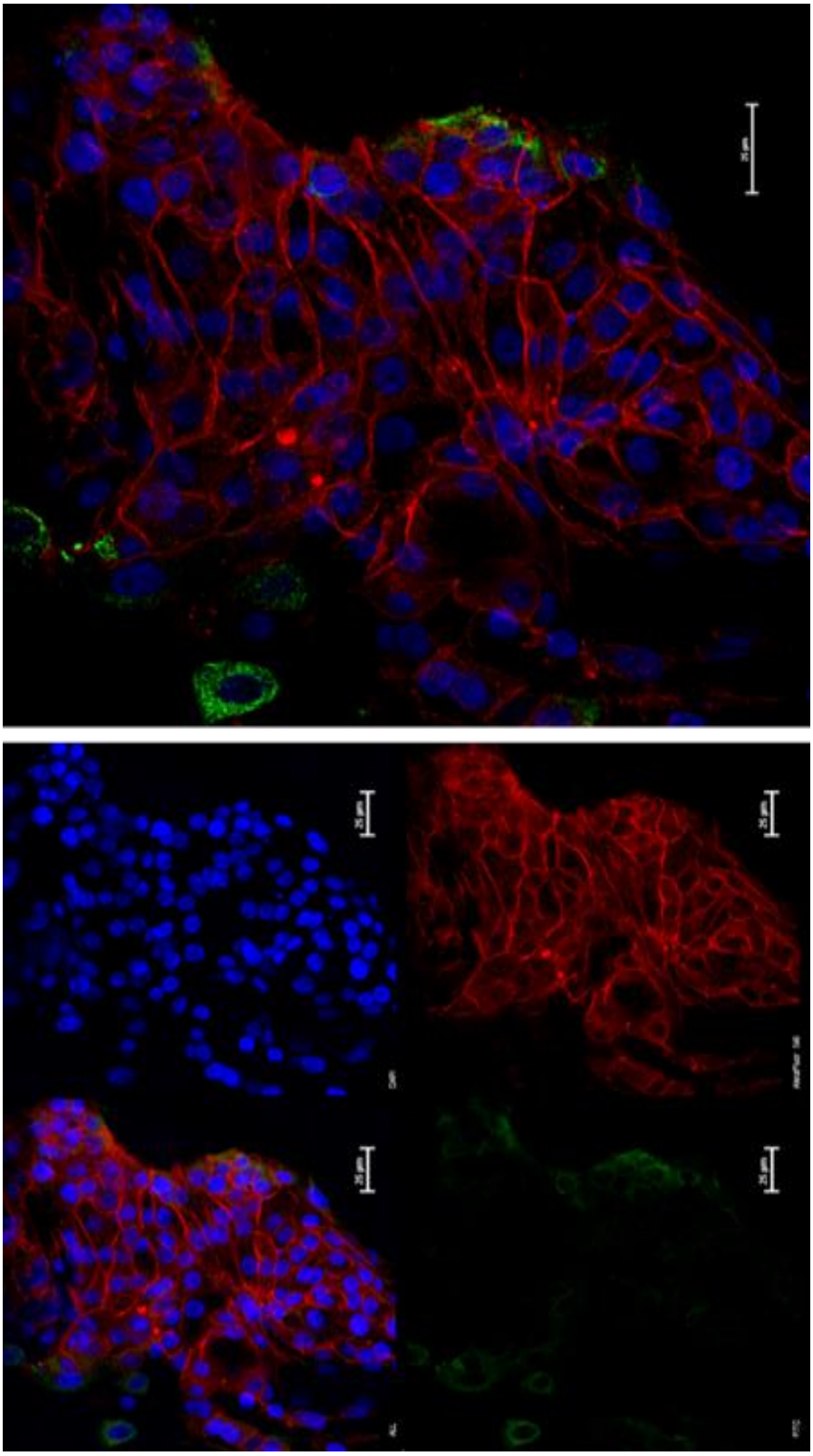
A.


100 µm

B.


TEC
Freshly isolated tracheal epithelial cells
DF-1
GAPDH
RAR
Keratin 14
Keratin 5
Cyt P-450

C.



D.

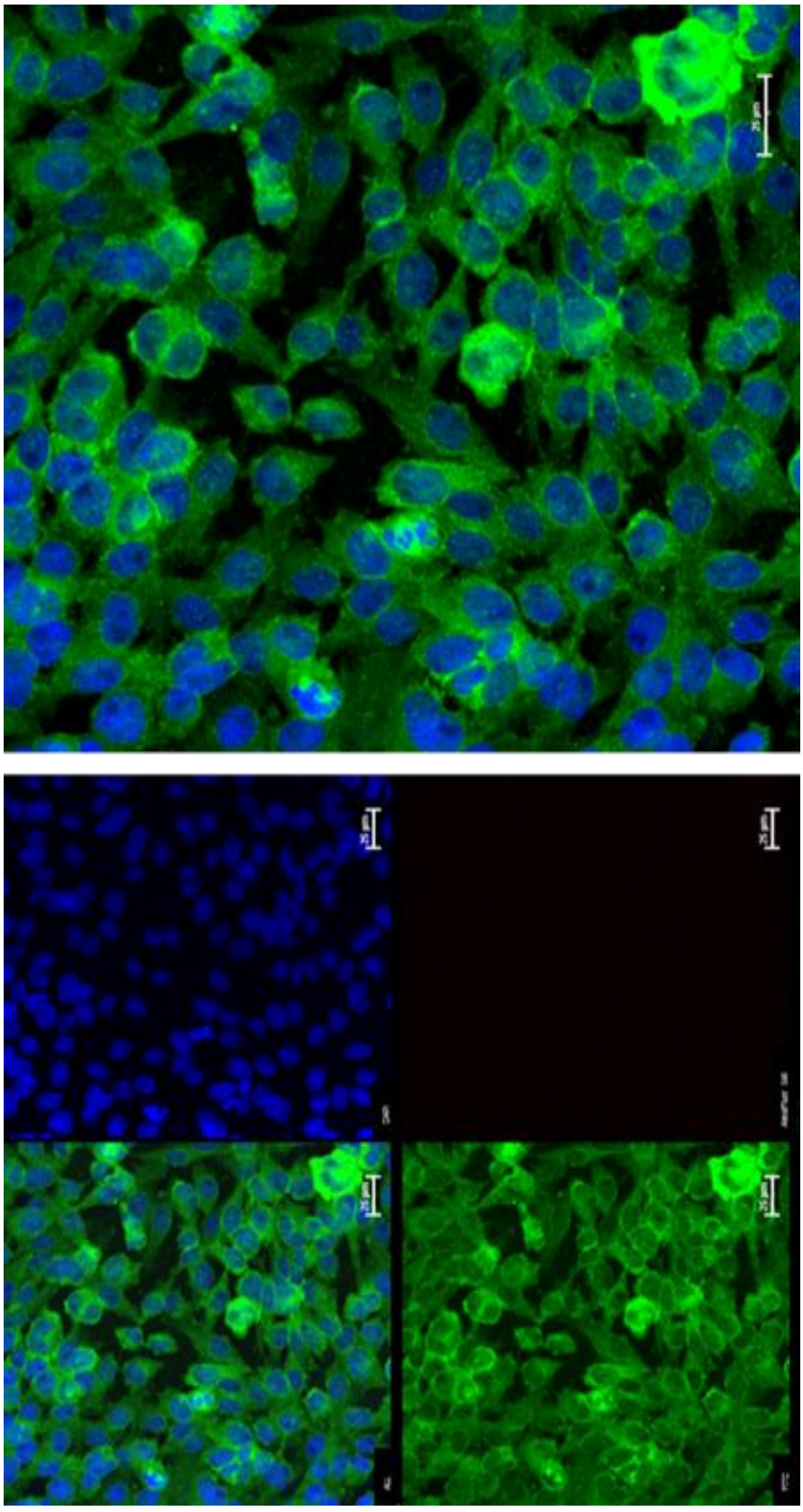
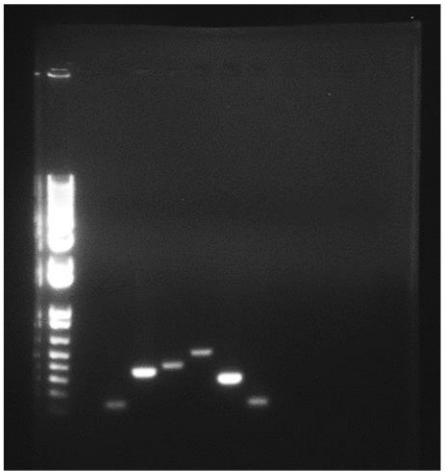


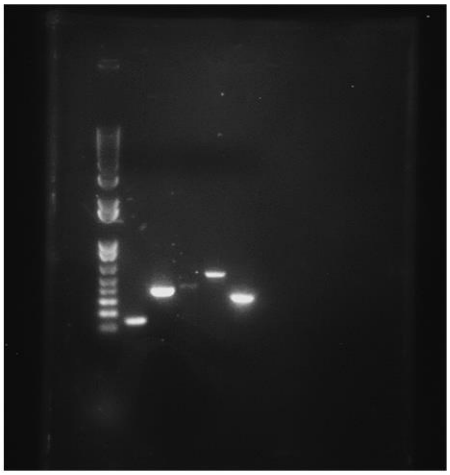
Figure 2.2

A



Lane 1: 10kb plus DNA ladder
Lane 3: GAPDH - TEC
Lane 4: RAR – TEC
Lane 5: Keratin 14 - TEC
Lane 6: Keratin 75 – TEC
Lane 7: Cytochrome p450 – TEC
Lane 8: GAPDH – DF-1
Lane 9: RAR – DF-1
Lane 10: Keratin 14 – DF-1
Lane 11: Keratin 75 – DF-1
Lane 12: Cytochrome p450 – DF-1

B



Lane 1: 10kb plus DNA ladder
Lane 2: GAPDH
Lane 3: RAR
Lane 4: Keratin 14
Lane 5: Keratin 75
Lane 6: Cytochrome p450

FIGURE 2.3:

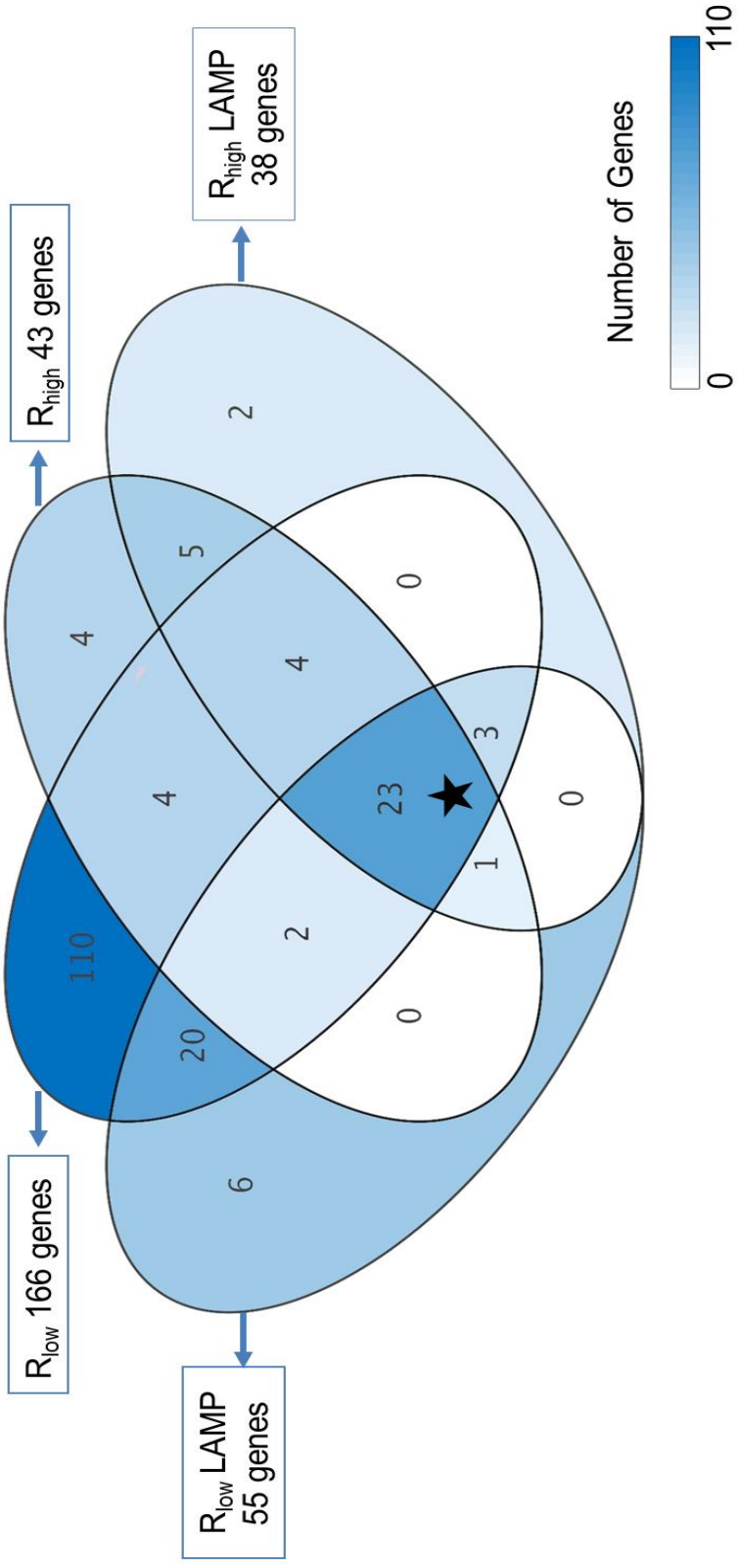

R_{high} LAMP
38 genes
R_{high} 43 genes
R_{low} 166 genes
R_{low} LAMP
55 genes
Number of Genes
110
0
2
5
0
4
4
3
23
0
1
110
2
20
0
6

Figure 2.4

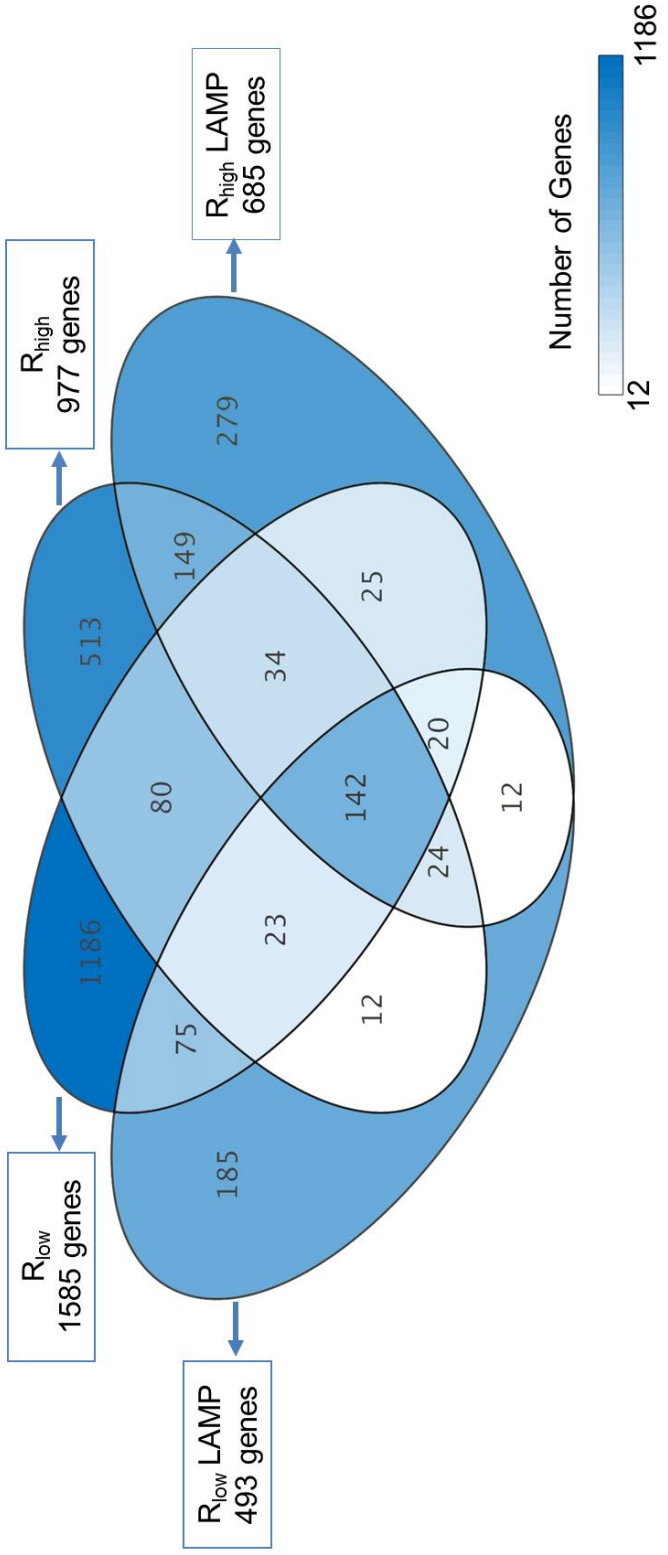

R_high 977 genes
R_high LAMP 685 genes
R_low 1585 genes
R_low LAMP 493 genes
Number of Genes
1186
12
513
149
279
25
34
80
142
20
12
24
1186
23
12
75
185

Figure 2.5

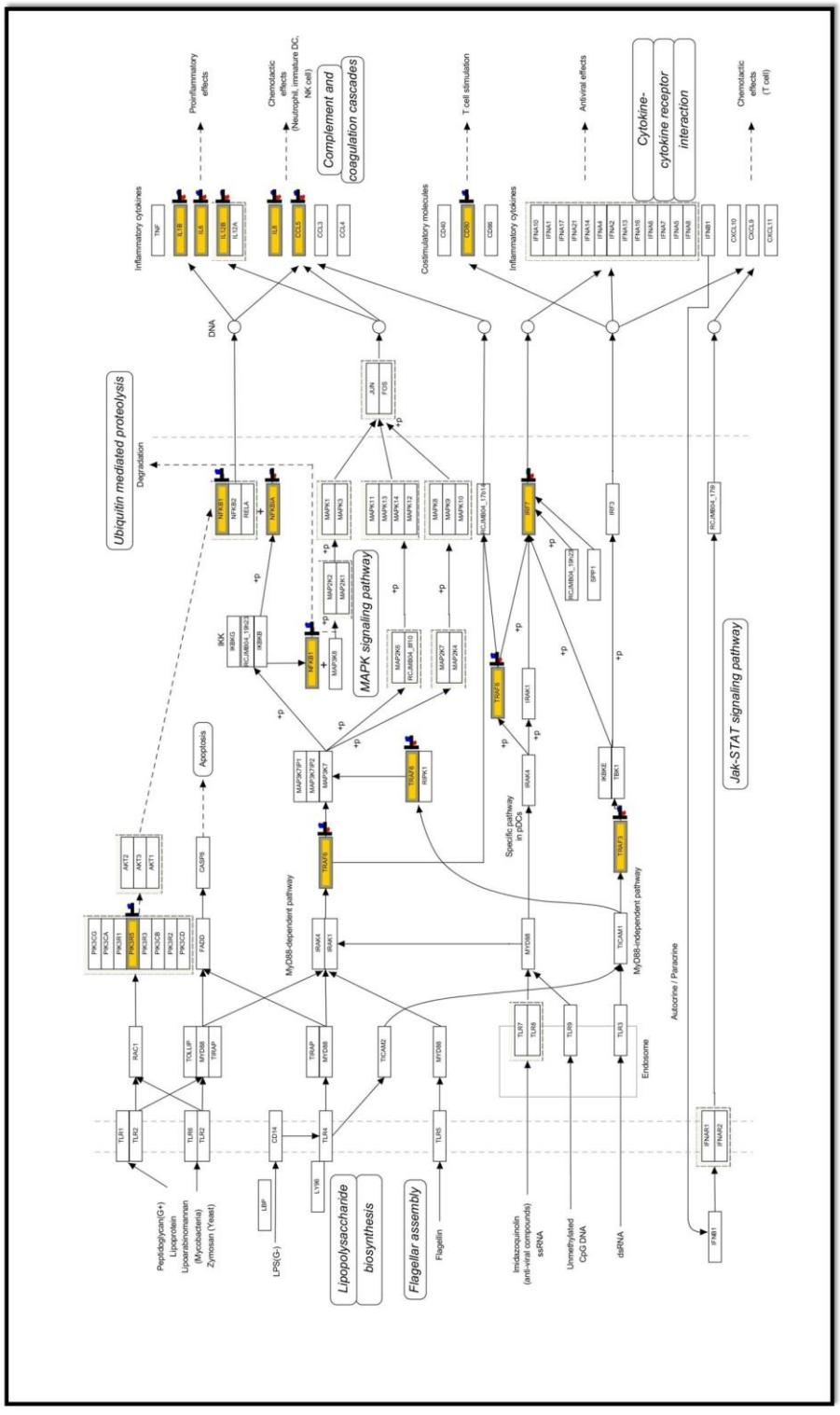
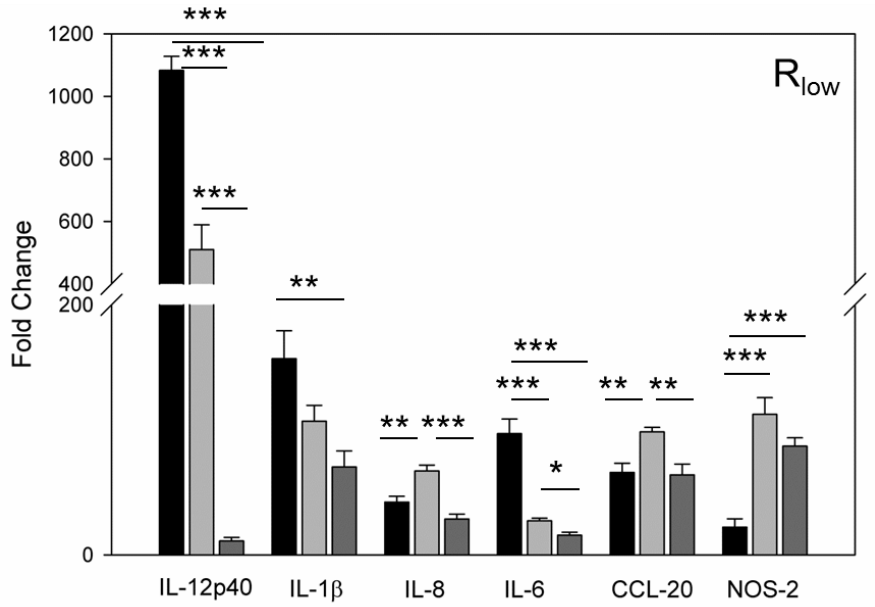
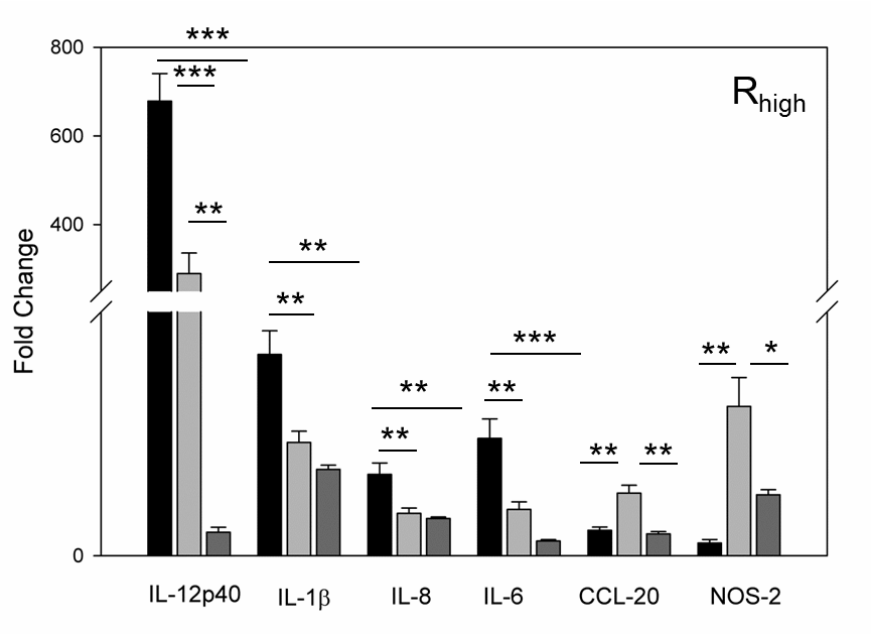

Ubiquitin mediated proteolysis
Degradation
MAPK signaling pathway
Jak-STAT signaling pathway
Cytokine-cytokine receptor interaction
Complement and coagulation cascades
Lipopolysaccharide biosynthesis
Flagellar assembly
MyD88-dependent pathway
MyD88-independent pathway
Specific pathway in pDCs
Endosome
Autocrine / Paracrine
Apoptosis
DNA
Inflammatory cytokines
Proinflammatory effects
Chemotactic effects (Neutrophil, immature DC, NK cell)
Costimulatory molecules
T cell stimulation
Antiviral effects
Chemotactic effects (T cell)
Peptidoglycan(G+)
Lipoprotein
Lipoarabinomannan (Mycobacteria)
Zymosan (Yeast)
LPS(G-)
Flagellin
Imidazoquinolin (anti-viral compounds)
ssRNA
Unmethylated CpG DNA
dsRNA

Figure 2.6:

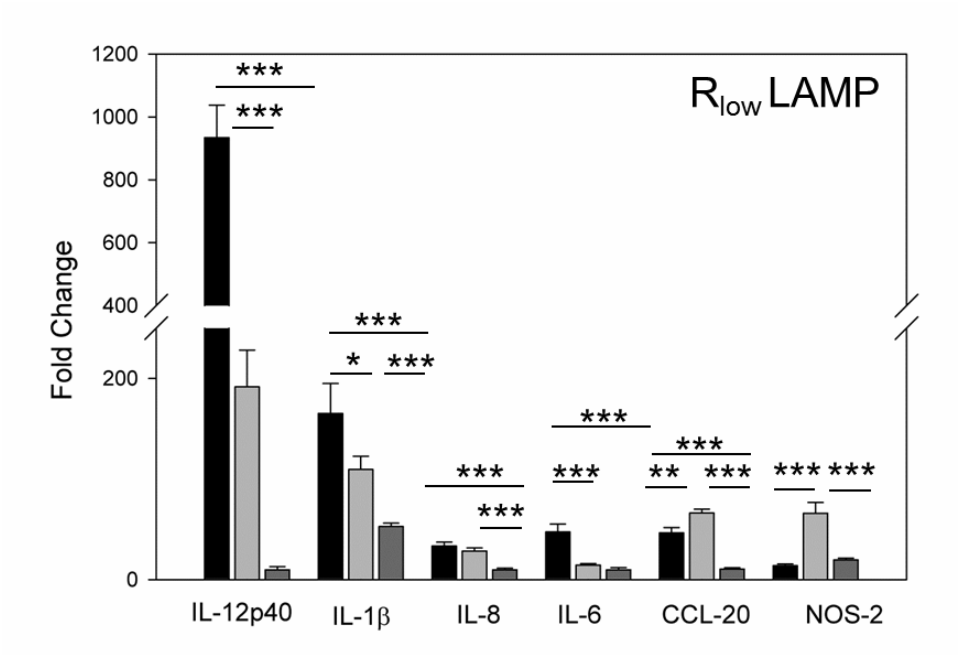
A.


Fold Change
1200
1000
800
600
400
200
0
R_{low}
IL-12p40
IL-1β
IL-8
IL-6
CCL-20
NOS-2

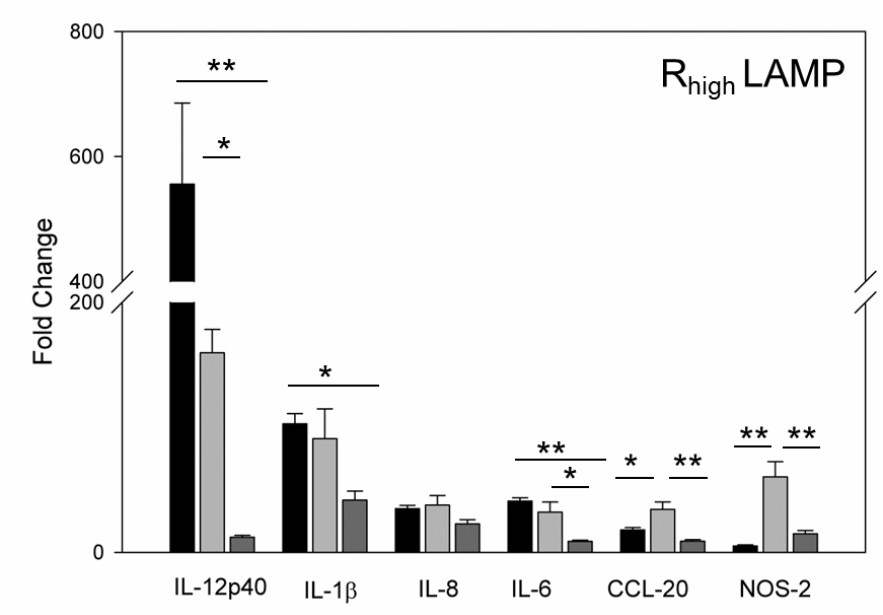
B.


Fold Change
800
600
400
0
R_{high}
IL-12p40
IL-1β
IL-8
IL-6
CCL-20
NOS-2

C.


R_low LAMP
Fold Change
1200
1000
800
600
400
200
0
IL-12p40
IL-1β
IL-8
IL-6
CCL-20
NOS-2

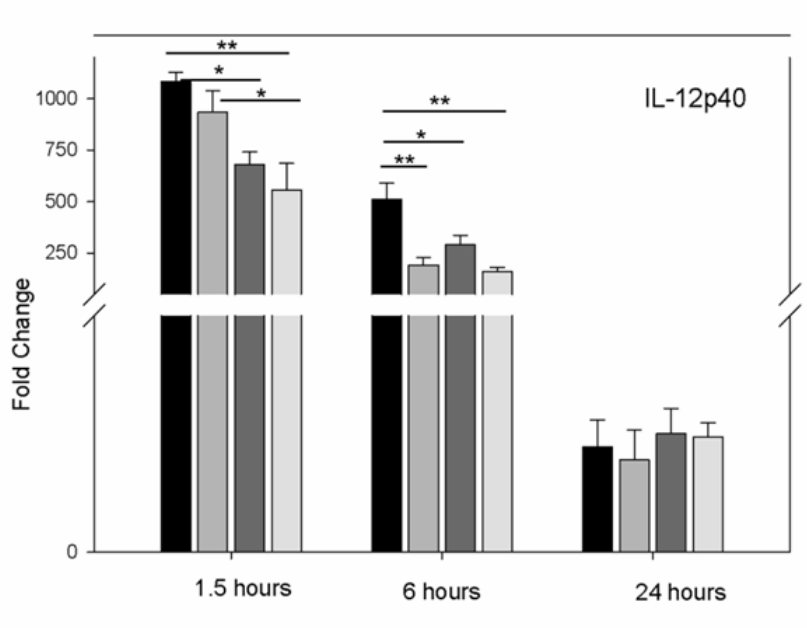
D.


R_high LAMP
Fold Change
800
600
400
200
0
IL-12p40
IL-1β
IL-8
IL-6
CCL-20
NOS-2

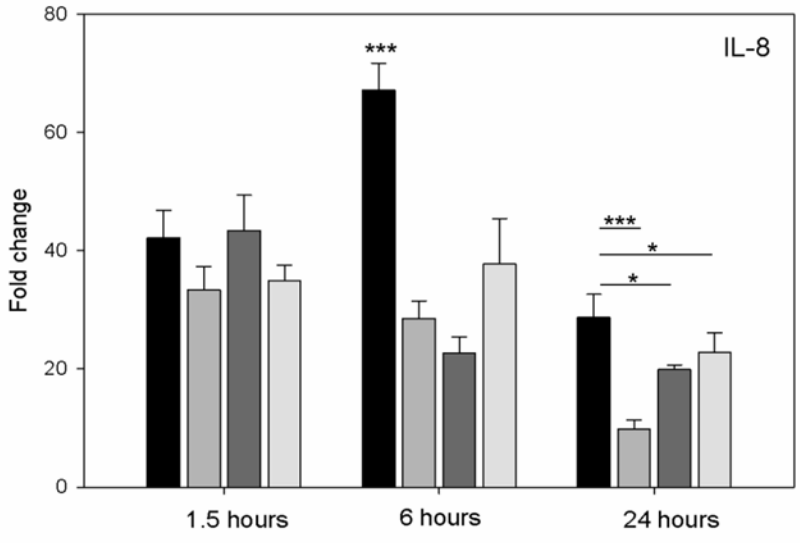

1.5 hours
6 hours
24 hours

FIGURE 2.7:

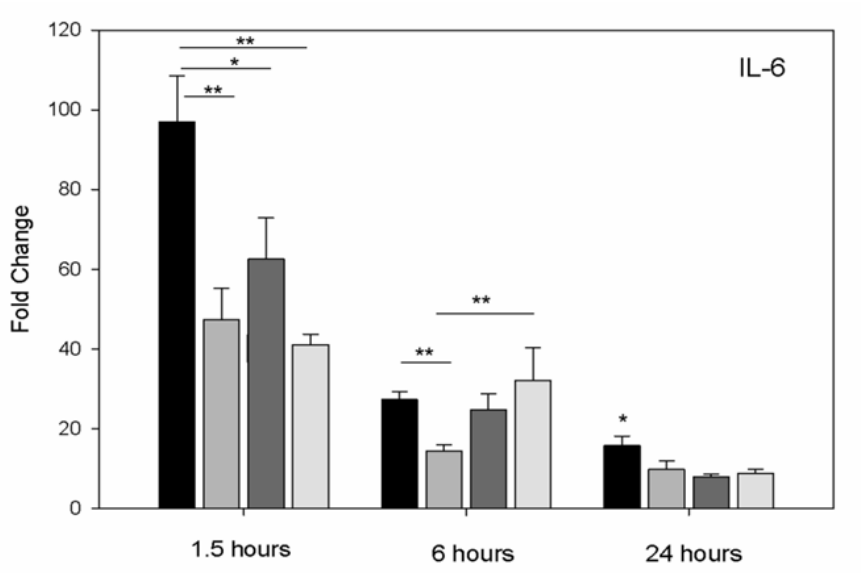
A.


IL-12p40
Fold Change
1000
750
500
250
0
**
*
*
**
*
**
1.5 hours
6 hours
24 hours

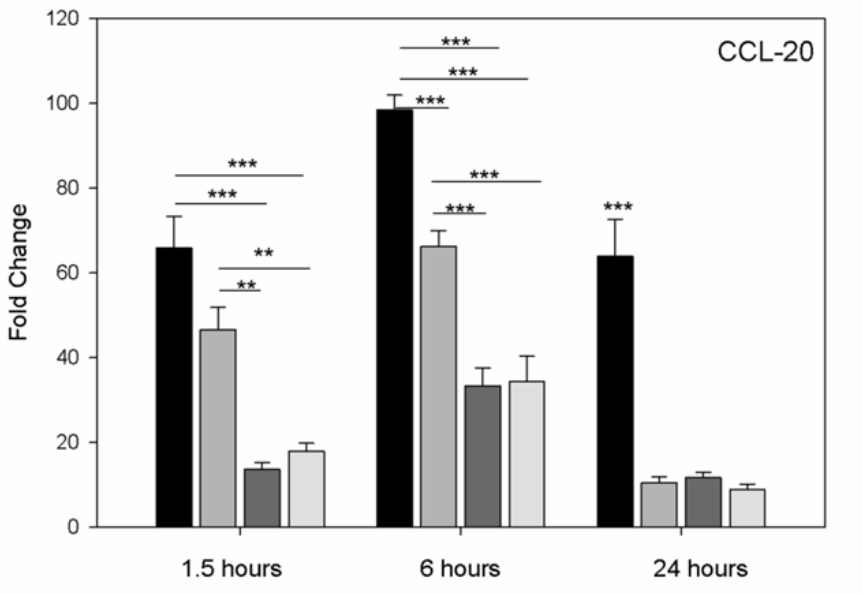
B.


IL-8
Fold change
80
60
40
20
0
***
***
*
*
1.5 hours
6 hours
24 hours

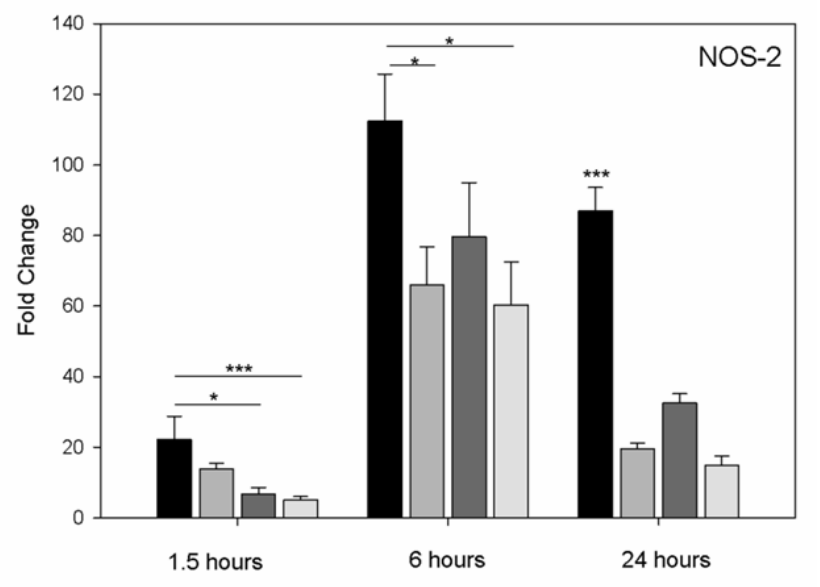
C.


IL-6
Fold Change
120
100
80
60
40
20
0
1.5 hours
6 hours
24 hours

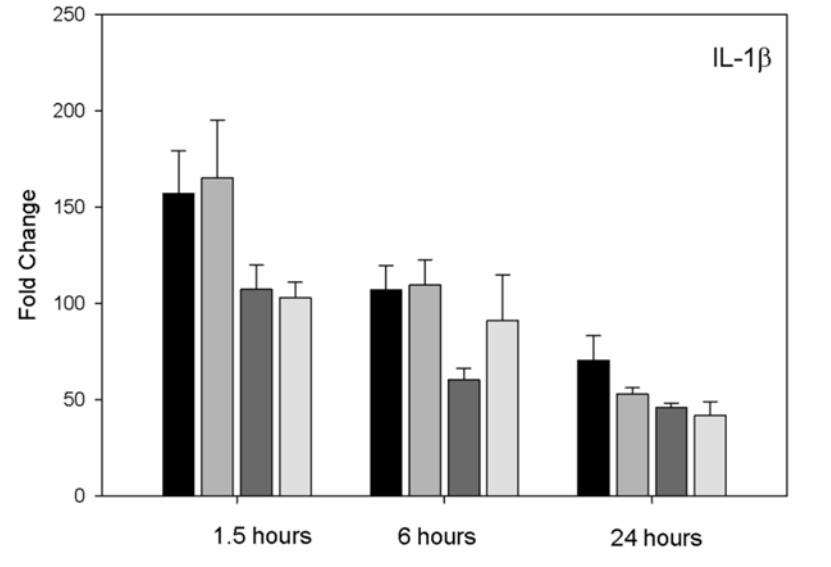
D.


CCL-20
Fold Change
120
100
80
60
40
20
0
1.5 hours
6 hours
24 hours

E.


NOS-2
Fold Change
140
120
100
80
60
40
20
0
*
*
*
***
***
1.5 hours
6 hours
24 hours

F.


IL-1β
Fold Change
250
200
150
100
50
0
1.5 hours
6 hours
24 hours

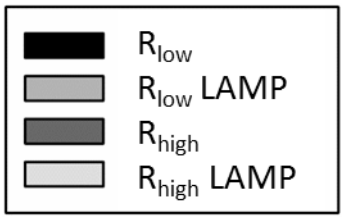
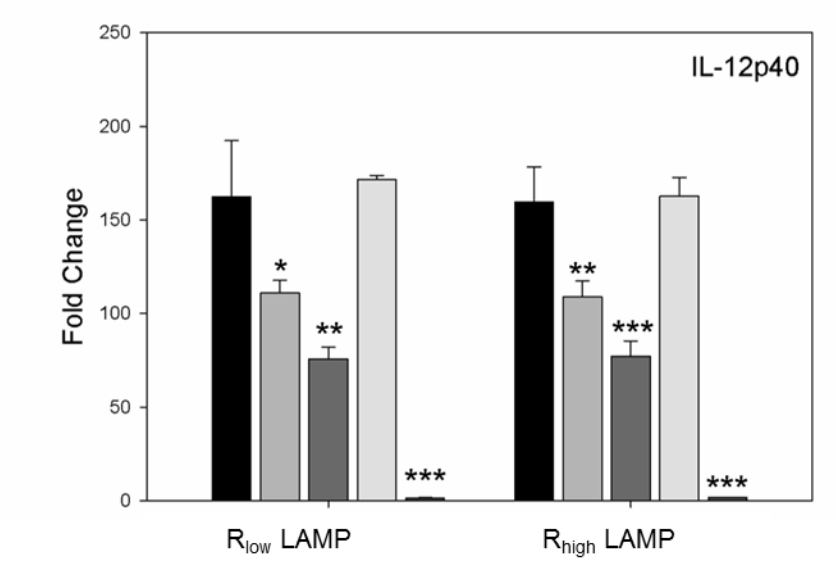
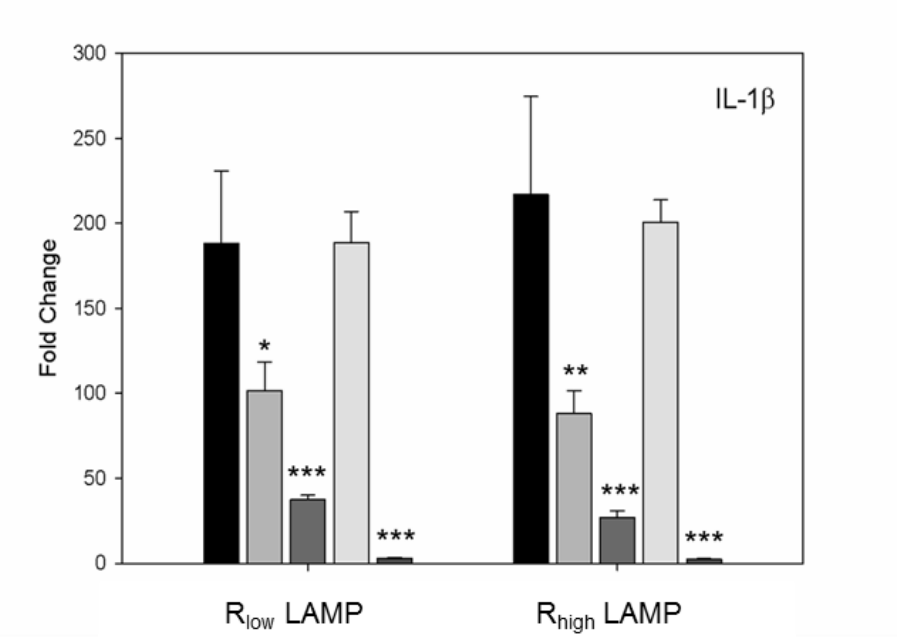

R low
R low LAMP
R high
R high LAMP

FIGURE 2.8:

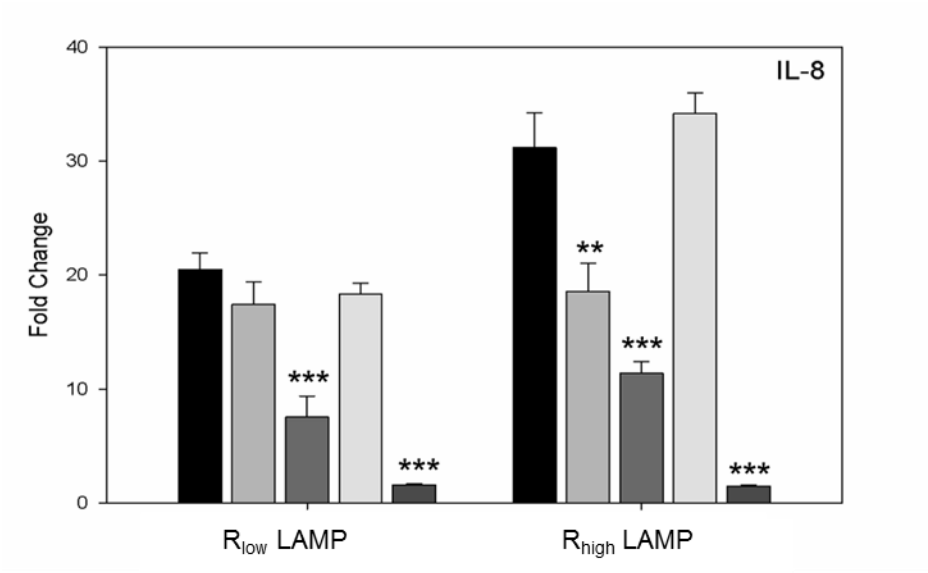
A.


IL-12p40
Fold Change
250
200
150
100
50
0
*
**
***
**
***
***
R_{low} LAMP
R_{high} LAMP

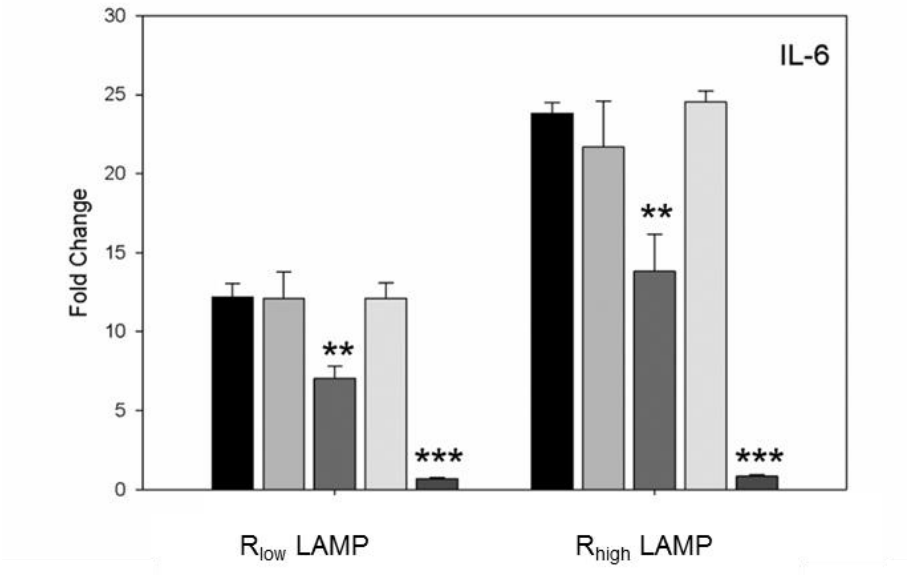
B.


IL-1β
Fold Change
300
250
200
150
100
50
0
*
***
***
**
***
***
R_{low} LAMP
R_{high} LAMP

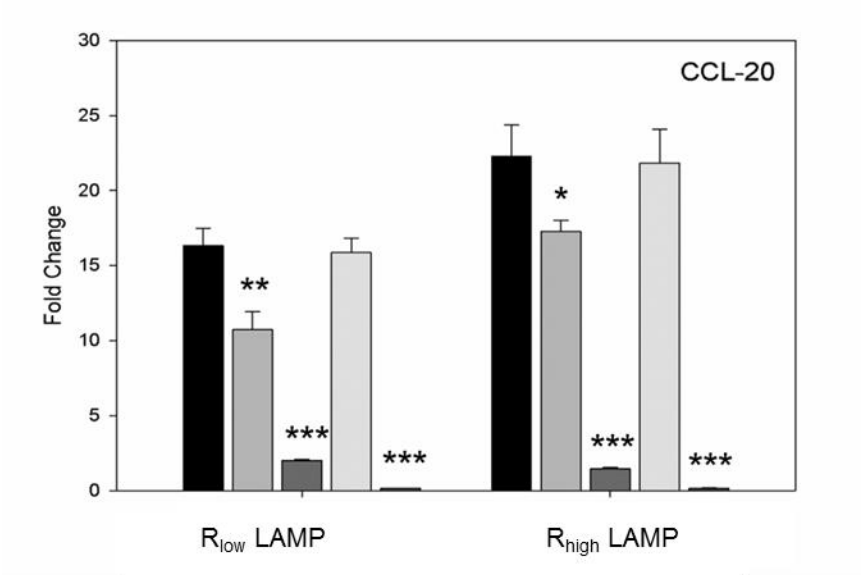
C.


IL-8
Fold Change
40
30
20
10
0
**
***
***
***
***
R_low LAMP
R_high LAMP

D.


IL-6
Fold Change
30
25
20
15
10
5
0
**
**
***
***
R_low LAMP
R_high LAMP

E.


CCL-20
Fold Change
30
25
20
15
10
5
0
**
***
***
*
***
***
R_low LAMP
R_high LAMP

F.

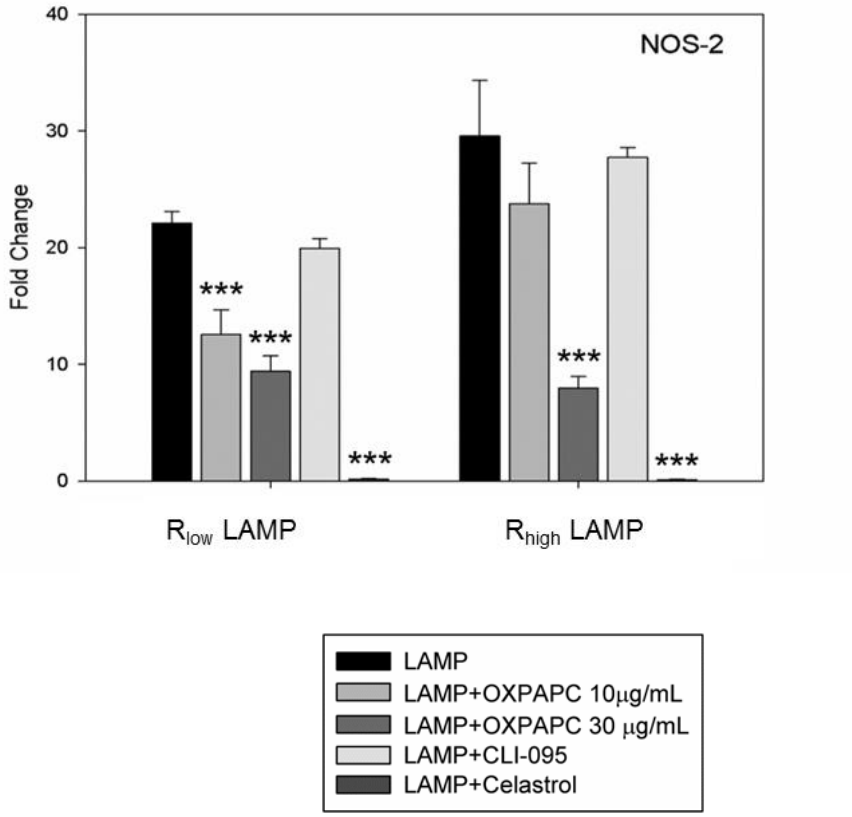
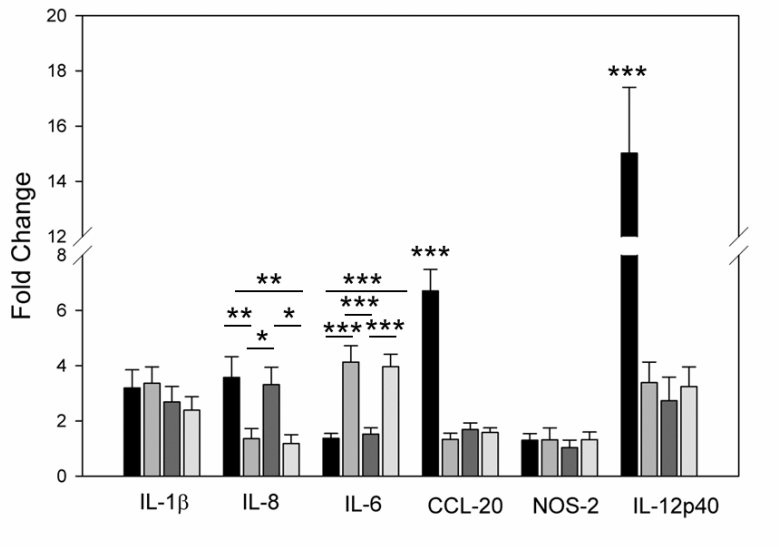

NOS-2
Fold Change
40
30
20
10
0
***
***
***
***
***
R_low LAMP
R_high LAMP
LAMP
LAMP+OXPAPC 10μg/mL
LAMP+OXPAPC 30 μg/mL
LAMP+CLI-095
LAMP+Celastrol

FIGURE 2.9:

A.


Fold Change
20
18
16
14
12
8
6
4
2
0
IL-1β
IL-8
IL-6
CCL-20
NOS-2
IL-12p40

B.

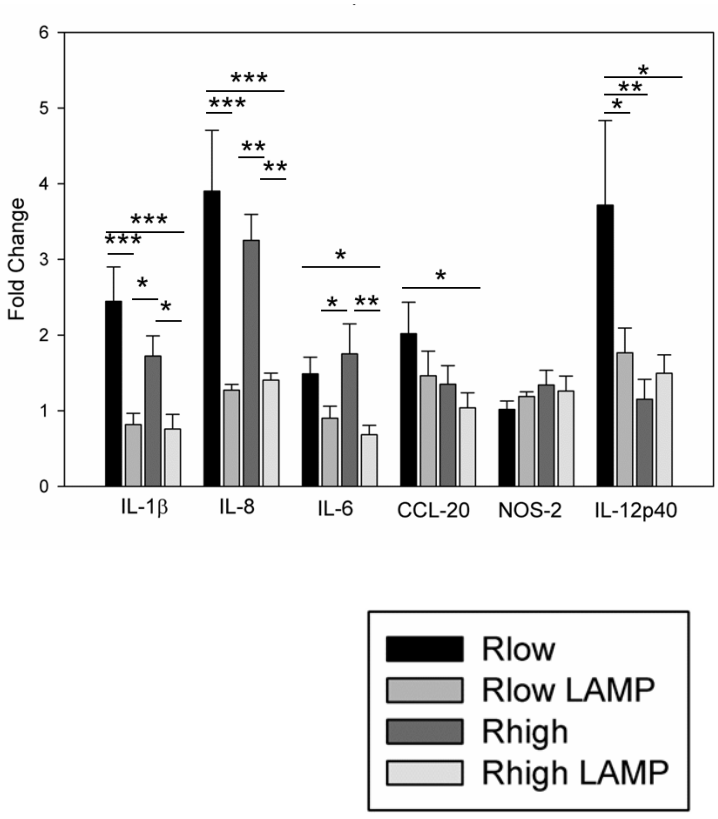
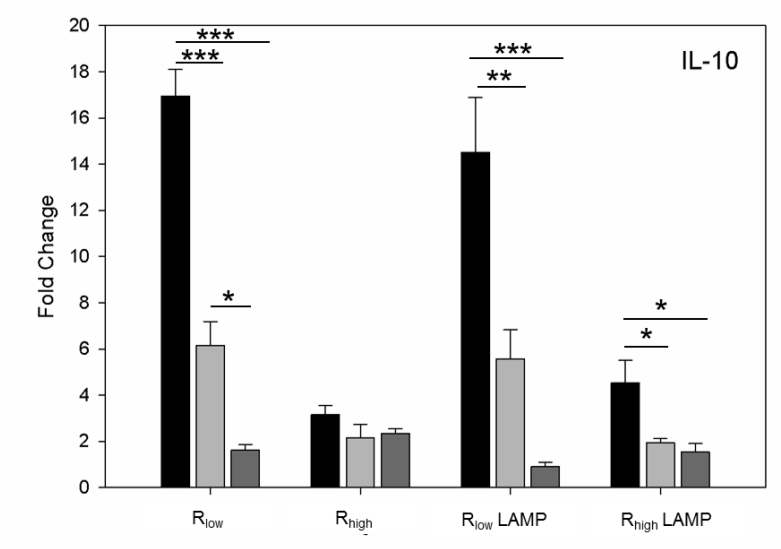
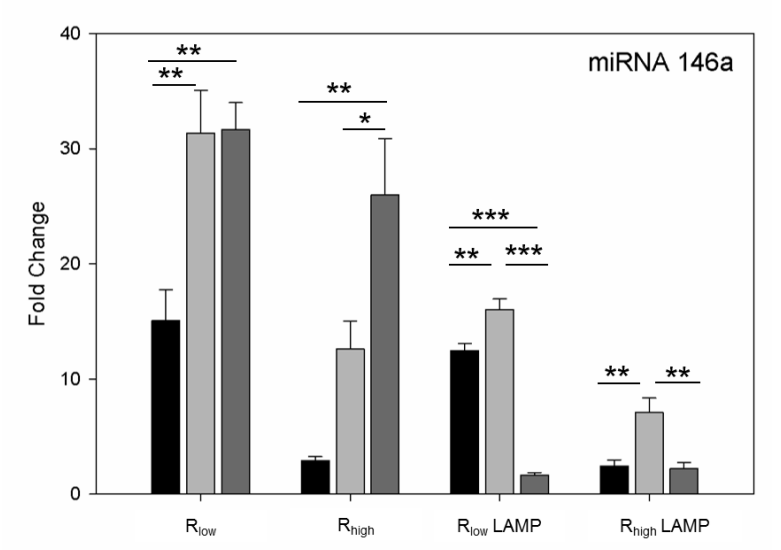

Fold Change
6
5
4
3
2
1
0
IL-1β
IL-8
IL-6
CCL-20
NOS-2
IL-12p40
Rlow
Rlow LAMP
Rhigh
Rhigh LAMP

FIGURE 2.10:

A.



B.



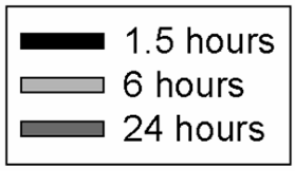
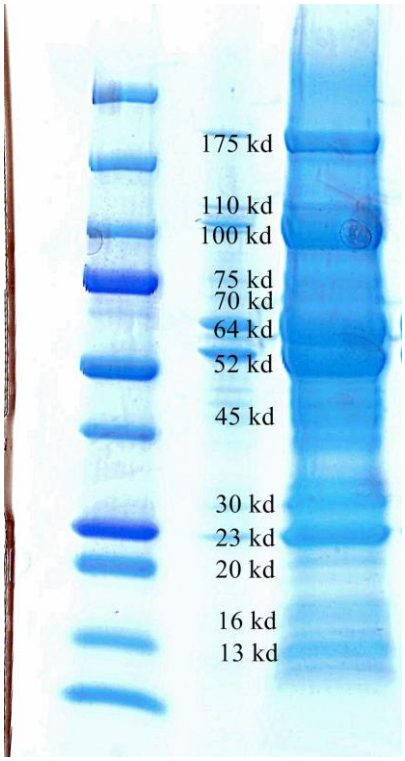
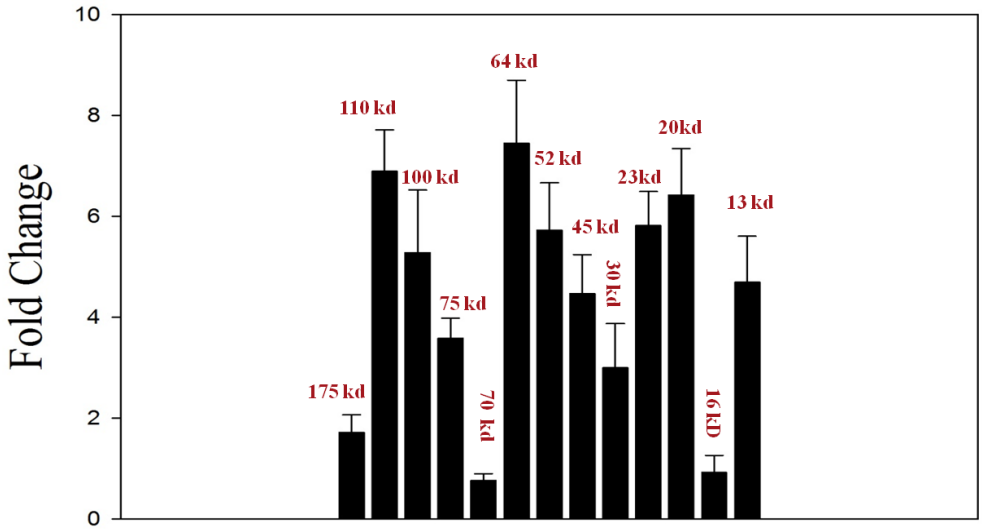


Figure 2.11

A.



B.



Chapter III

Role of *Mycoplasma gallisepticum* and Chicken Tracheal Epithelial Cell Interaction in Macrophage Chemotaxis and Activation.

Sanjukta Majumder [1, 2], Lawrence K. Silbart [1, 2, 3,*]

Department of Animal Science[1], Center of Excellence for Vaccine Research[2], Department of Allied Health Sciences[3],The University of Connecticut, Storrs, CT- 06269, USA;

3.1 Abstract

Mycoplasma gallisepticum (*M. gallisepticum*) mediates severe leukocyte influx into the tracheal submucosa after attachment and colonization of the epithelium. We recently reported that *M. gallisepticum* mediates up-regulation of chemokine and cytokine genes from chicken tracheal epithelial cells (TEC). The current study identifies how this interaction might lead to the further inflammatory process. Conditioned medium from TECs exposed to *M. gallisepticum* aided in macrophage (HD-11) chemotaxis with virulent R_{low} strain being more efficient compared to non-virulent R_{high}. Co-culture assay of HD-11 cells with mycoplasma exposed TECs lead to up-regulation of several pro-inflammatory genes in HD-11 cells including IL-1β, IL-6, IL-8, CCL20, MIP-1β, CXCL-13 and RANTES. R_{low} was found to be significantly more efficient in up-regulating these genes and aided in stable expression of chemokine genes. When HD-11 cells were exposed directly to live mycoplasmas they elaborated expression of all above mentioned genes as well as IFN-γ, but R_{low} did not show higher efficiency in the response. However in both cases the average kinetic trend identified initial up-regulation of cytokine genes followed by dramatic decline within 24 hours, whereas chemokine genes except CCL-20 remained significantly above control level until 24 hours. Taken together our data supports the notion that *M. gallisepticum* and host respiratory epithelial cell interaction leads to macrophage chemotaxis and activation leading to expression of inflammatory genes, and higher efficiency of R_{low} in the process resonates with what is seen *in vivo*. The kinetics of expression of chemokine and cytokine genes may also suggest the mechanism of leukocyte infiltration to the tracheal submucosa without successful

clearance of the pathogen; however detailed *in vivo* studies are required to confirm the molecular events during *M. gallisepticum* pathogenesis.

3.2 Introduction

Mycoplasma gallisepticum is primarily known as an extracellular pathogen, however in recent years there has been an accumulation of evidences indicating invasion of a variety of cell types including RBCs, HeLa cells and fibroblasts [1-6]. The primary site of *M. gallisepticum* attachment and colonization is the respiratory epithelium; but no studies have reported invasive capabilities of this pathogen inside tracheal epithelial cells. In fact one study reported that *M. gallisepticum* was unable to invade ciliated tracheal epithelial cells but were sometimes found inside phagocytic vacuoles [7]. In our laboratory we also tried to identify cellular localization *M. gallisepticum* in chicken trachea by in-situ hybridization utilizing 16S riboprobes and identified their location to be extracellular (unpublished, see appendix).

Due to the absence of a cell wall, mycoplasmas do not possess any endotoxin and despite being present in certain mycoplasmas, no exotoxins have been identified in *M. gallisepticum* [8-11]. However after attachment to the respiratory epithelium, *M. gallisepticum* mediates a severe inflammatory response, initially comprised of heterophils and macrophages and followed later by infiltrating lymphocytes including both B and T cells, into the submucosa. Previous studies reported that macrophages play a significant role in mycoplasma clearance, whereas polymorphonuclear leukocytes may in fact aid in dissemination of mycoplasma to other tissues [12, 13]. Both B and T cells were also found to play critical role in clearance and prevention of dissemination of mycoplasma

[13-15]. But as these events play important role in anti-mycoplasmal defense, they may also contribute to the significant immunopathology associated with the disease.

Chemokines and cytokines are often major factors in determining the outcome of immune responses and complex interplay between cells and cytokines often determine disease outcome. These molecules have been found to play significant role in mycoplasmal disease pathogenesis. Majority of studies conducted on interaction of mycoplasmas with macrophages and monocytes found expression and release of several chemokines and cytokines including but not limited to IL-4, IL-5, IL-6, IFN-γ, TNF-α, IL-1β, MIP-1β and presence these cytokines was highly correlated with the presence of gross inflammatory lesions [16-24]. Epithelial cell derived inflammatory mediators are known for their role in recruiting leukocytes to the site of infection [25] and during *Mycoplasma genitalium* infection, human cultured endocervical epithelial cells were found to produce IL-6, IL-7, IL-8, MCP-1 and GM-CSF [26-28] and *Mycoplasma pneumoniae* infection resulted in the release of IL-8 and RANTES from human nasal epithelial cells and up-regulation of IL-1β, IL-8 and TNF-α genes from A549 cells [29]. A recently published study from our laboratory identified, up-regulation of CCL-20, IL-1β, IL-6, IL-8 and IL-12p40 in chicken tracheal epithelial cells within 1.5 hours of exposure to *M. gallisepticum* and their lipid associated membrane proteins (LAMP). Exposure to the virulent R_{low} strain of *M. gallisepticum* was significantly more efficient in up-regulating these genes, and also up-regulated over 100 additional genes by 5- or more fold [30] compared to non-virulent R_{high} strain and LAMPs. However A previous study from our laboratory reported down regulation of CCL-20, IL-1β, IL-8 and IL-12p40 in chicken trachea at day 1 upon experimental infection with *M. gallisepticum* with

CCL-20 and IL-12p40 expression remaining low throughout the time course and expression of IFN-γ, CXCL-13, RANTES, MIP-1β and lymphotactin to be up-regulated [31]. Since *M. gallisepticum* is able to induce this severe inflammatory response just by colonization of epithelial cell surface, without the presence of any endotoxin and known exotoxin led us to hypothesize that chemokines and cytokines produced by epithelial cells upon interaction with virulent *M. gallisepticum* aids in chemotactic migration of macrophages into the submucosa. And the above mentioned discrepancy in inflammatory gene expression also led us tohypothesize that the migrated macrophages in-turn express a unique set of inflammatory mediators which possibly results in an aberrant and sustained signal for further leukocyte recruitment leading to the observed dysregulated/"over-exuberant" immune response and the ensuing immunopathological changes in the tracheal tissue. To test this hypothesis we utilized conditioned medium from epithelial cells exposed to a virulent and a non-virulent strain of *M. gallisepticum*, to identify their macrophage chemotactic properties *in vitro*. A co-culture study of macrophages with *M. gallisepticum* exposed tracheal epithelial cells was performed to identify macrophage activation leading to expression of inflammatory genes.

3.3 Material and Methods

Mycoplasma gallisepticum culture conditions

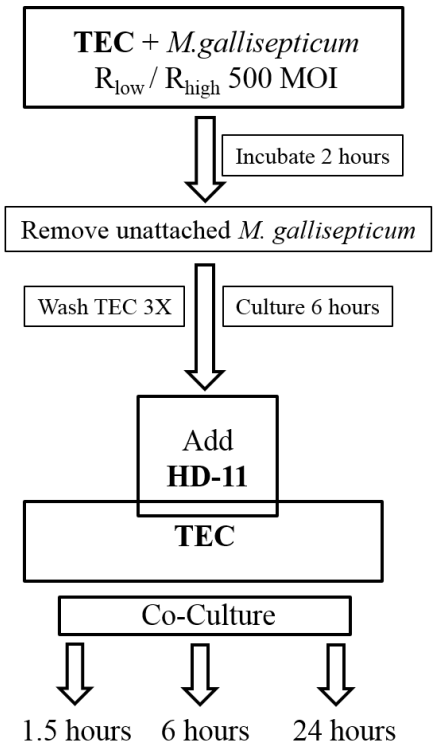
M. gallisepticum, low passage virulent strain R_{low} or high passage non-virulent strain R_{high} were grown in modified Hayflick's medium until change of phenol red dye in media from red to orange or log phase. $0.7 \times 10^8 - 1.3 \times 10^8$ cfu of mycoplasma per milliliter of culture were used in all studies to avoid use of overgrown culture.

HD-11 and tracheal epithelial cell culture

Primary chicken tracheal epithelial cells (TEC) were cultured in ATE medium as previously described [30]. All exposures were done 72 hours post plating, when TECs reached about 70-80% confluency. Chicken HD-11 macrophage cell line was kindly provided by Dr. Steven C. Ricke at University of Arkansas, Department of Food Science. HD-11 cells were cultured in RPMI supplemented with 5% FBS and 5% chicken serum at 37^{o}C with 5% CO_2. HD-11 cells were plated at a concentration 10^4 cells/well in a 6.5mm Transwell with 0.4µm Pore Polycarbonate Membrane Insert (Corning Inc.; Tewksbury MA) 18 hours prior to the co-culture assay. For other assays, HD-11 cells were plated at a concentration of 10^5 cells/well in 24 well plates 18 hours prior to exposure.

Co-culture Study and exposure of HD-11 cells to live mycoplasma

For the co-culture study chicken TECs were exposed to 10^8 cfu. *M.gallisepticum* (Approximately 500 MOI) strain R_{low} or R_{high} or media alone for 2 hours to allow cytadherence. Supernatants containing unattached mycoplasmas were then removed and cells were washed thrice with sterile PBS. Cells were then supplemented with 500 µL fresh ATE medium and cultured for 6 hours. Transwell supports containing HD-11 cells were then placed over each well of the 24 well plates. Co-culture was allowed for 1.5, 6 and 24 hours.



HD-11 cells were also exposed to 25, 50, 100 or 200 MOI of live *M.gallisepticum* strain R_{low} or R_{high} per well as positive controls and cultured for 1.5, 6 and 24 hours. All experiments were done with 6 biological replicates.

RNA isolation, cDNA synthesis and Real time quantitative PCR

RNA was isolated from HD-11 cells using Qiagen RNeasy Mini (Qiagen; Valencia, CA) kit following manufacturer's instruction. RNA quality and quantity were analyzed in Agilent 2100 Bioanalyzer using Agilent RNA 6000 Pico kit (Agilent technologies; Mendon,MA). All cDNA were synthesized from 1µg total RNA using iScript Reverse Transcription Supermix for RT-qPCR (Bio Rad; Hercules, CA). All RT-qPCR were performed using 1µL of cDNA with iTaq Universal SYBR Green Supermix (Bio Rad) in a total 20µL reaction. All amplfications were performed in a 7900HT Fast Real Time PCR system (Life Technogies; Grand Island,NY) using the protocol 50^{o}C for 2 minutes,

95 ºC for 1 min, followed by 40 cycles of 95 ºC for 15 seconds and 58–60 ºC (depending on Tm values of specific primers) for 60 seconds. Melt curve analysis was performed at 65-95 ºC with 0.5 ºC increment and 2 sec/step. Agarose gel electrophoresis was used to confirm all amplicon sizes after RT-qPCR. Reference gene for all real time reactions was GAPDH and fold differences were determined using the ΔΔCT method. Primer sequences for all genes are listed in **Table 3.1**.

Chemotaxis Assay

TECs were exposed to 10^8 cfu (approximately 500 MOI) *M.gallisepticum* R_{low} or R_{high} suspended in ATE medium and adherence was allowed for 2 hours. ATE medium alone was used as control. After 2 hours unattached mycoplasmas were removed along with the supernatant and the TECs were washed thrice with PBS. TEC culture was allowed for another 6 hours, and the culture medium was used as the conditioned medium. Chemotaxis assay was done using Neuro Probe 48 well micro-chemotaxis chamber and Neuro-probe 5µM pore size, polycarbonate membranes (Neuro Probe Inc.; Gaithersburg, MD). Chicken recombinant MIP-1β (Kingfisher Biotech; St Paul, MN) at a concentration of 100 ng/mL was used as positive control and DMEM-F12 as negative control. Conditioned medium from mycoplasma exposed TECs or control TECs were used for the assay. 30µL chemo-attractants were added in the bottom chamber and 50 µL HD-11 cells ($2X10^7$ cells/mL) were added on the top chamber. Chemotaxis assay was allowed to run for 4 hours at 37^0C incubator with 5% CO_2. After completion of the assay, filters were collected, and cells that did not move towards the chemotaxis gradient through the pores were scraped. Cells that moved towards the chemotactic gradient through the pores were then stained using a Hema3 Stat Pack stain kit (Thermo Fisher

Scientific Inc.; Hudson, NH) according to manufacturer's recommendations. The membrane was then air dried and cells were counted under a light microscope at 10X magnification. All experiments were performed with 6 biological replicates.

Statistical Analysis

All statistical analyses were performed using the Sigmaplot 11.0 software. Multiple pairwise comparison of number of cells migrated during the chemotaxis assay and mRNA fold differences during RT-qPCR studies were analyzed using one-way repeated measures ANOVA, using Shapiro-Wilk normality tests. Post-hoc pairwise multiple comparison analyses were done using Student-Newman-Keuls Method. Results were denoted as fold change ± SD. Differences were considered significant if $p<0.05$. Significant differences were denoted as * = $P< 0.05$, **= $P <0.01$, *** = $P < 0.001$.

3.4 Results

Chemotaxis Assay

Chemotaxis assay was run as described in the methods section. Chicken recombinant MIP-1β and conditioned media from R_{low} exposed TECs showed comparable levels of chemotactic activity towards HD-11 cells, with an average of 178 and 152 cells, which was significantly higher than the number of cells migrating in response to conditioned medium from R_{high} exposed TECs ($p<0.001$), conditioned medium from control TECs ($p<0.001$) and DMEM-F12 alone ($p<0.001$). Conditioned media from R_{high} exposed TEC chemoattracted larger numbers of cells than control TECs ($p<0.05$) and DMEM-F12 ($p<0.01$). However no significant difference was observed between conditioned media obtained from control TECs and DMEM-F12 alone **(Figure 3.1)**.

Gene expression in HD-11 cells upon Co-culture with mycoplasma exposed TECs

Expression of IL-1β, IL-6, CCL-20, MIP-1β, CXCL-13, IL-8 and RANTES increased significantly in HD-11 cells during co-culture with both R_{low} and R_{high} exposed TECs **(Figure 3.2 A-H)**. Expression of IL-1β, IL-6 and CCL-20 peaked at 6 hours; reducing significantly thereafter in all samples **(Figure 3.2 B-D)**. Expression of MIP-1β and IL-8 peaked at 6 hours only during co-culture with R_{high} exposed TECs but stabilized after 6 hours during co-culture with R_{low} exposed TECs **(Figure 3.2 E,G)** and CXCL-13 peaked at 6 hours during co-culture with R_{low} exposed TECs and at 1.5 hours during R_{high} **(Figure 3.2F)**. RANTES expression on the other hand had a stable expression after 6 hours in all samples **(Figure 3.2H)**. IFN-γ expression did not differ between any time points during any exposure **(Figure 3.2A).** When the magnitude of gene expression were compared between HD-11 co-cultured with R_{low} or R_{high} exposed TECs, expression of all 7 genes were found to be significantly higher in the R_{low} exposed TEC for at least two or more time points **(Figure 3.3 A-H)**.

Gene expression in HD-11 cells upon exposure to live mycoplasma

In HD-11 cells exposed to both live *M. gallisepticum* strains R_{low} and R_{high} at various MOI, expression of IFN-γ, IL-1β and IL-6 and CCL20 peaked at 6 hours reducing significantly thereafter with IFN-γ and IL-6 reduced to baseline levels by 24 hours **(Figure 3.4A-D and Figure 3.5A-D).** MIP-1b, IL-8 and RANTES expression increased over time during all exposures **(Figure 3.4E, G, H and Figure 3.5E, G, H)**. CXCL-13 expression peaked at 6 hours reducing slightly by 24 hours, but still remained significantly up-regulated compared to control **(Figure 3.4F and Figure 3.5F)**. No notable differences in gene expression were observed between exposures to various

MOIs. Surprisingly, when HD-11 cells were exposed to either live R_{low} or R_{high}, unlike the previous studies R_{low} did not up-regulate expression of genes at a higher level than R_{high} **(Figure 3.6A-H)**.

3.5 Discussion

M. gallisepticum infection leads to inflammation of trachea, air sacs and lungs in chickens, and is associated with severe cytopathic effects and sub-epithelial infiltration leukocytes [14, 32, 33]. Cytadhesion to the respiratory epithelium is the primary and most critical step for this pathogenesis therefore cytadhesion deficient strains fail to induce a disease state in the host [34-36]. As invasive capabilities of *M. gallisepticum* into the tracheal epithelium have not yet been identified, it still remains elusive as to how this pathogen is able to mediate the severe inflammatory response just by adhering to the epithelium. We recently published a study identifying both R_{low} and R_{high} strains to be able to up-regulate several inflammatory genes from tracheal epithelial cells. However R_{low} was found to be more efficient in up-regulating inflammatory genes as well as differentially regulating several unique genes in tracheal epithelial cells not observed during R_{high} exposure [30]. The current study reports how *M. gallisepticum* and chicken tracheal epithelial cell interaction might play a role in the overall inflammatory response, and why virulent strains are more efficient in the process.

During the chemotaxis assay we identified that conditioned medium from tracheal epithelial cells exposed to R_{low} was significantly more efficient in chemoattracting macrophages when compared to conditioned medium from epithelial cells exposed to R_{high}. The co-culture study that was designed to identify effect of cell derived mediators from mycoplasma exposed epithelial cells on HD-11 cells, identified up-regulation of

several chemokine and cytokine genes including IL-1β, IL-6, IL-8, CCL-20, MIP-1β, CXCL-13 and RANTES but not IFN-γ and HD-11 cells co-cultured with R_{low} exposed TECs showed significantly higher expression of all genes at two or more time points. We did not observe any significant changes in mRNA expression of IL-12p40 during any exposure at any time point. Activated macrophages are well known for their production of IL-12p40, which is essential for Th1 development [37], and are known to dramatically alter their secretory profile leading to production of chemokines and cytokines like IL-1β, IL-6, IL-8, MIP-1β, TNF-α, CXCL-13 and RANTES [38-42]. Therefore lack of up-regulation of IL-12p40 in this study and also during the previously published study identifying down regulation of this gene in chicken trachea [31] might suggest an improper activation of macrophages during *M. gallisepticum* infection. No observable difference in expression of IFN-γ, also suggest failure of epithelial cell derived mediators to properly activate macrophages [43]. However when HD-11 cells were exposed to live mycoplasma, IFN-γ along with all above mentioned genes were significantly up-regulated in all samples, but surprisingly unlike the co-culture study, R_{low} failed to induce higher gene up-regulation when compared to R_{high} and neither varied significantly between various MOIs.

When the kinetics of expression were analyzed, expression of IL-1β, IL-6, CXCL-13 and CCL-20 was found to peak at 6 hours, significantly reducing thereafter during co-culture of HD-11 cells to both R_{low} and R_{high} exposed TECs, however expression of MIP-1β and IL-8 sustained at significantly high level in HD-11 cells co-cultured with R_{low} exposed TECs and RANTES during both co-cultures. When HD-11 cells were exposed to various MOI of live *M. gallisepticum* R_{low} or R_{high}, expression of Expression of IFN-γ and

IL-6 levels had a dramatic decrease after peak expression at 6 hours and reached baseline levels, and expression of IL-1β and CCL-20 was also significantly reduced by 24 hours. Expression of the other chemokine genes on the other hand stayed significantly above control level throughout the time course.

Taken together these results support our notion that interaction of *M. gallisepticum* with host respiratory epithelial cells lead to macrophage chemotaxis and activation leading to expression of inflammatory chemokine and cytokine genes. Higher efficiency of R_{low} exposed epithelial cells in all gene expression, as well as induction of sustained chemokine gene expression in macrophages also indicate why virulent strains are more efficient in establishing the inflammatory response, whereas non virulent strains fail to do so. Expression of inflammatory genes from macrophages therefore might not require invasion of live mycoplasma into the epithelium, and our results indicating both virulent and non-virulent mycoplasma at various MOIs, to be equally capable in differential gene expression from macrophages strengthens this notion.

Whereas chemokines are known for their leukocyte recruitment properties, chemokines are involved in activation of leukocytes [44]. Therefore the observed kinetic trend of chemokine and cytokine gene expression in the study may indicate the severe immunopathology associated with *M. gallisepticum* infection in chickens without successful clearance of the pathogen, however more detailed in vivo studies are required to confirm these findings and better understand the cellular and molecular events during *M. gallisepticum* pathogenesis.

3.6 Acknowledgements

We acknowledge Dr. Steven C. Ricke of University of Arkansas for kindly providing us with the HD-11 cell lines. We thank Dr. Steven J. Geary for providing us with the *Mycoplasma gallisepticum* strains and Dr. Micheal A. Lynes for providing his laboratory space and equipment for the chemotaxis assay. We acknowledge Ms. Sadikshya Bhandari and Dr. Kathryn M. Pietrosimone for their help with the chemotaxis assay. We also thank Ms. Debra Rood for her maintenance of the laboratory.

3.7 References

1. Rosengarten, R., et al., *Host-pathogen interactions in mycoplasma pathogenesis: virulence and survival strategies of minimalist prokaryotes.* Int J Med Microbiol, 2000. **290**(1): p. 15-25.
2. Winner, F., R. Rosengarten, and C. Citti, *In vitro cell invasion of Mycoplasma gallisepticum.* Infect Immun, 2000. **68**(7): p. 4238-44.
3. Much, P., et al., *Mycoplasma gallisepticum: Influence of cell invasiveness on the outcome of experimental infection in chickens.* FEMS Immunol Med Microbiol, 2002. **34**(3): p. 181-6.
4. Vogl, G., et al., *Mycoplasma gallisepticum invades chicken erythrocytes during infection.* Infect Immun, 2008. **76**(1): p. 71-7.
5. Furnkranz, U., et al., *Factors influencing the cell adhesion and invasion capacity of Mycoplasma gallisepticum.* Acta Vet Scand, 2013. **55**: p. 63.
6. Indikova, I., et al., *Role of the GapA and CrmA cytadhesins of Mycoplasma gallisepticum in promoting virulence and host colonization.* Infect Immun, 2013.
7. Tajima, M., T. Nunoya, and T. Yagihashi, *An ultrastructural study on the interaction of Mycoplasma gallisepticum with the chicken tracheal epithelium.* Am J Vet Res, 1979. **40**(7): p. 1009-14.
8. Chambaud, I., H. Wroblewski, and A. Blanchard, *Interactions between mycoplasma lipoproteins and the host immune system.* Trends Microbiol, 1999. **7**(12): p. 493-9.
9. Cole, B.C., *The immunobiology of Mycoplasma arthritidis and its superantigen MAM.* Curr Top Microbiol Immunol, 1991. **174**: p. 107-19.
10. D'Orazio, J.A., B.C. Cole, and J. Stein-Streilein, *Mycoplasma arthritidis mitogen up-regulates human NK cell activity.* Infect Immun, 1996. **64**(2): p. 441-7.
11. Medina, J.L., et al., *Mycoplasma pneumoniae CARDS toxin induces pulmonary eosinophilic and lymphocytic inflammation.* Am J Respir Cell Mol Biol, 2012. **46**(6): p. 815-22.
12. Hickman-Davis, J.M., *Role of innate immunity in respiratory mycoplasma infection.* Front Biosci, 2002. **7**: p. d1347-55.
13. Cartner, S.C., et al., *Roles of innate and adaptive immunity in respiratory mycoplasmosis.* Infect Immun, 1998. **66**(8): p. 3485-91.
14. Javed, M.A., et al., *Correlates of immune protection in chickens vaccinated with Mycoplasma gallisepticum strain GT5 following challenge with pathogenic M. gallisepticum strain R(low).* Infect Immun, 2005. **73**(9): p. 5410-9.
15. Gaunson, J.E., et al., *The cellular immune response in the tracheal mucosa to Mycoplasma gallisepticum in vaccinated and unvaccinated chickens in the acute and chronic stages of disease.* Vaccine, 2006. **24**(14): p. 2627-33.
16. Lam, K.M., *The macrophage inflammatory protein-1beta in the supernatants of Mycoplasma gallisepticum-infected chicken leukocytes attracts the migration of chicken heterophils and lymphocytes.* Dev Comp Immunol, 2002. **26**(1): p. 85-93.
17. Shimizu, T., Y. Kida, 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): p. 121-9.

18. Shimizu, T., Y. Kida, and K. Kuwano, *A dipalmitoylated lipoprotein from Mycoplasma pneumoniae activates NF-kappa B through TLR1, TLR2, and TLR6.* J Immunol, 2005. **175**(7): p. 4641-6.
19. Shimizu, T., Y. Kida, and K. Kuwano, *Triacylated lipoproteins derived from Mycoplasma pneumoniae activate nuclear factor-kappaB through toll-like receptors 1 and 2.* Immunology, 2007. **121**(4): p. 473-83.
20. Shimizu, T., Y. Kida, and K. Kuwano, *Mycoplasma pneumoniae-derived lipopeptides induce acute inflammatory responses in the lungs of mice.* Infect Immun, 2008. **76**(1): p. 270-7.
21. Shimizu, T., Y. Kida, and K. Kuwano, *A triacylated lipoprotein from Mycoplasma genitalium activates NF-kappaB through Toll-like receptor 1 (TLR1) and TLR2.* Infect Immun, 2008. **76**(8): p. 3672-8.
22. Faulkner, C.B., et al., *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.* Infect Immun, 1995. **63**(10): p. 4084-90.
23. Nishimoto, M., et al., *Gene expression of tumor necrosis factor alpha and interferon gamma in the lungs of Mycoplasma pulmonis-infected mice.* Microbiol Immunol, 1994. **38**(5): p. 345-52.
24. Blanchard, A. and G. Browning, *Mycoplasmas: Molecular Biology Pathogenicity and Strategies for Control*. 2005: CRC Press. 600.
25. Gomez, M.I. and A. Prince, *Airway epithelial cell signaling in response to bacterial pathogens.* Pediatr Pulmonol, 2008. **43**(1): p. 11-9.
26. McGowin, C.L., et al., *Persistent Mycoplasma genitalium infection of human endocervical epithelial cells elicits chronic inflammatory cytokine secretion.* Infect Immun, 2012. **80**(11): p. 3842-9.
27. McGowin, C.L., et al., *Mycoplasma genitalium-encoded MG309 activates NF-kappaB via Toll-like receptors 2 and 6 to elicit proinflammatory cytokine secretion from human genital epithelial cells.* Infect Immun, 2009. **77**(3): p. 1175-81.
28. McGowin, C.L., V.L. Popov, and R.B. Pyles, *Intracellular Mycoplasma genitalium infection of human vaginal and cervical epithelial cells elicits distinct patterns of inflammatory cytokine secretion and provides a possible survival niche against macrophage-mediated killing.* BMC Microbiol, 2009. **9**: p. 139.
29. Yang, J., et al., *Regulation of proinflammatory cytokines in human lung epithelial cells infected with Mycoplasma pneumoniae.* Infect Immun, 2002. **70**(7): p. 3649-55.
30. Majumder, S., F. Zappulla, and L.K. Silbart, *Mycoplasma gallisepticum Lipid Associated Membrane Proteins Up-regulate Inflammatory Genes in Chicken Tracheal Epithelial Cells via TLR-2 Ligation through an NF-kappaB Dependent Pathway.* PLoS One, 2014. **9**(11): p. e112796.
31. Mohammed, J., et al., *Chemokine and cytokine gene expression profiles in chickens inoculated with Mycoplasma gallisepticum strains Rlow or GT5.* Vaccine, 2007. **25**(51): p. 8611-21.

32. Levisohn, S., et al., *Comparison of in vivo and in vitro methods for pathogenicity evaluation for Mycoplasma gallisepticum in respiratory infection.* Avian Pathol, 1986. **15**(2): p. 233-46.
33. Gaunson, J.E., et al., *Lymphocytic infiltration in the chicken trachea in response to Mycoplasma gallisepticum infection.* Microbiology, 2000. **146 (Pt 5)**: p. 1223-9.
34. Papazisi, L., et al., *GapA and CrmA coexpression is essential for Mycoplasma gallisepticum cytadherence and virulence.* Infect Immun, 2002. **70**(12): p. 6839-45.
35. Dykstra, M.J., et al., *Evaluation of cytopathologic changes induced in chicken tracheal epithelium by Mycoplasma gallisepticum in vivo and in vitro.* Am J Vet Res, 1985. **46**(1): p. 116-22.
36. Levisohn, S., *Early stages in the interaction between Mycoplasma gallisepticum and the chick trachea, as related to pathogenicity and immunogenicity.* Isr J Med Sci, 1984. **20**(10): p. 982-4.
37. Kobayashi, T., et al., *NFIL3 is a regulator of IL-12 p40 in macrophages and mucosal immunity.* J Immunol, 2011. **186**(8): p. 4649-55.
38. Duffield, J.S., *The inflammatory macrophage: a story of Jekyll and Hyde.* Clin Sci (Lond), 2003. **104**(1): p. 27-38.
39. Luster, A.D., *The role of chemokines in linking innate and adaptive immunity.* Curr Opin Immunol, 2002. **14**(1): p. 129-35.
40. Gordon, S., *Alternative activation of macrophages.* Nat Rev Immunol, 2003. **3**(1): p. 23-35.
41. Mosser, D.M. and X. Zhang, *Activation of murine macrophages.* Curr Protoc Immunol, 2008. **Chapter 14**: p. Unit 14 2.
42. Mosser, D.M., *The many faces of macrophage activation.* J Leukoc Biol, 2003. **73**(2): p. 209-12.
43. Bashyam, H., *IFNgamma: issuing macrophages a license to kill.* J Exp Med, 2007. **204**(1): p. 3.
44. Borish, L.C. and J.W. Steinke, *2. Cytokines and chemokines.* J Allergy Clin Immunol, 2003. **111**(2 Suppl): p. S460-75.

3.8 Figure Legends

Figure 3.1: Chemotaxis assay results.

Results denoted as mean of total number of cells migrated towards chemotactic gradient ± SD.

Figure 3.2: Kinetic analysis of differentially regulated genes in HD-11 cells during co-culture study

Kinetics of mRNA fold difference in HD-11 cells co-cultured with TECs exposed to R_{low} or R_{high} at 1.5, 6 and 24 hours respectively. Samples normalized to housekeeping gene GAPDH and HD-11 co-cultured with un-exposed TECs as control. n=6 for all experiments. Results are denoted as fold change ± SD with all control values set at 1. Significant differences denoted as * = P< 0.05, **= P <0.01, *** = P < 0.001. **A:** IFN-γ mRNA **B:** IL-1β mRNA **C** IL-6 mRNA **D:** CCL-20 mRNA **E.** MIP-1β mRNA. **F:** CXCL-13 mRNA **G:** IL-8 mRNA **H:** RANTES mRNA

Figure 3.3: Comparison of differential gene expression in HD-11 cells during co-culture study

mRNA fold difference comparison between HD-11 cells co-cultured with TECs exposed to either R_{low} or R_{high} at 1.5, 6 and 24 hours respectively. Samples normalized to housekeeping gene GAPDH and HD-11 co-cultured with un-exposed TECs as control. n=6 for all experiments. Results are denoted as fold change ± SD with all control values set at 1. Significant differences denoted as * = P< 0.05, **= P <0.01, *** = P < 0.001. **A:** IFN-γ mRNA **B:** IL-1β mRNA **C** IL-6 mRNA **D:** CCL-20 mRNA **E.** MIP-1β mRNA. **F:** CXCL-13 mRNA **G:** IL-8 mRNA **H:** RANTES mRNA

Figure 3.4: Kinetic analysis of differentially regulated genes in HD-11 cells during exposure to live R_{low}

Kinetics of mRNA fold difference in HD-11 cells exposed to various MOI of R_{low} at 1.5, 6 and 24 hours respectively. Samples normalized to housekeeping gene GAPDH and unexposed HD-11 as control. n=6 for all experiments. Results are denoted as fold change ± SD with all control values set at 1. Significant differences denoted as * = P< 0.05, **= P <0.01, *** = P < 0.001. **A:** IFN-γ mRNA **B:** IL-1β mRNA **C** IL-6 mRNA **D:** CCL-20 mRNA **E.** MIP-1β mRNA. **F:** CXCL-13 mRNA **G:** IL-8 mRNA **H:** RANTES mRNA

Figure 3.5: Kinetic analysis of differentially regulated genes in HD-11 cells during exposure to live R_{high}

Kinetics of mRNA fold difference in HD-11 cells exposed to various MOI of R_{high} at 1.5, 6 and 24 hours respectively. Samples normalized to housekeeping gene GAPDH and unexposed HD-11 as control. n=6 for all experiments. Results are denoted as fold change ± SD with all control values set at 1. Significant differences denoted as * = P< 0.05, **= P <0.01, *** = P < 0.001. **A:** IFN-γ mRNA **B:** IL-1β mRNA **C** IL-6 mRNA **D:** CCL-20 mRNA **E.** MIP-1β mRNA. **F:** CXCL-13 mRNA **G:** IL-8 mRNA **H:** RANTES mRNA

Figure 3.6: Comparison of differential gene expression in HD-11 cells exposed to R_{low} or R_{high}

mRNA fold difference comparison between HD-11 cells exposed to various MOI of R_{low} and R_{high} at 1.5, 6 and 24 hours respectively. Samples normalized to housekeeping gene GAPDH and unexposed HD-11 as control. n=6 for all experiments. Results are denoted as fold change ± SD with all control values set at 1. Significant differences

denoted as * = P< 0.05, **= P <0.01, *** = P < 0.001. **A:** IFN-γ mRNA **B:** IL-1β mRNA **C** IL-6 mRNA **D:** CCL-20 mRNA **E.** MIP-1β mRNA. **F:** CXCL-13 mRNA **G:** IL-8 mRNA **H:** RANTES mRNA

3.9 Table 1: Gene specific primers for RT-qPCR

Gene Name	Primer sequence 5'-3'
GAPDH Forward	ATTCTACACACGGACACTTCA
GAPDH Reverse	CACCAGTGGACTCCACAACATA
IL-1β Forward	GCTGGAACTGGGCAG AT
IL-1β Reverse	GGTAGAAGATGAAGCGGGTC
IL-8 Forward	GTGCATTAGCACTCATTCTAAGTT
IL-8 Reverse	GGCCATAAGTGCCTTTACG
IL-6 Forward	CCTGTTCGCCTTTCAGACCTA
IL-6 Reverse	AGTCTGGGATGACCACTTC
CCL-20 Forward	GCCAGAAGCTCAAGAGGATG
CCL-20 Reverse	TCCAGAAGTTCAACGGTTCC
IFN-γ Forward	TAGCTGACGGTGGACCTA
IFN-γ Reverse	CTCAGATATGTGTTTGATGTGCG
MIP-1β Forward	CTGCTTCACCTACATCTCCC
MIP-1β Reverse	GTCCTGTACCCAGTCGTT
CXCL-13 Forward	GGACCTCCCGAAGCTGAA
CXCL-13 Reverse	TCTGCCTTTCCACGGATACAT
RANTES Forward	TATTTCTACACCAGCAGCAAATG
RANTES Reverse	GCAGACACCTCAGGTCC

Figure 3.1:

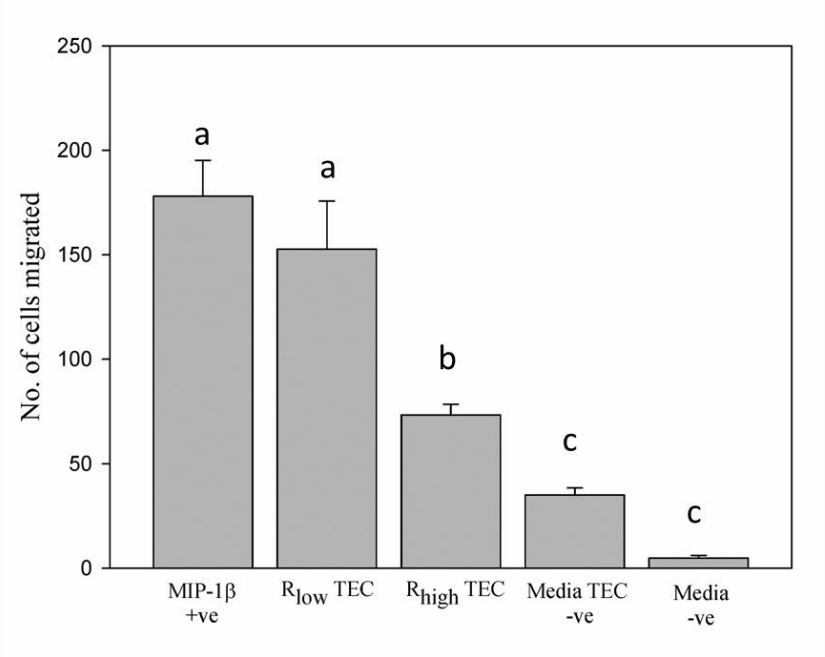
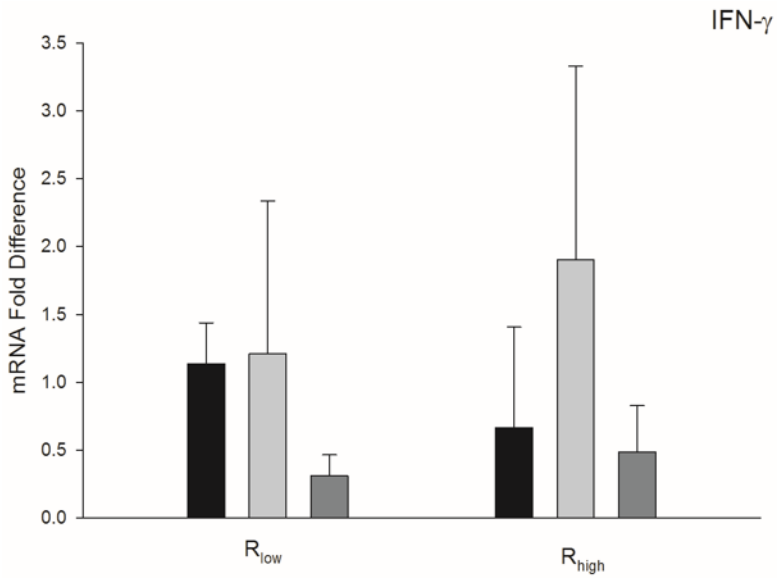
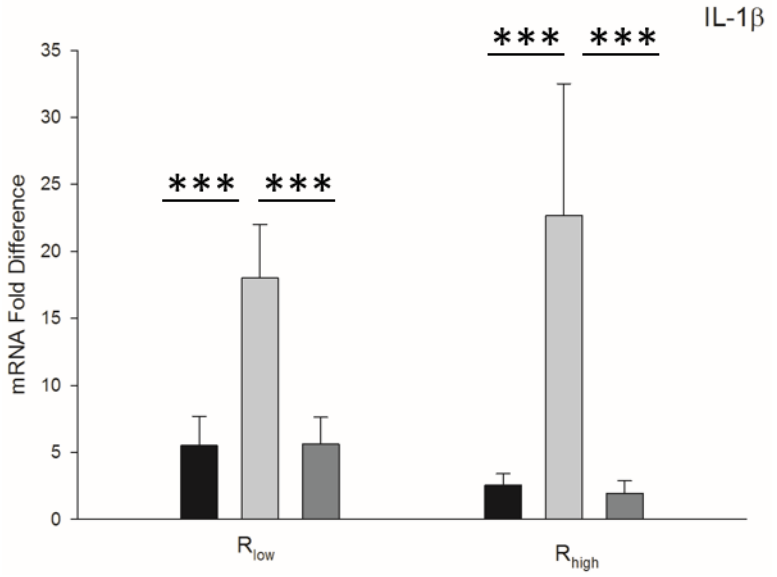

250
200
150
100
50
0
No. of cells migrated
a
a
b
c
c
MIP-1β +ve
R low TEC
R high TEC
Media TEC -ve
Media -ve

Figure 3.2:

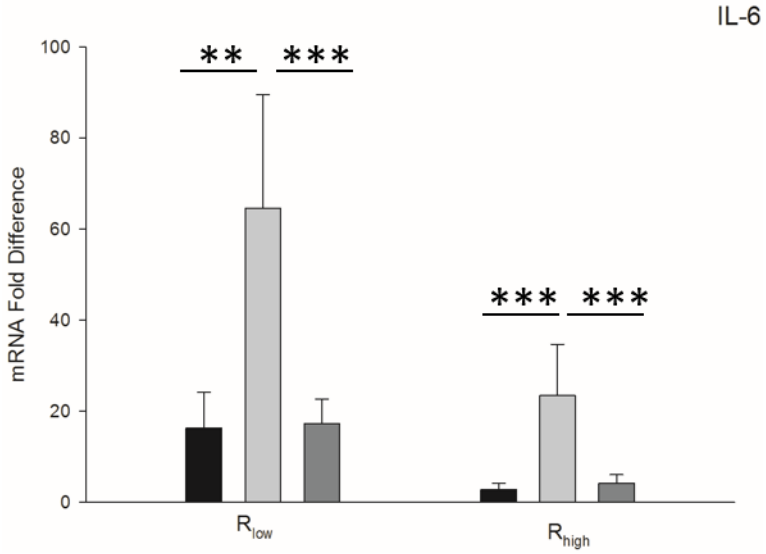
A.


IFN-γ
mRNA Fold Difference
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
R low
R high

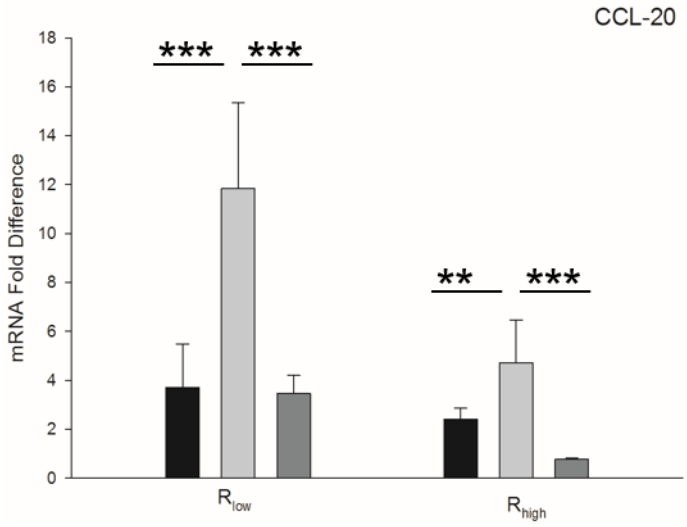
B.


IL-1β
mRNA Fold Difference
35
30
25
20
15
10
5
0
***
***
***
***
R low
R high

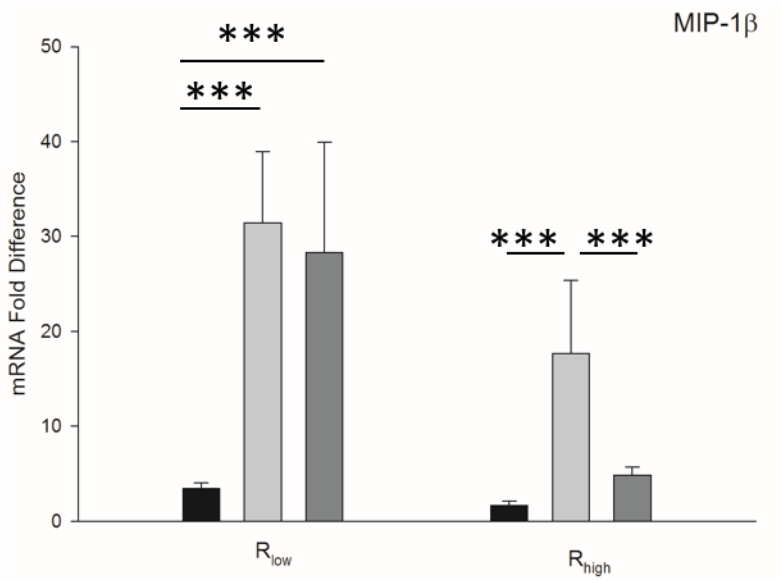
C.


IL-6
mRNA Fold Difference
100
80
60
40
20
0
**
***
***
***
R low
R high

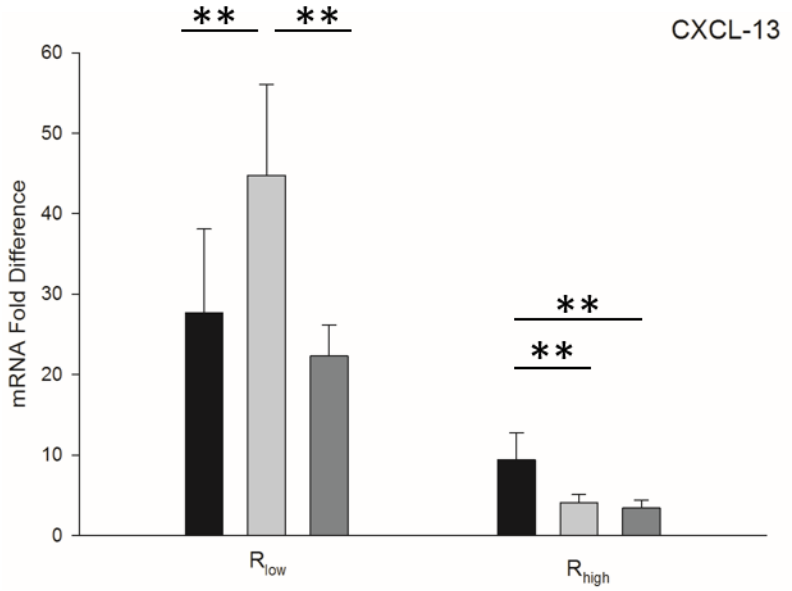
D.


CCL-20
mRNA Fold Difference
18
16
14
12
10
8
6
4
2
0
***
***
**
***
R low
R high

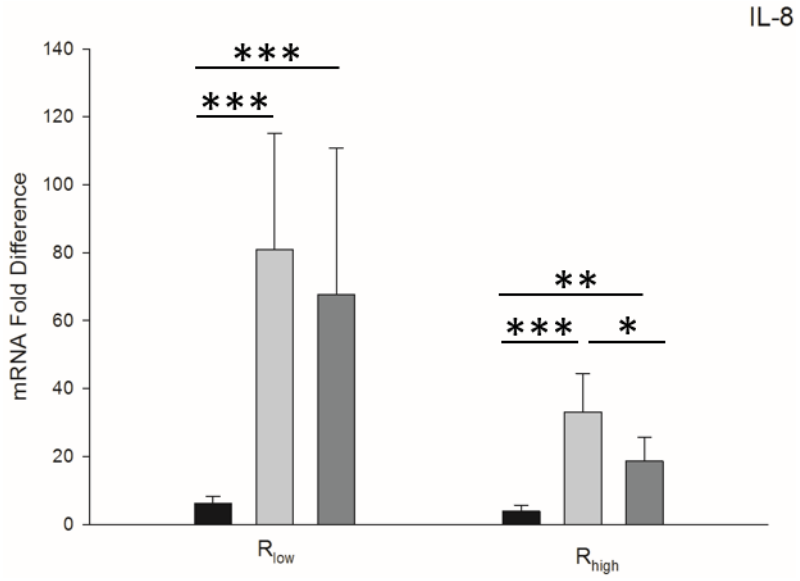
E.


MIP-1β
mRNA Fold Difference
50
40
30
20
10
0
***
***
***
***
R low
R high

F.


CXCL-13
mRNA Fold Difference
60
50
40
30
20
10
0
**
**
**
**
R low
R high

G.


IL-8
mRNA Fold Difference
140
120
100
80
60
40
20
0
***
***
**
***
*
R low
R high

H.

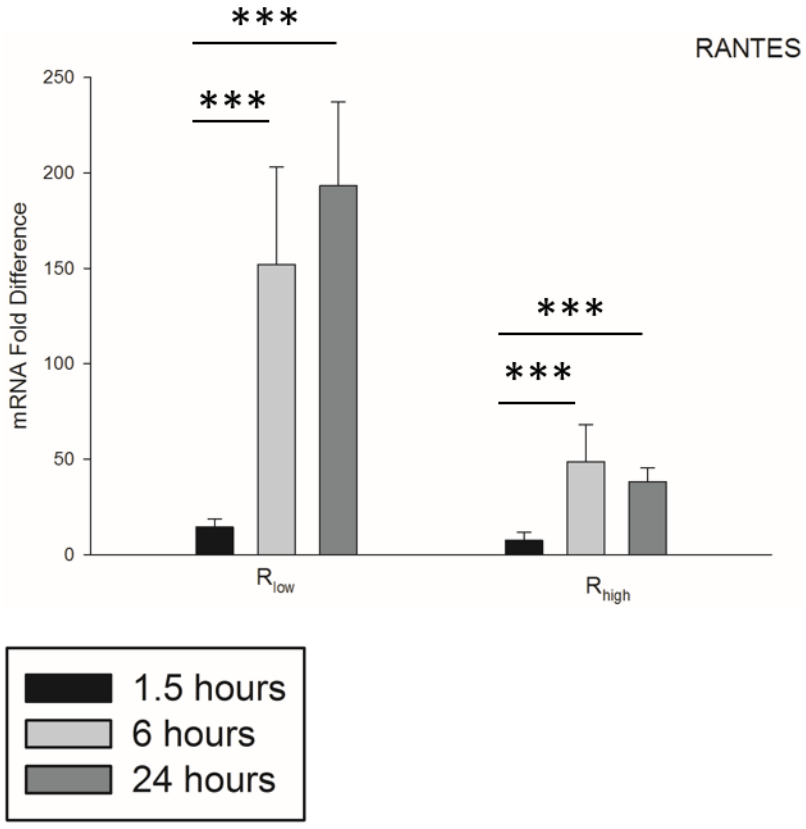
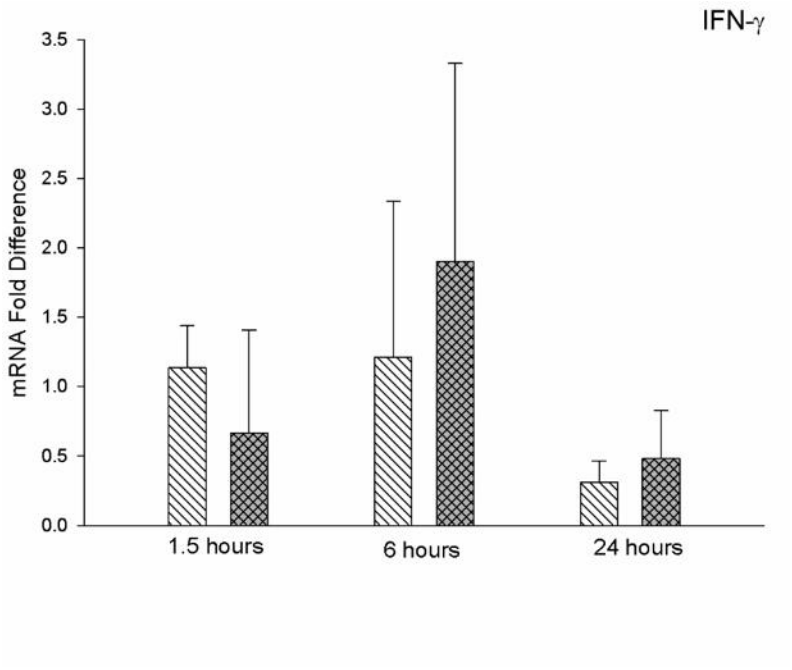
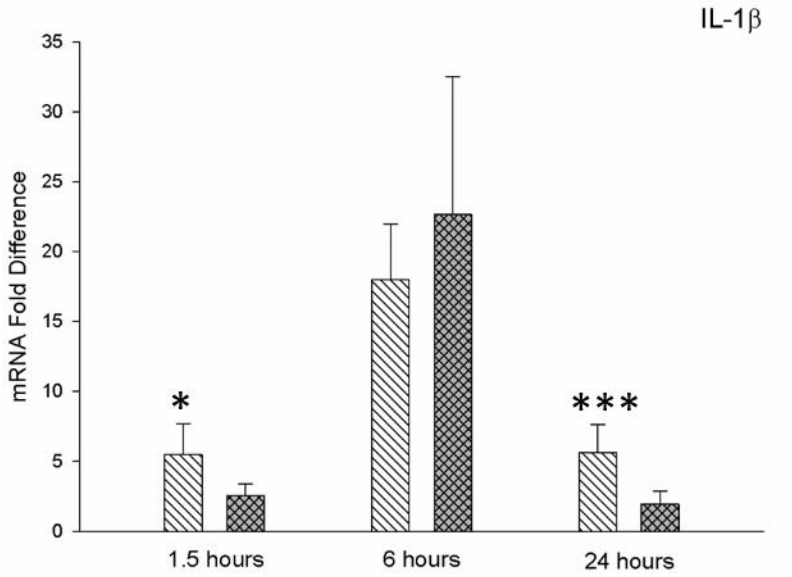

RANTES
mRNA Fold Difference
250
200
150
100
50
0
***
***
***
***
R low
R high
1.5 hours
6 hours
24 hours

Figure 3.3:

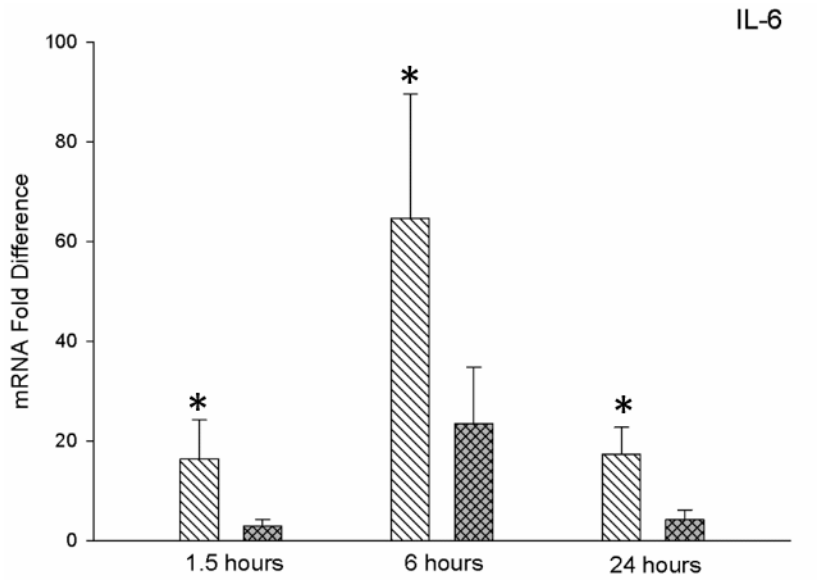
A.


IFN-γ
mRNA Fold Difference
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1.5 hours
6 hours
24 hours

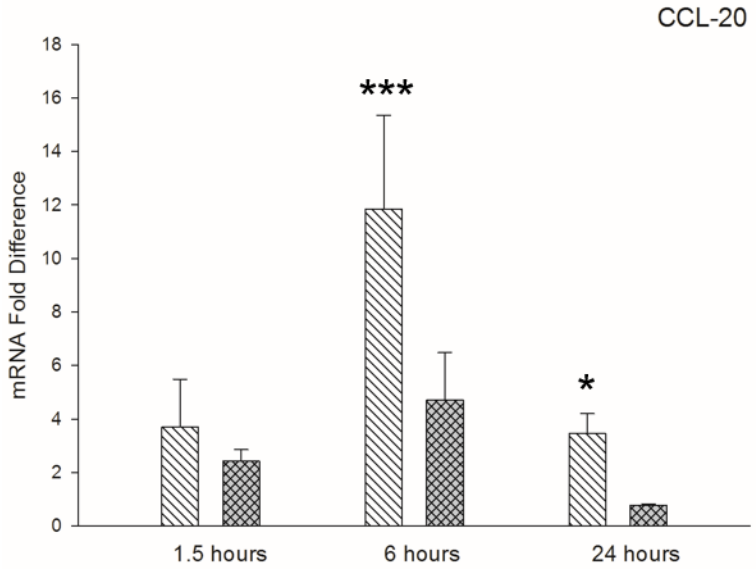
B.


IL-1β
mRNA Fold Difference
35
30
25
20
15
10
5
0
*
***
1.5 hours
6 hours
24 hours

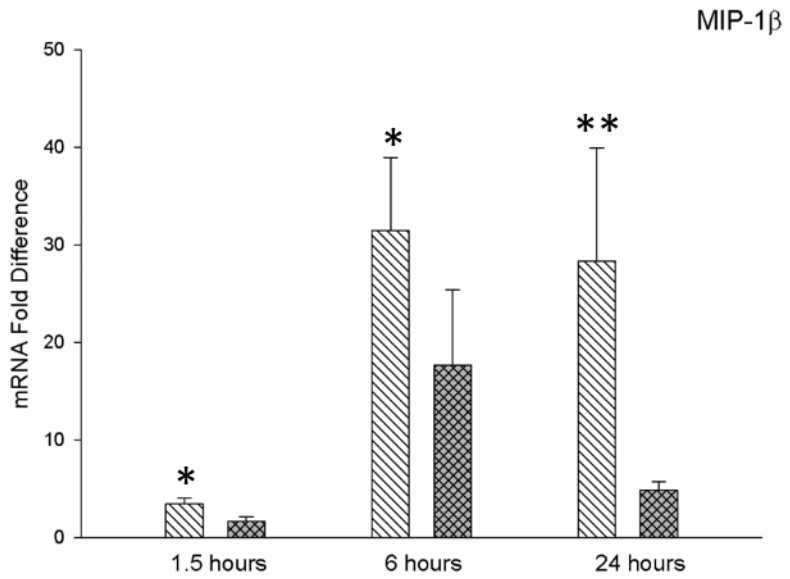
C.


IL-6
mRNA Fold Difference
100
80
60
40
20
0
*
*
*
1.5 hours
6 hours
24 hours

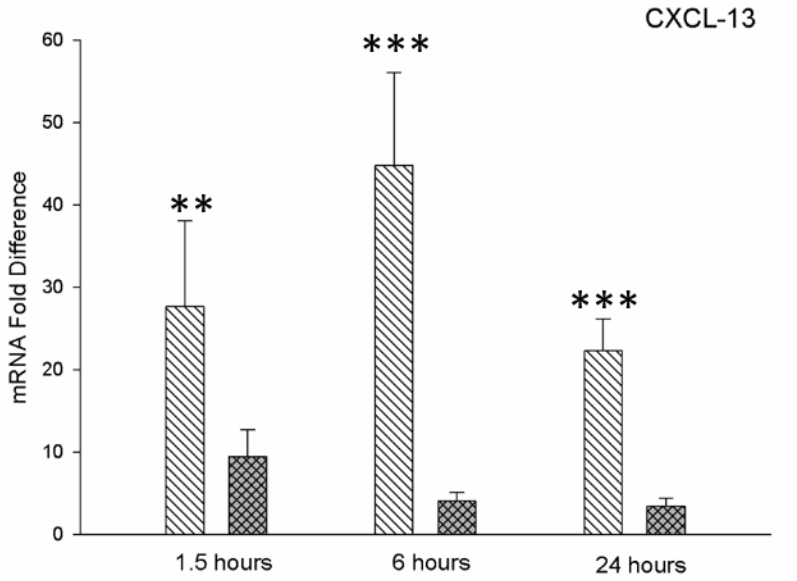
D.


CCL-20
mRNA Fold Difference
18
16
14
12
10
8
6
4
2
0
***
*
1.5 hours
6 hours
24 hours

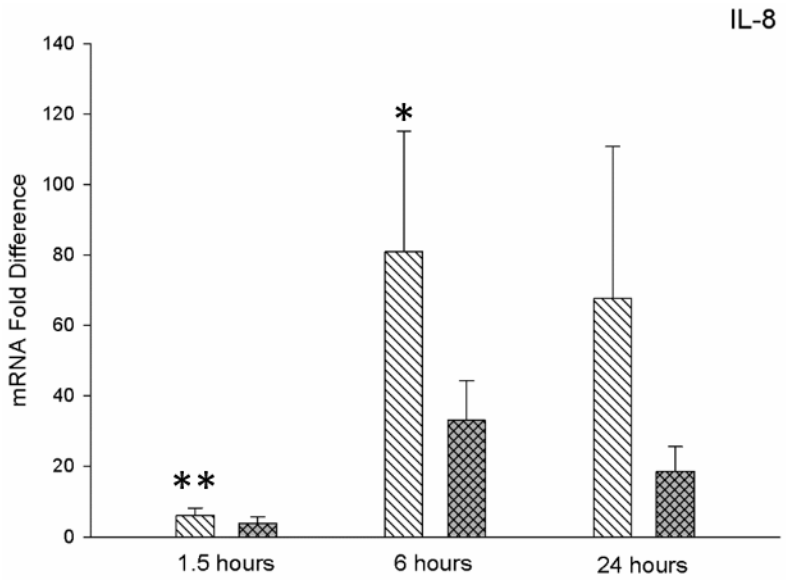
E.


MIP-1β
mRNA Fold Difference
50
40
30
20
10
0
*
*
**
1.5 hours
6 hours
24 hours

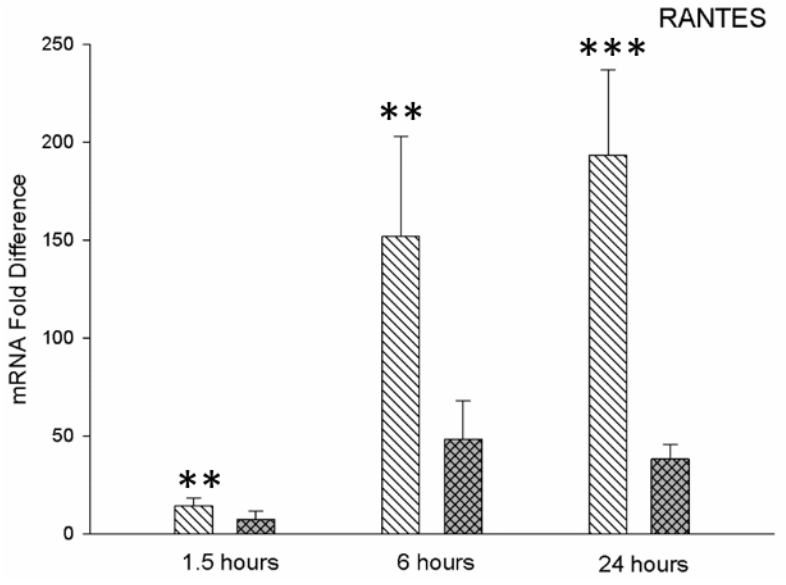
F.


CXCL-13
mRNA Fold Difference
60
50
40
30
20
10
0
**
***
***
1.5 hours
6 hours
24 hours

G.


IL-8
mRNA Fold Difference
140
120
100
80
60
40
20
0
**
*
1.5 hours
6 hours
24 hours

H.


RANTES
mRNA Fold Difference
250
200
150
100
50
0
**
**
***
1.5 hours
6 hours
24 hours

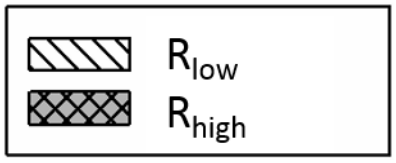
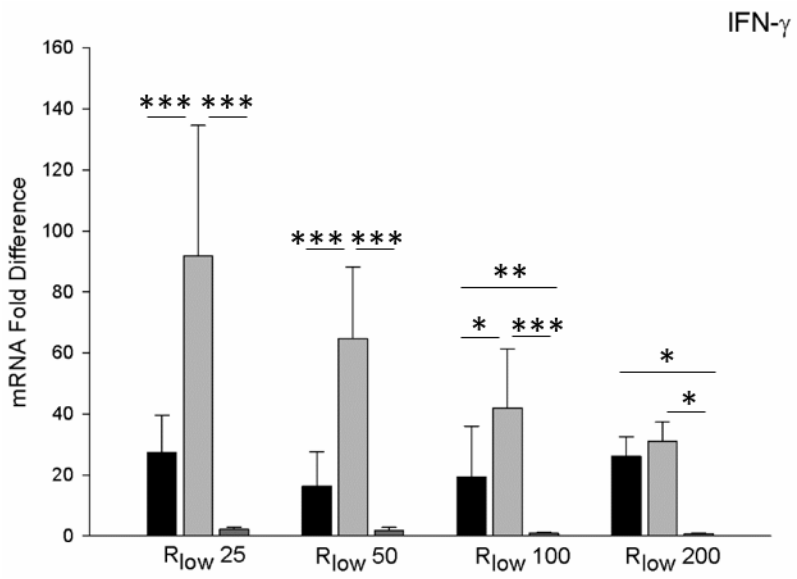
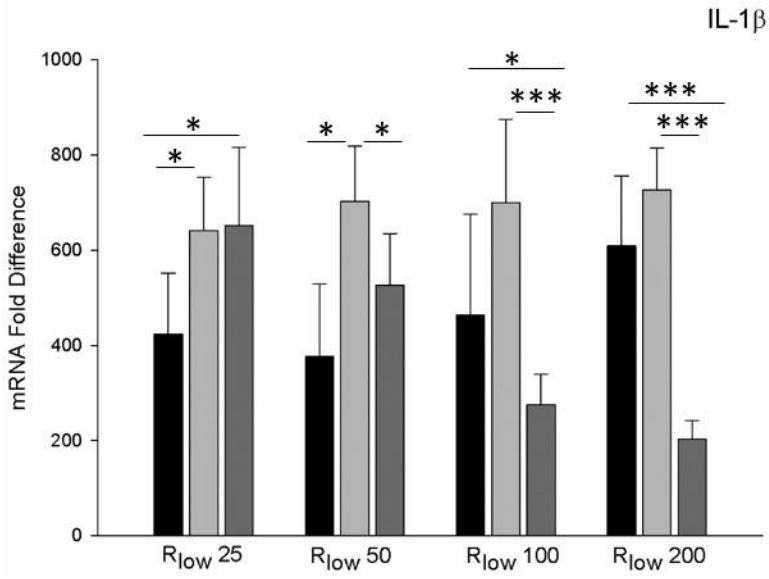

R low
R high

Figure 3.4:

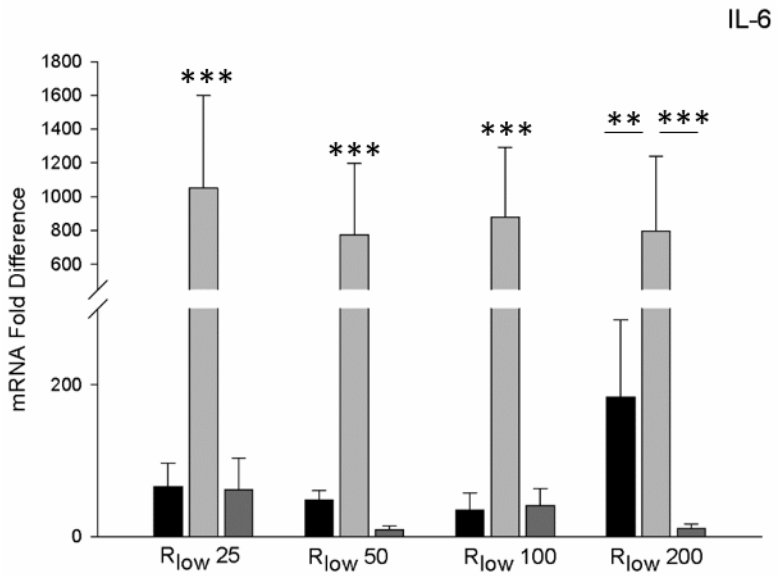
A.


IFN-γ
mRNA Fold Difference
160
140
120
100
80
60
40
20
0
*** ***
*** ***
**
* ***
*
*
R low 25
R low 50
R low 100
R low 200

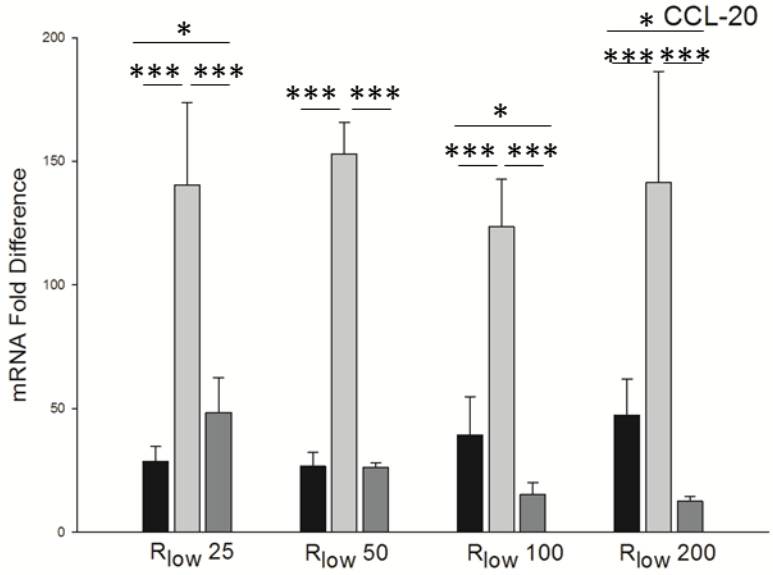
B.


IL-1β
mRNA Fold Difference
1000
800
600
400
200
0
*
*
*
*
*
***
***
***
R low 25
R low 50
R low 100
R low 200

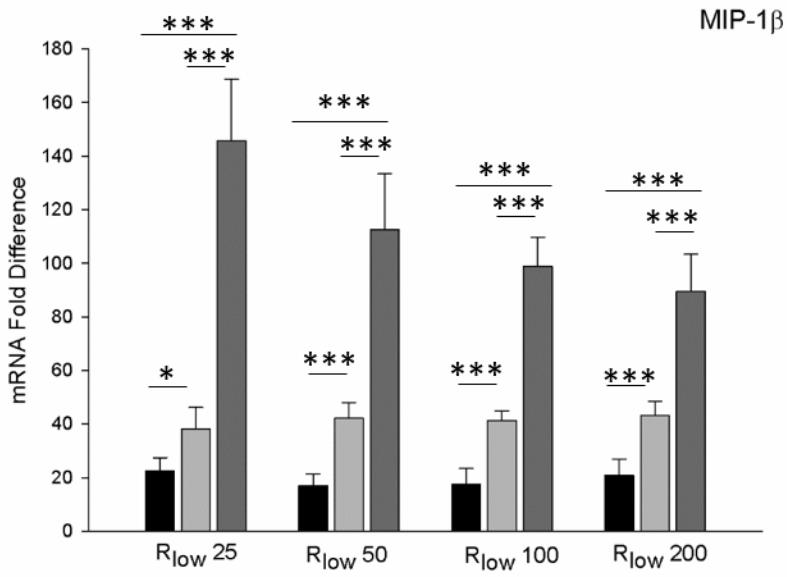
C.


IL-6
mRNA Fold Difference
1800
1600
1400
1200
1000
800
600
200
0
***
***
***
** ***
R low 25
R low 50
R low 100
R low 200

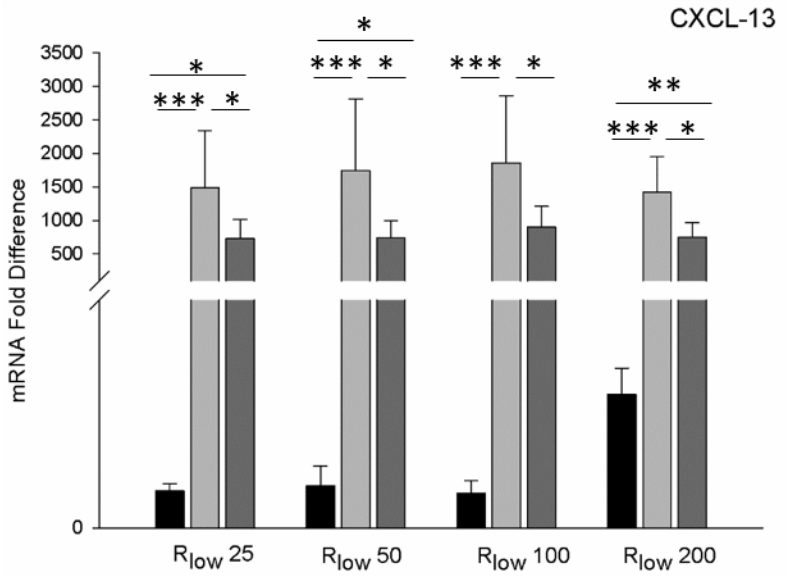
D.


* CCL-20
mRNA Fold Difference
200
150
100
50
0
*
*** ***
*** ***
*
*** ***
*
*** ***
R low 25
R low 50
R low 100
R low 200

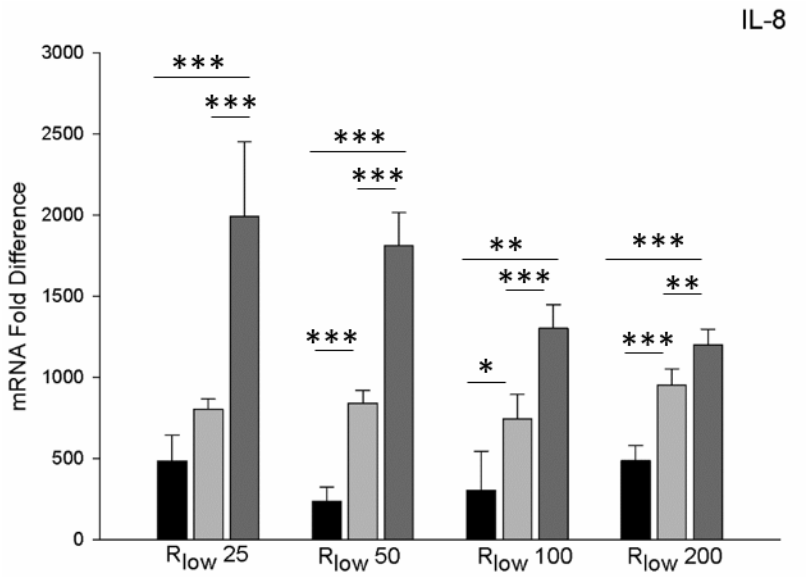
E.


MIP-1β
mRNA Fold Difference
180
160
140
120
100
80
60
40
20
0
R_{low} 25
R_{low} 50
R_{low} 100
R_{low} 200

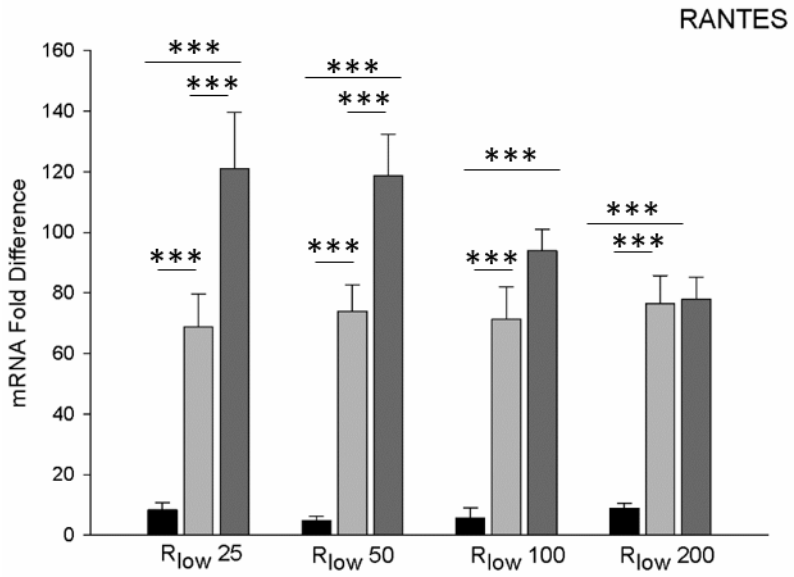
F.


CXCL-13
mRNA Fold Difference
3500
3000
2500
2000
1500
1000
500
0
R_{low} 25
R_{low} 50
R_{low} 100
R_{low} 200

G.


IL-8
mRNA Fold Difference
3000
2500
2000
1500
1000
500
0
***
***
***
***
***
**
***
*
***
**
***
R low 25
R low 50
R low 100
R low 200

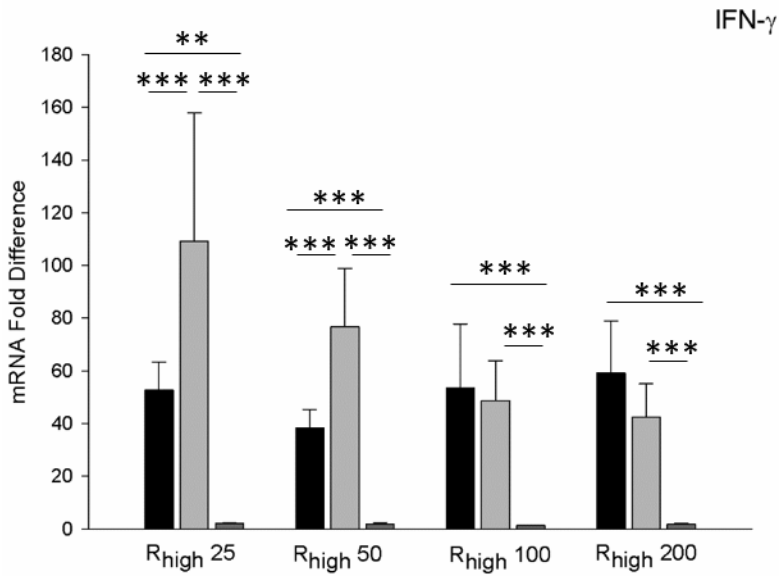
H.


RANTES
mRNA Fold Difference
160
140
120
100
80
60
40
20
0
***
***
***
***
***
***
***
***
***
***
R low 25
R low 50
R low 100
R low 200

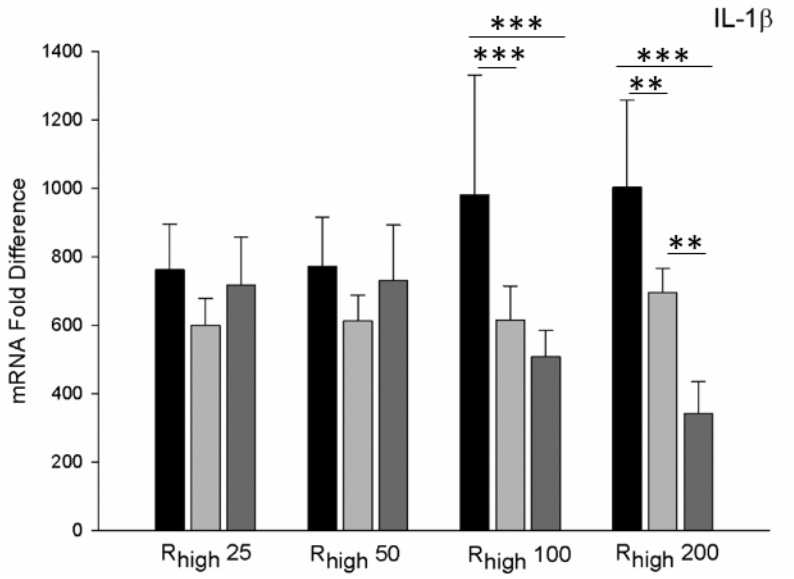

1.5 hours
6 hours
24 hours

Figure 3.5:

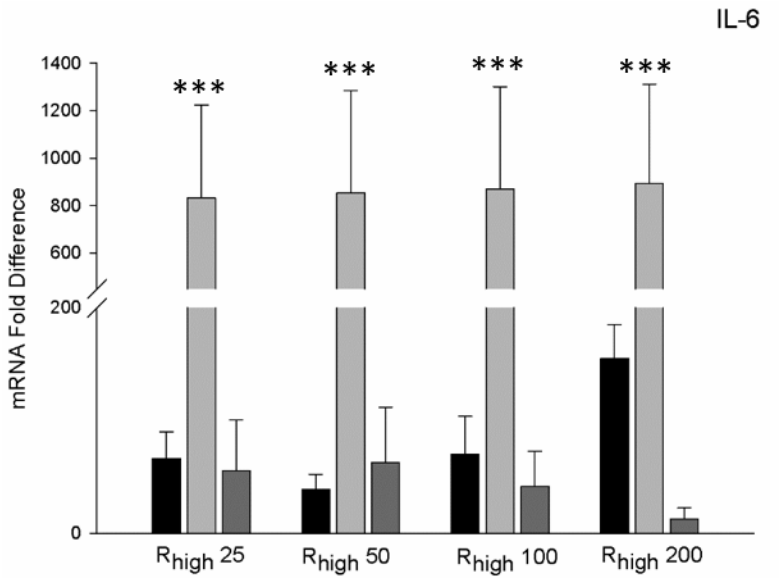
A.


IFN-γ
mRNA Fold Difference
180
160
140
120
100
80
60
40
20
0
**
*** ***
***
*** ***
***
***
***
***
R_{high} 25
R_{high} 50
R_{high} 100
R_{high} 200

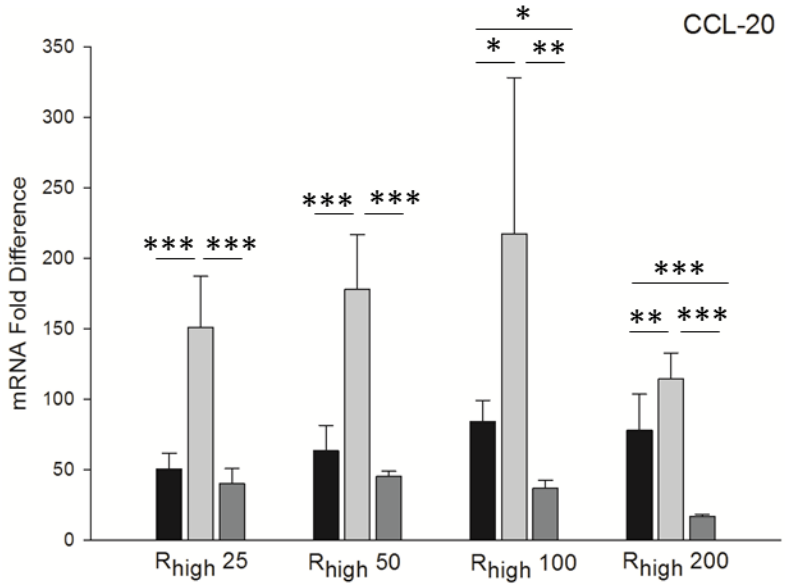
B.


IL-1β
mRNA Fold Difference
1400
1200
1000
800
600
400
200
0
***
***
***
**
**
R_{high} 25
R_{high} 50
R_{high} 100
R_{high} 200

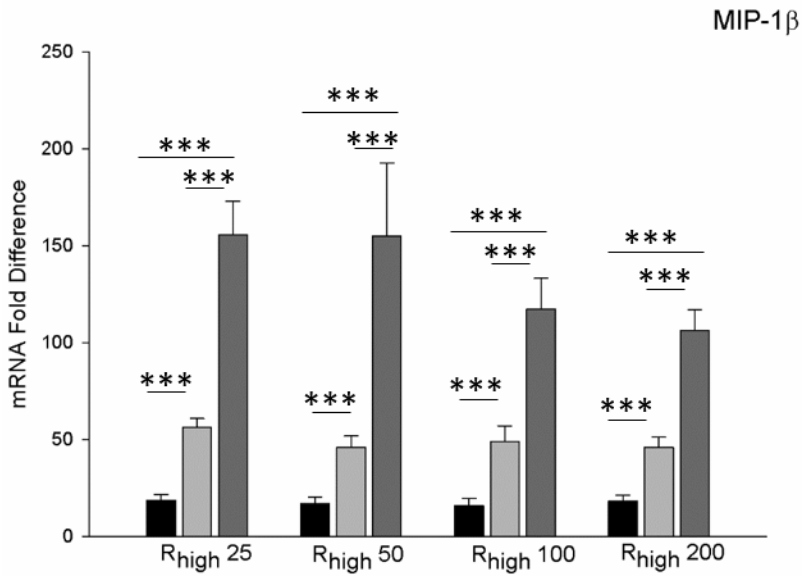
C.


IL-6
mRNA Fold Difference
1400
1200
1000
800
600
200
0
***
***
***
***
R_{high} 25
R_{high} 50
R_{high} 100
R_{high} 200

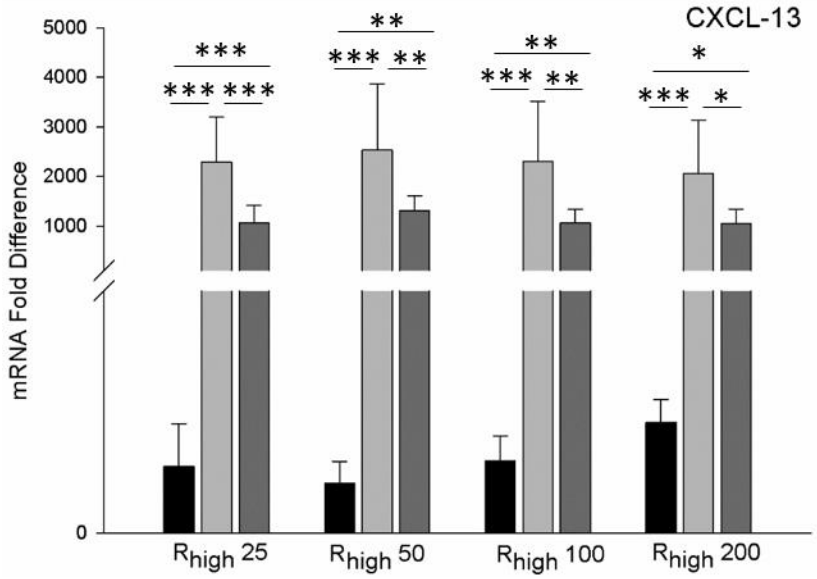
D.


CCL-20
mRNA Fold Difference
350
300
250
200
150
100
50
0
*
*
**
***
***
***
***
**
***
R_{high} 25
R_{high} 50
R_{high} 100
R_{high} 200

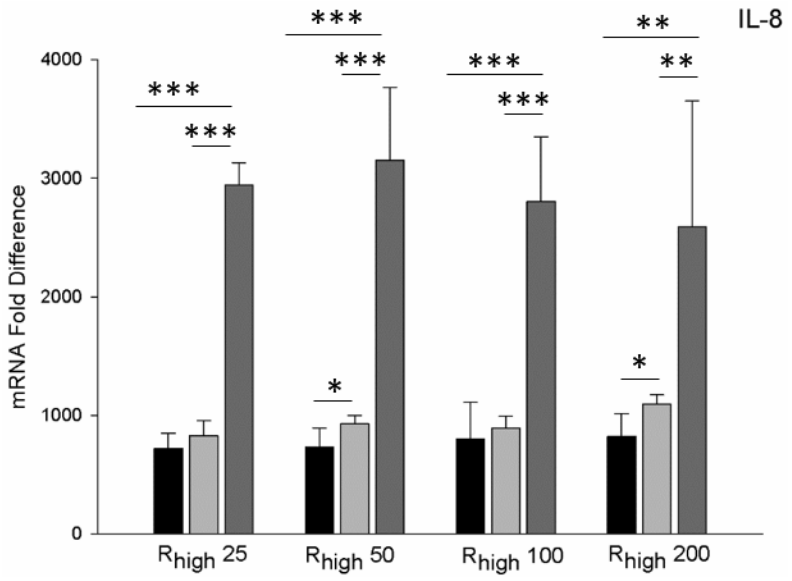
E.


MIP-1β
mRNA Fold Difference
250
200
150
100
50
0
***
R_{high} 25
R_{high} 50
R_{high} 100
R_{high} 200

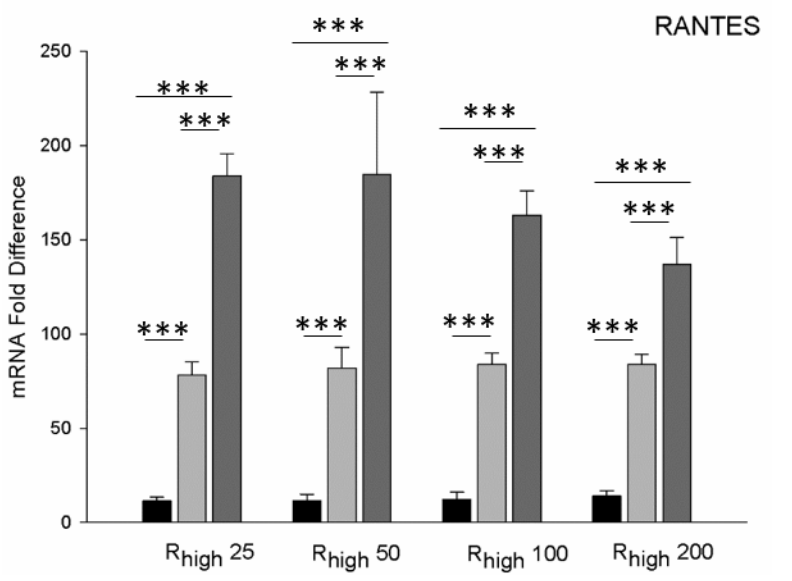
F.


CXCL-13
mRNA Fold Difference
5000
4000
3000
2000
1000
0
***
**
*
R_{high} 25
R_{high} 50
R_{high} 100
R_{high} 200

G.


IL-8
mRNA Fold Difference
4000
3000
2000
1000
0
***
***
***
***
*
***
***
**
**
*
R high 25
R high 50
R high 100
R high 200

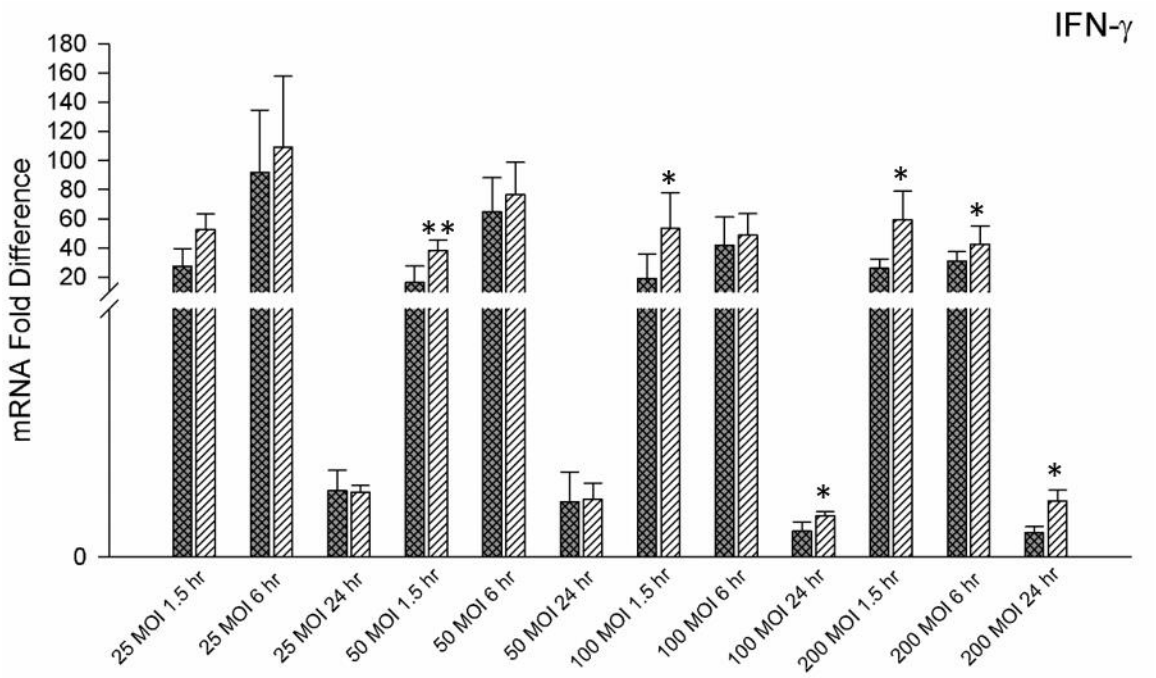
H.


RANTES
mRNA Fold Difference
250
200
150
100
50
0
***
***
***
***
***
***
***
***
***
***
***
***
R high 25
R high 50
R high 100
R high 200

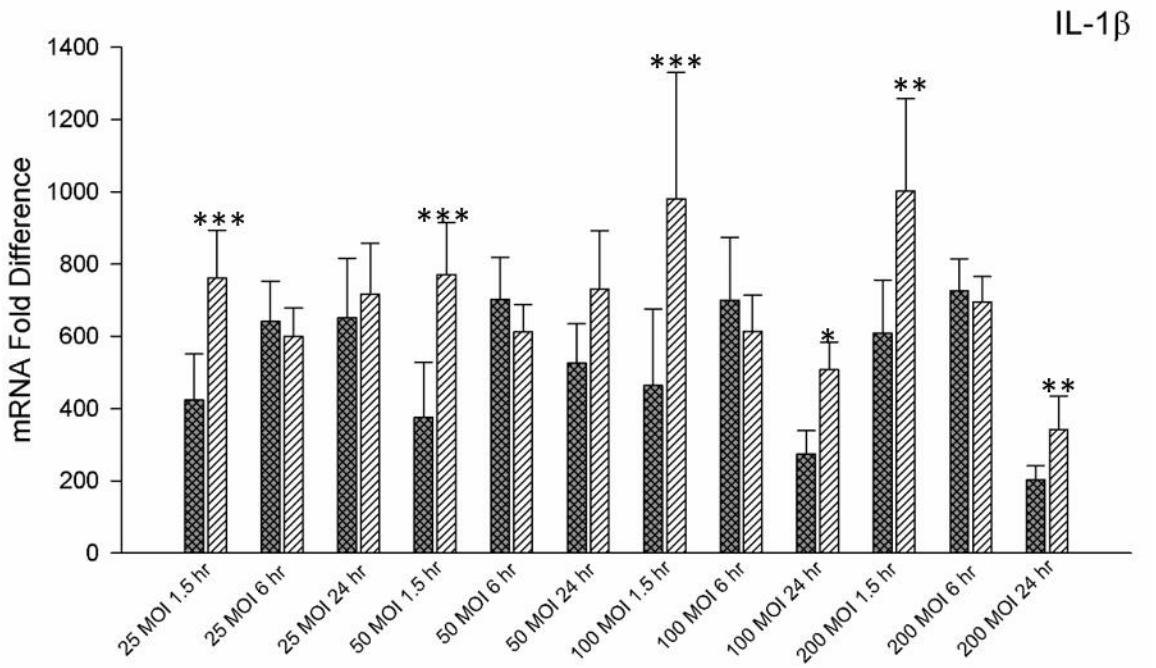

1.5 hours
6 hours
24 hours

Figure 3.6:

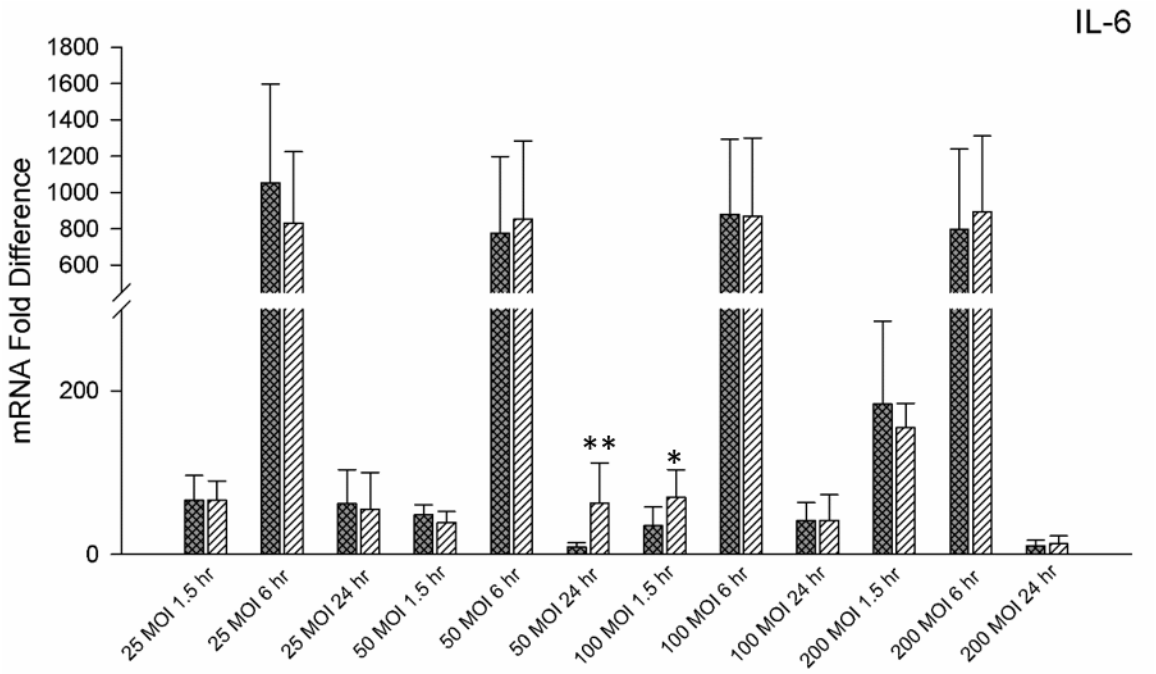
A.


IFN-γ
mRNA Fold Difference
180
160
140
120
100
80
60
40
20
0
25 MOI 1.5 hr
25 MOI 6 hr
25 MOI 24 hr
50 MOI 1.5 hr
50 MOI 6 hr
50 MOI 24 hr
100 MOI 1.5 hr
100 MOI 6 hr
100 MOI 24 hr
200 MOI 1.5 hr
200 MOI 6 hr
200 MOI 24 hr

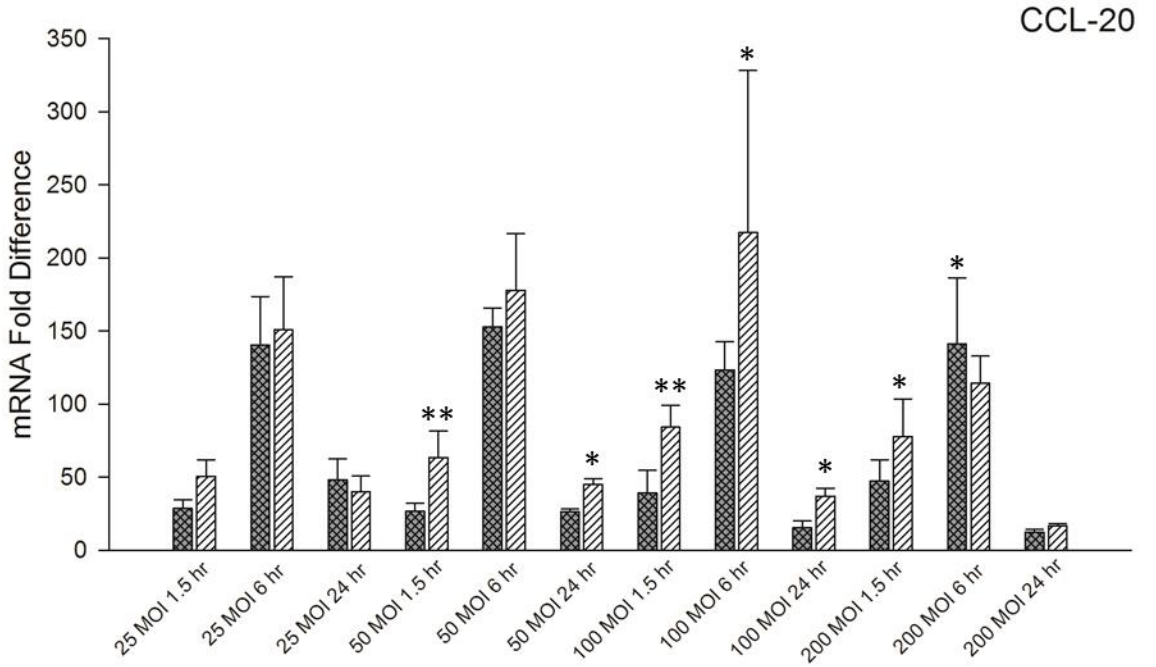
B.


IL-1β
mRNA Fold Difference
1400
1200
1000
800
600
400
200
0
25 MOI 1.5 hr
25 MOI 6 hr
25 MOI 24 hr
50 MOI 1.5 hr
50 MOI 6 hr
50 MOI 24 hr
100 MOI 1.5 hr
100 MOI 6 hr
100 MOI 24 hr
200 MOI 1.5 hr
200 MOI 6 hr
200 MOI 24 hr

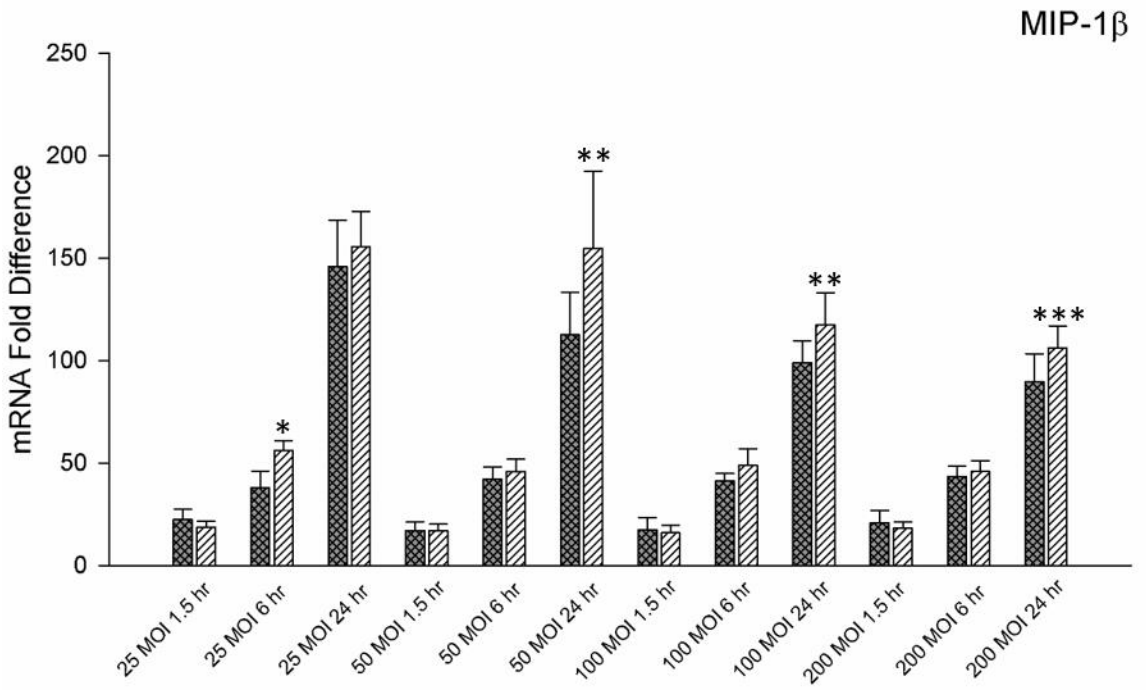
C.


IL-6
mRNA Fold Difference
1800
1600
1400
1200
1000
800
600
200
0
**
*
25 MOI 1.5 hr
25 MOI 6 hr
25 MOI 24 hr
50 MOI 1.5 hr
50 MOI 6 hr
50 MOI 24 hr
100 MOI 1.5 hr
100 MOI 6 hr
100 MOI 24 hr
200 MOI 1.5 hr
200 MOI 6 hr
200 MOI 24 hr

D.


CCL-20
mRNA Fold Difference
350
300
250
200
150
100
50
0
**
*
25 MOI 1.5 hr
25 MOI 6 hr
25 MOI 24 hr
50 MOI 1.5 hr
50 MOI 6 hr
50 MOI 24 hr
100 MOI 1.5 hr
100 MOI 6 hr
100 MOI 24 hr
200 MOI 1.5 hr
200 MOI 6 hr
200 MOI 24 hr

E.


MIP-1β
mRNA Fold Difference
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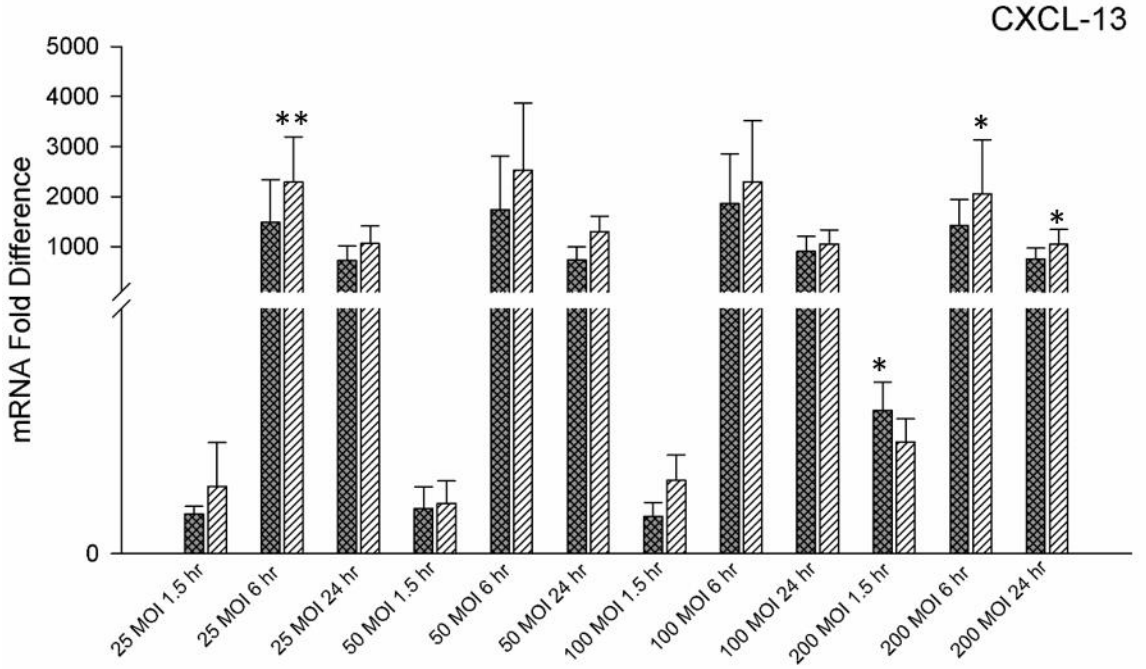
200
150
100
50
0
*
**
**
***
25 MOI 1.5 hr
25 MOI 6 hr
25 MOI 24 hr
50 MOI 1.5 hr
50 MOI 6 hr
50 MOI 24 hr
100 MOI 1.5 hr
100 MOI 6 hr
100 MOI 24 hr
200 MOI 1.5 hr
200 MOI 6 hr
200 MOI 24 hr

F.


CXCL-13
mRNA Fold Difference
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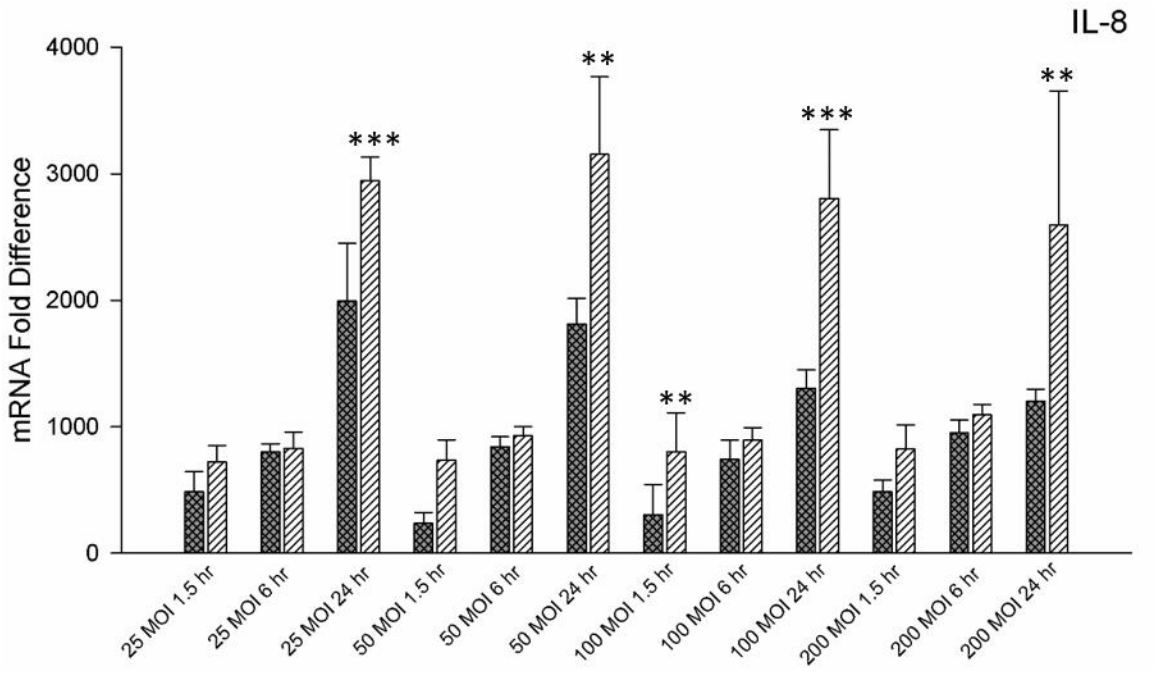
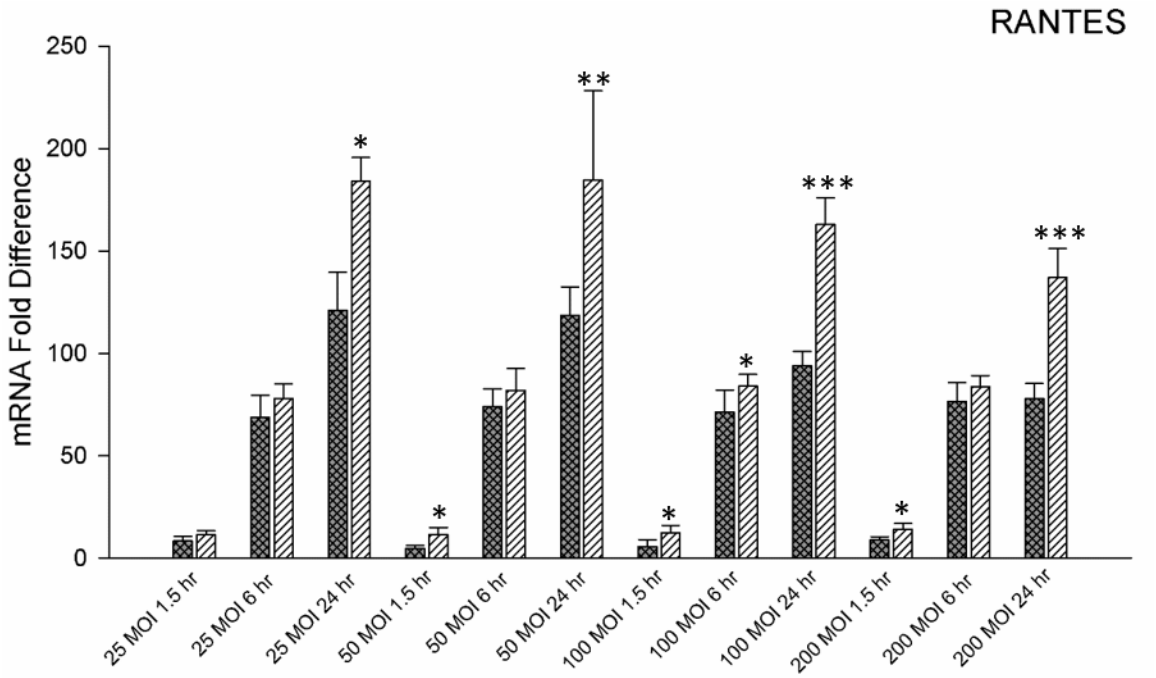
4000
3000
2000
1000
0
**
*
*
*
25 MOI 1.5 hr
25 MOI 6 hr
25 MOI 24 hr
50 MOI 1.5 hr
50 MOI 6 hr
50 MOI 24 hr
100 MOI 1.5 hr
100 MOI 6 hr
100 MOI 24 hr
200 MOI 1.5 hr
200 MOI 6 hr
200 MOI 24 hr

G.


IL-8
mRNA Fold Difference
4000
3000
2000
1000
0
***
**
**
***
**
25 MOI 1.5 hr
25 MOI 6 hr
25 MOI 24 hr
50 MOI 1.5 hr
50 MOI 6 hr
50 MOI 24 hr
100 MOI 1.5 hr
100 MOI 6 hr
100 MOI 24 hr
200 MOI 1.5 hr
200 MOI 6 hr
200 MOI 24 hr

H.


RANTES
mRNA Fold Difference
250
200
150
100
50
0
*
**
*
*
***
***
25 MOI 1.5 hr
25 MOI 6 hr
25 MOI 24 hr
50 MOI 1.5 hr
50 MOI 6 hr
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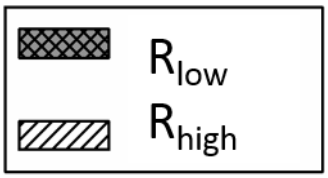
100 MOI 6 hr
100 MOI 24 hr
200 MOI 1.5 hr
200 MOI 6 hr
200 MOI 24 hr


R_{low}
R_{high}

Chapter IV

SUMMARY

4.1 Study I

Mycoplasma gallisepticum-mediated respiratory inflammation in chickens is associated with accumulation of leukocytes in the tracheal submucosa. However the molecular mechanisms underpinning these changes are not yet well understood. The first study was based on the hypothesis that the inflammatory events are initiated upon ligation of mycoplasma lipid associated membrane proteins (LAMP) to TLRs expressed on chicken tracheal epithelial cells (TEC).

Microarray analysis identified that TECs exposed to live R_{low} and R_{high} as well as LAMPs from both strains differentially regulated several genes. A total of 166, 43, 55 and 38 genes were differentially regulated ≥ 5-fold in TECs within 1.5 hour of exposure to either live R_{low} or $R_{high,}$ or to 5μg/mL R_{low} or R_{high} LAMPs respectively. 23 genes were commonly up-regulated ≥ 5-fold in TECs in all four exposure groups, which included IL-1β, IL-6, IL-8, IL-12p40 and CCL-20. Gene ontology and pathway analyses identified genes from several ontological categories and pathways to be up-regulated. Also exposure to viable R_{low} resulted in differential expression of 110 unique genes by a factor of ≥ 5- fold.

Kinetic analysis of 1β, IL-6, IL-8, IL-12p40, CCL-20 and NOS-2 identified their peak expression at 1.5 or 6 hours reducing significantly by 24 hours, suggesting these act as early responders to *M. gallisepticum* infection in context of epithelial cells. Also live R_{low} was significantly more efficient in up-regulating expression of these genes when compared to R_{high} or LAMPs from both strains.

When gene expression in TECs after exposure to LAMPs in presence of various signaling inhibitors was analyzed, expression of all six genes reduced significantly in presence of a TLR-2/4 inhibitor but not TLR-4 inhibitor alone. In presence of a NF-κB inhibitor, gene expression reduced to baseline levels. Preliminary studies on various LAMP fractions and their role in up-regulating IL-1β from TECs identified possible important role of VlhA 3.03 and MslA.

This study also analyzed for host regulatory mechanisms acting against lipoprotein and live mycoplasma mediated inflammatory gene up-regulation. A regulatory micro RNA, miRNA146a was found to be up-regulated over time during exposure to live mycoplasma and negatively correlated with expression of pro-inflammatory genes.

Therefore taken together this study suggest that *M. gallisepticum* lipid associated lipoproteins up-regulate inflammatory genes from tracheal epithelial cells via ligation to TLR-2 in a NF-κB dependent pathway. Live virulent strain R_{low} however, was found to be significantly more efficient in differential gene expression from TECs. This study also identified presence of host compensatory mechanisms that most likely aid in regulating this pro-inflammatory response.

Although this study identified the role of *M. gallisepticum* LAMPs in initiating inflammatory response from TECs *in vitro* and *ex vivo*, more detailed *in vivo* studies are required to elucidate similarities and/or differences in this response within a live host. It would also be important to identify specific lipoproteins involved in this response leading to a better understanding of *M. gallisepticum* virulence factors and host inflammatory responses.

4.2 Study II

Whereas the first study identified role of live *M. gallisepticum* and their lipid associated membrane proteins in differentially regulating inflammatory genes from host respiratory epithelial cells, the second study was sought to identify how this interaction might play a role in the inflammatory response observed during infection.

The results of this study indicated that interaction of tracheal epithelial cells with the virulent R_{low} strain lead to significantly more efficient in macrophage chemotaxis when compared to the non-virulent R_{high} strain. Expression of various chemokine and cytokine genes were analyzed, including IFN-γ, IL-1β, IL-6, IL-8, CCL-20, MIP-1β, CXCL-13 and RANTES. IFN-γ expression did not differ significantly in macrophages (HD-11) co-cultured with *M. gallisepticum* exposed tracheal epithelial cells, but expression of rest of the genes were significantly up-regulated. Expression of all analyzed genes were also observed to be significantly higher in HD-11 cells co-cultured with TECs exposed to R_{low} compared to R_{high} at least during two or more time point.

The study also analyzed the effect of live *M. gallisepticum* on HD-11 cells. Similarly as the co-culture study, chemokine and cytokine genes were significantly up-regulated during both exposures. But R_{low} did not show higher efficacy compared to R_{high}, in fact in certain cases R_{high} was found to be more efficient. It was also observed that various MOI of mycoplasma were almost equally capable in up-regulating these genes.

During kinetic analysis of the chemokine and cytokine genes the average trend showed, that the cytokine genes peaked at 6 hours and significantly reduced by 24 hours,

but the chemokine genes except CCL-20 peaked at 24 hours or had a sustained expression after 6 hours especially in presence of R_{low}.

Therefore this study supports the notion that interaction of *M. gallisepticum* with host respiratory epithelial cells leads to macrophage chemotaxis and up-regulation of inflammatory genes. The observation that virulent strains are more efficient in both these processes may also explain why these strains lead to a severe inflammatory response and the non-virulent strains fail to do so. However since during exposure of macrophages to live mycoplasma, virulent strains fail to show higher efficacy compared to a non-virulent strain, might indicate irrelevance to what is actually is observed *in vivo*, and suggest that mycoplasmal invasion of epithelial cells might not be required for precipitation of the inflammatory response. The observed kinetic trend of chemokine and cytokine genes may also indicate the severe immunopathology associated with *M. gallisepticum* infection in chickens without successful clearance of the pathogen.

But it should be noted that this research had certain limitations. First these studies were done *in vitro* to characterize involvement of each cell type in the inflammatory process during *M. gallisepticum* infection and did not involve any *in vivo* models. Therefore more detailed *in vivo* studies are required to confirm these findings and better understand the cellular and molecular events during *M. gallisepticum* pathogenesis. Other limitations to this study include usage of a macrophage cell line and we should point out that a cell line may not respond the same way as primary cells and also may not be similar to what would be observed *in vivo*.

Finally, all our studies analyzed mRNA expression, but abundance of mRNA in cytosol may not absolutely correlate with protein expression as up to a 40% variation in

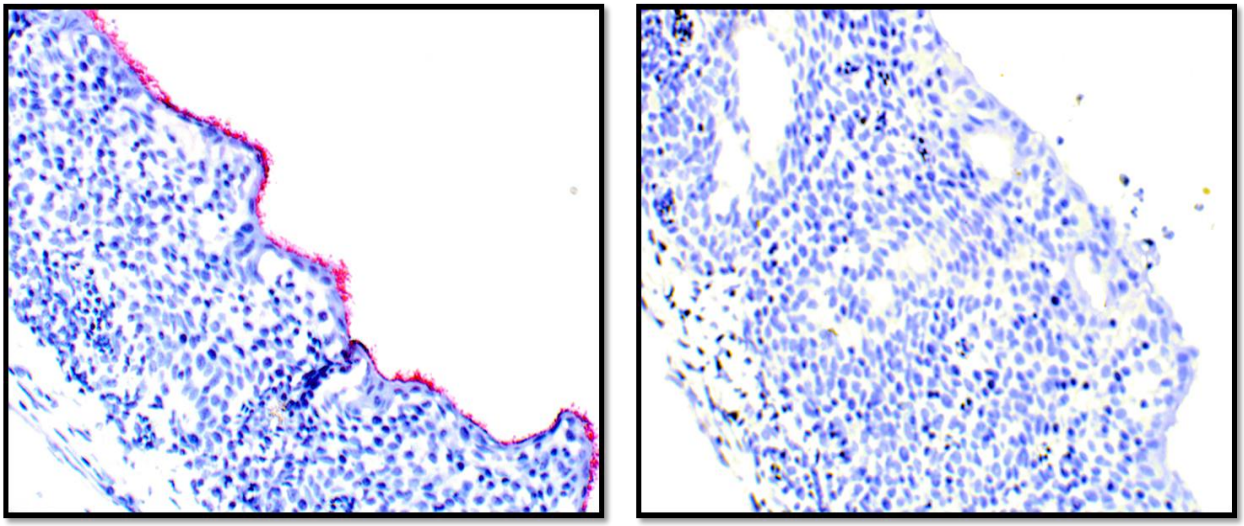
protein concentration has been observed. Besides post transcriptional and post translational modifications play a key role in protein expression and release. This observation has previously been made in mycoplasma literature. A cytadhesion deficient non-virulent *M. pneumonia* strain was found to up-regulate mRNA expression of IL-1β in monocytes at a similar level as a virulent strain but lacked release of mature IL-1β like the virulent strain. This was dependent on caspase-1 mediated post translational processing [1, 2]. Therefore further analyses of protein expression of the chemokine and cytokine genes are required to make a stronger correlation to the inflammatory response observed during *M. gallisepticum* infection.

4.3 References

1. Shimizu, T., Y. Kida, and K. Kuwano, *Cytoadherence-dependent induction of inflammatory responses by Mycoplasma pneumoniae.* Immunology, 2011. **133**(1): p. 51-61.
2. Vogel, C. and E.M. Marcotte, *Insights into the regulation of protein abundance from proteomic and transcriptomic analyses.* Nat Rev Genet, 2012. **13**(4): p. 227-32.

APPENDIX

Figure 1: Epithelial Surface Localization of *M. gallisepticum*



Mycoplasma gallisepticum 16S Riboprobe | Negative Control

In-situ hybridization of *M.gallisepticum* 16S riboprobe in paraffin embedded chicken tracheal ring, 4 day post *M. gallisepticum* exposure at 400X magnification. Dark pink staining of the left figure indicates localization of mycoplasmas. No sub-epithelial mycoplasma could be detected.