ROLE OF TOLL-LIKE RECEPTOR 9 IN MOUSE LUNG INFLAMMATION IN RESPONSE TO CHICKEN BARN AIR

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By

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ABSTRACT

Lung dysfunction due to exposure to air in high intensity livestock barn operations is a common problem for workers in these facilities. Exposure to this air has been linked to disorders such as chronic bronchitis, occupational asthma, organic dust toxic syndrome, and chronic cough and phlegm. These symptoms have been linked to higher levels of endotoxins in air in chicken and swine barns. However, there are many other toxic molecules such as bacterial DNA and gases capable of inducing respiratory inflammation. Bacterial molecules are recognized through highly conserved pattern recognition molecules called Toll-like receptors (TLR). While lipopolysaccharides are recognized by TLR4, bacterial unmethylated DNA binds to and signals through TLR9. As a prelude to understanding the biology of TLR9 in lung inflammation, it is important to precisely clarify their *in situ* expression in the lung.

I determined expression of TLR9 in intact lungs from cattle, pigs, dogs, horses, mice, and humans. Two samples from normal lungs of cattle, pigs, dogs, three from horses, and two from inflamed calf lungs were tested. Five normal mouse and three normal human lungs were similarly tested as well as 5 human lungs with diagnosis of asthma. The expression was determined with multiple methods such as Western blots, immunohistology, immunogold electron microscopy and *in situ* hybridization. Lungs from all the species showed TLR9 expression in the bronchial epithelium, vascular endothelium, alveolar septa, alveolar macrophages, and type-II alveolar epithelial cells. Immuno-electron microscopy detected TLR9 on the plasma membrane, cytoplasm and the nucleus of various cells including macrophages. *In situ* hybridization demonstrated TLR9 mRNA in the bronchial epithelium, vascular endothelium, alveolar septa, alveolar macrophages, and type-II alveolar epithelial cells of mouse and human. Asthmatic human lungs showed many more inflammatory cells expressing TLR9 compared to healthy lungs. In cattle and horses, pulmonary intravascular macrophages showed robust expression of TLR9. Depletion of pulmonary intravascular macrophages in horses resulted in significant reduction in total TLR9 mRNA in the lungs. Having determined that TLR9 expression is similarly expressed on many lung cell types in mice and humans, I determined the role of TLR9 in barn air induced lung inflammation by exposing $TLR9^{-/-}$ and wild-type mice (6 per group) to single or multiple days (5 and 20) in a chicken barn. Each exposure was of 8 hours/day duration. The $TLR9^{-/-}$ mice exposed 5 and 20 times showed significant reductions in TNF-α and IFN-γ expression in lung lavages as well as cellular changes consistent with reduced lung inflammation such as reductions in the number of lung neutrophils. This suggests that barn dust DNA, acting through TLR9, contributes to lung inflammation seen in response to exposure to chicken barn air.

These fundamental data advance our knowledge on the cell-specific expression of TLR9 across a range of species including the humans and demonstrate that $TLR9^{-/-}$ partially regulates lung inflammation induced following exposure to chicken barn air.

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LIST OF ABBREVIATIONS

ALI	Acute lung inflammation
AM	Alveolar macrophages
ANOVA	Analysis of variance
ARDS	Acute respiratory distress syndrome
BAL	Bronchiolar alveolar lavage
BSA	Bovine serum albumin
CCR	Chemokine receptor
CCL	Chemokine ligand
CD	Cluster of differentiation
cDNA	Complimentary DNA
CFU	Colony forming units
CO_2	Carbon dioxide
COPD	Chronic obstructive pulmonary disease
CpG	Unmethylated cytosine-guanocine
CTLA	Cytotoxic T-lymphocyte antigen
CXCR	α-chemokine receptor
DC	Dendritic cell
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
EM	Electronmicroscopy
GADPH	Glyceraldehyde-3-phosphate dehydrogenase
H_2S	Hydrogen sulfide
HMGB1	High-mobility group protein B1
ICAM	Intercellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IκB	Inhibitor of κB
IP-10	Interferon gamma-induced protein 10kDa
IRAK	Interleukin receptor-associated kinase

IRF	Interferon regulatory factor
ISH	In-situ hybridization
KLH	Keyhole limpet hemocyanin
LFA	Lymphocyte function-associated antigen
LPS	Lipopolysaccharide
Mac-1	Macrophage-1 antigen
MCP	Monocyte chemoattractant protein
MHC	Major histocompatibility complex
MIF	Macrophage migration inhibitory
MIP	Macrophage inflammatory protein
MKK	Mitogen-activated protein kinase kinase
mRNA	Messenger ribonucleic acid
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TWA	Time-weighted average
RNA	Ribonucleic acid
NF-κB	Nuclear factor kappa beta
NH_3	Ammonia
NKT	Natural killer T-cell
NIOSH	National Institute for Occupational Safety and Health
ODN	Oligodinucleotide
ODTS	Organic dust toxic syndrome
OSHA	Occupational Safety and Health Administration
OVA	Ovalbumen
PAF	Platelet-activating factor
PAR	Thrombin receptor
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cell
PI3K	Phosphoinositide 3-kinase
PIM	Pulmonary intravascular macrophage

ppb	Parts per billion
ppm	Parts per million
RAGE	Receptor for advanced glycation endproducts
RANTES	Regulated upon activation, normal T-cell expressed and secreted (CCL5)
TAB	TAK associated binding protein
TAK	TGF-β activated kinase 1
TGF	Tumor growth facor
TH	T-helper cell type
TRAF	Tumor necrosis factor receptor associated factor
Treg	Regulatory T cell
Tris	Tris(hydroxymethyl)aminomethane
TSLP	Thymic stromal lymphopoietin
VEGF	Vascular endothelial growth factor
vWF	von Willebrand Factor
WT	Wild type

CHAPTER1: REVIEW OF LITERATURE

1.1. Introduction

The shift in livestock production from single family farms to high-intensity feed and growth operations housed in large barn environments has led to improved efficiency in the industry. However, with this move have come health problems, particularly for farm workers in such operations (Simpson et al., 1998), though there are also concerns for the local environment as well as nearby settlements (Radon et al., 2007). Many of the lung problems faced by barn workers are believed to be caused by organic dust, and in particular on levels of microbial molecules in these facilities (Donham et al., 2000; Larsson et al., 1999; Reynolds et al., 1994; Thelin et al., 1984; Zejda et al., 1994). Although there are many inflammatory molecules in the barn air, endotoxin which binds to Toll-like receptor (TLR)- 4 has been studied the most, and has been shown to contribute to lung inflammation and airway hyperresponsiveness in the workers (Dosman et al., 2006; Zejda et al., 1994). Among other molecules, unmethylated DNA recognized by TLR9 (Hemmi et al., 2000) may also impact lung physiology in barn workers.

1.2 Worker Illness

Workers in high-intensity livestock operations have a higher prevalence of lower and upper respiratory problems compared to other agricultural fields (Simpson et al., 1998). Of these groups chicken barn workers appear to be the most susceptible to respiratory problems such as upper respiratory problems and lower respiratory lung dysfunction and chronic bronchitis (Kirychuk et al., 2006; Radon et al., 2001; Rylander & Carvalheiro, 2006). Some symptoms mentioned include chest tightness, chronic cough, phlegm, wheezing, dyspnea, irritation of the

eyes and other symptoms (Just et al., 2009). Workers may experience diseases of the lung such as organic dust toxic syndrome, hypersensitivity pneumonitis (Farmer's Lung), toxin fever, as well as allergic and non-allergic forms of rhinitis or asthma (Iversen et al., 2000; Just et al., 2009; Kimbell-Dunn et al., 1999). Lung function in general was lower in poultry barn workers (Donham et al., 1990; Morris et al., 1991; Rees et al., 1998; Reynolds et al., 1993; Zuskin et al., 1995). A number of these changes were associated with dust and/or LPS levels in these facilities (Donham et al., 2000; Kirychuk et al., 2006). This is in keeping with work that has shown ODTS to be caused by LPS (Hagmar et al., 1990; Zejda et al., 1994). While much work has established the role of LPS in this and other lung dysfunction conditions, the question is do other receptors also play a role?

1.3. Barn Environment

The barn environment is highly complex containing many elements such as dust, noxious gases and microbial molecules that could potentially affect lung physiology. Surveys of high intensity livestock operations have shown that several gasses such as carbon dioxide (CO_2), hydrogen sulfide (H_2S), and ammonia (NH_3) may be elevated (Koerkamp et al., 1998; Liang et al., 2005).

CO_2 produced from animal respiration and manure is more an indicator of ventilation problems than a specific health problem and can induce mild symptoms at levels above 10000ppm (Liang et al., 2005 ; Carbon dioxide as a fire suppressant: Examining the risks ; *Chemical sampling information*). Current AIKA 8hr TWA exposure levels are 5000ppm (ACGIH, 2010).

H_2S is produced from reduction of sulfur-containing compounds by bacteria under anaerobic conditions and is a particular problem with pig operations (Arogo et al., 2000). Chemically H_2S is heavier than air, soluble in water, and detectable by smell at about 30ppb (*Chemical sampling information: Hydrogen sulfide*). Although H_2S is most often found at low levels in confined animal operations, certain activities such as manure manipulation can produce lethal levels (Zhu et al., 2000). Current 8hr TWA exposure levels are 100ppm with 15ppm short term exposure levels (ACGIH, 2010).

NH_3 has been shown to have deleterious effects on both humans as well as animals (Kirkhorn & Garry, 2000; Portejoie et al., 2002). Early studies linking NH_3 with reduced appetite as well as reduced immune resistance to infections (Anderson et al., 1966; Charles & Payne, 1966) have been disputed and more rigorous studies have not shown any effects (Doig & Willoughby, 1971). Current National Institute for Occupational Safety and Health (NIOSH) recommendations are for 25ppm weighted 8hr average and short term limits of 35ppm (*Safety and health topics: Ammonia*). The high solubility of ammonia in water means that much of it will be dissolved in excretions of the upper respiratory tract, and possibly does not even reach the bronchi or alveoli. Even if absorbed through the system, estimates are that it would take about 500ppm to see a significant increase in blood ammonia levels (Ryer-Powder, 1991). However acute exposures of 100-400ppm are reported to be irritating to eyes and throat with higher levels of 1750-2500ppm induce coughing, bronchospasm and chest pain and shortness of breath, fluid in the lungs, chest pains and airway spasms with 2500-5000ppm (Ryer-Powder, 1991). Lower levels however are possible, with chicken barns being the worst of the high intensity livestock facilities for ammonia.

1.3.1. Dust Particulate Size and Composition

Dust particles can induce cell damage directly through their own size and composition or through combined delivery with other toxic molecules such as LPS. The shape and size of the particles determine their delivery and localization in the lungs (Vincent & Mark, 1981). Therefore, it is important to consider total dust and well as inhalable/respirable dust.

In humans, inhalable particles are those smaller than 100µm (ACGIH, 2010) and are defined as those particles deposited anywhere in the respiratory tract. Particles of less than 10µm are called respirable and can deposit anywhere in the trachea, bronchi, or bronchiolar region while those in the range of 5-0.1mm enter the alveoli (Whyte, 1993, Ruzer & Harley, 2005). The particles are deposited by impaction in the nasopharyngeal region, sedimentation at the bronchial level, and diffusion at the alveolar level (Sanchez et al., 2009). These distinctions of size can be crucial, particularly if there are characteristics of one particle size that differ from another. For example, in cage housed chicken barns a greater percentage of total recovered LPS from dust is present in the respirable fraction compared to floor housed chicken barn facilities (Kirychuk et al., 2010). Another study had previously concluded that some symptoms such as current and chronic phlegm were significantly higher in cage barn workers (Kirychuk et al., 2006), suggesting that these differences in LPS loading onto respirable particles may lead to clinical differences in workers.

Chemical composition of dust particles may also play a role in their capacity to stimulate cells of the lung (Fubini & Areán, 1999). In the barns the primary source of dust will be organic materials. While this can be a complex mixture, some constituents can include dried fecal matter, skin flakes, dried urine, pollens, feed material, housing litter, feathers, mites and spores

(Oppliger et al., 2008; Ellen et al., 2000). These items may act to carry a wide variety of microorganisms such as bacteria, viruses, mould, and the chemical remnants of these organisms (Just et al., 2009; Merchant & Ross, 2002). The dust solids can further act as a transport vector not only for microorganisms but also for noxious gasses (Pedersen et al., 2000). Chicken barn dusts consists of 900 g/kg dry matter, 95 g/kg ash, 150 g/kg nitrogen, 6.5g/kg phosphorus, 30 g/kg potassium, 4 g/kg chlorine, and 3 g/kg sodium (Ellen et al., 2000).

Dust concentration in the air will depend on several other physical factors such as aersolization velocity, settling velocity, and resuspension rate, factors which can be influenced by a number of choices made by the individual producer (Davis & Morishita, 2005; Just et al., 2009; Oppliger et al., 2008; Pedersen et al., 2000). Such choices include facility ventilation, relative humidity, stage of feed and methods of feeding, sources/locations of dust, animal density and general facility management and cleanliness. Variables can also be due to the animals themselves, such as age, weight, and size (Ellen et al., 2000; Oppliger et al., 2008; Whyte, 1993).

Methods to mitigate dust, dust movement, or the microbial population are not always easy, and may counter each other. For example, circulation of air within a barn is vital, however, this will resuspend dust in the barn environment (Pedersen et al., 2000). Relative humidity as well poses several challenges. While at a certain level (75%) humidity can reduce inhalable dust, it does little for respirable dust, and increases to moisture on the litter can increase ammonia production and presumably microbial growth (Ellen et al., 2000). Similarly, steps to reduce airborne dust by spraying with water or oil (Pedersen et al., 2000; Senthilselvan et al., 1997) can run into similar problems of altering microbial growth by adding moisture or providing better conditions for anaerobic organisms, or by encouraging ammonia production (Whyte, 1993).

1.3.2. Microbial population

The microbial ecology in animal barns is influenced by the construction, animal composition, feed composition, heat, humidity, farming practices, and other such possible variables. Chicken barns most commonly have aerobes such as *Micrococcus, Proteus, Pseudomonas*, *Escherichia coli*, and *Staphylococcus* species (Just et al., 2009). Interestingly, despite the importance of LPS in many worker health studies, only about 7-17% of the bacteria in chicken barns were gram negative bacteria capable of producing LPS (Just et al., 2009). Studies have also revealed significant differences between cage and floor-based chicken barns dusts (Kirychuk et al., 2006; Kirychuk et al., 2010), as well as between barn and house dust (Alenius et al., 2009). Further, differences in the collection site of samples has shown the presence of microenvironments that can differ greatly in bacterial composition within the same facility (Just et al., 2009). For example, litter constitutes a lower oxygen environment than airborne dusts, so coliform bacteria can better survive in the litter (Whyte, 1993). The impact of such lower oxygen, anaerobic, and more fastidious species of bacteria may have been underplayed in the past, as modern PCR and epifluorescence techniques have given estimates of bacterial numbers in the range of 10^8 or 10^9 bacteria/m^3 air compared to culture techniques which yielded in the range of 10^5 CFU/m^3 (S. A. Lee et al., 2006; Nehme et al., 2008; Oppliger et al., 2008; Radon et al., 2001). Likewise fungal spores may have also been under-estimated, showing 10^7 spores/m^3 via epifluorescence compared to 10^4 CFU/m^3 (S. A. Lee et al., 2006; Radon et al., 2001). Recently high levels of *Archaeobacteria* species have been recorded within these facilities (Nehme et al., 2009). While the role of barn LPS has been well studied (Donham et al., 2000; Kirychuk et al., 2006; Larsson et al., 1999; Thelin et al., 1984; Zejda et al., 1994), it is important that other bacterial components are also studied (Roy et al., 2003; Rylander & Carvalheiro, 2006). This is becoming especially important because of prevalence of spores of

moulds and fungi such as *Cladosporium, Aspergillus, Penicillium, Alternaria, Fusarium, Geotrichum*, and S*treptomyces* (Just et al., 2009; S. A. Lee et al., 2006; Sauter et al., 1981).

1.3.3. Lipopolysaccharides

Given the number of papers that have linked LPS to lung dysfunction, special mention should be given to this bacterial product. LPS is produced by gram negative bacteria that is ubiquitous in the environment (Rylander, 2002). The molecule is composed of a hydrophilic polysaccharide and a biologically active lipid A segment. LPS is a strongly pro-inflammatory molecule, and a causative agent of septic shock (Suffredini et al., 1989). In the discussion of barn air quality LPS is important because often lung function decline can be linked to the levels of this molecule (Dosman et al., 2006; Kirychuk et al., 2006; Senthilselvan et al., 2009), or to its receptor, TLR4 (Senthilselvan et al., 2009). DNA isolated from settled barn dust was found to stimulate monocytes in culture (Roy et al., 2003), but no information exists on exposure to DNA in the barn, or the role of its receptor, TLR9 in lung dysfunction.

1.4. Facility Construction

The type and construction of a particular facility bears a lot on the types of gasses generated. First, chicken production doesn't have to deal with production of H_2S that pig production has. Other changes however are more subtle, but nonetheless of importance. For example, in one study there was a significant gradient of NH_3 and CO_2 that followed the direction of manure belts in a cage barn (Chai et al., 2010). Likewise, there has been much study on cage compared to floor chicken barns which has shown significant differences in the levels of dust and LPS, as well as LPS loading onto different particle sizes (Kirychuk et al., 2006; Kirychuk et al., 2010; Senthilselvan et al., 2011) depending on the type of chicken barn. This is

accompanied, as might be expected, by differences in worker illness in different barns (Kirychuk et al., 2006). Animal age will also affect conditions as well, such as increases in ammonia, dust, and LPS in barns with older chickens, possibly the result of older litter and more active birds (Jones et al., 1984; Senthilselvan et al., 2011).

1.5. Toll-like Receptors

TLRs are cell receptors of the innate immune system that can detect molecules that are associated with microbial pathogens (Kumar et al., 2009). First found in Drosophila, these receptors were soon found to have a role in resistance to fungal infection (Lemaitre et al., 1996). Since then, analogous receptors have been found in many species with similar roles, initiating cell signaling cascades which in turn initiate innate immunity. In mammals TLR signaling can induce inflammation, or augment ongoing immune responses.

TLR4, one of the first recognized TLRs in mammals binds to LPS and can induce strong pro-inflammatory responses. Work by many groups has identified TLR4 and environmental LPS as critical factors in lung dysfunction seen in barn workers (Charavaryamath et al., 2008; Dosman et al., 2006; Kirychuk et al., 2006; Zejda et al., 1994). As more TLR receptors have been discovered however, questions have been raised about the contribution of these other receptors to overall immune responses to barn air.

1.6. Toll-Like Receptor 9 (TLR9)

TLR9 is a receptor that binds microbial DNA and induces cytokines, many similar to those seen with LPS ligation of TLR4. TLR9, along with TLR3, TLR7, and TLR8, is restricted to the endosome. Early studies established that blocking of endosomal acidification with chloroquine or bafilomycin A could block CpG stimulation (Hacker et al., 1998; Yi et al., 1998),

and immobilization on a surface also prevented CpG activity by not allowing internalization of DNA (Krieg et al., 1995; Manzel & Macfarlane, 1999). When TLR9 was later discovered, it wasn't surprising that confocal studies showed clear endosomal localization of TLR9 activation (Ahmad-Nejad et al., 2002). Work in macrophages and dendritic cells (Latz et al., 2004) clearly showed that TLR9 was synthesized and located in the endoplasmic reticulum, a result soon confirmed by others (Leifer et al., 2004). TLR9 expression was not seen at the cell surface, the Golgi apparatus, and early endosomes of unstimulated cells. Labeled CpG DNA was taken up into large motile vesicular structures known as tubular lysosomes that extended towards the nucleus and stained for an early endosomal marker. Clathrin-dependent endocytosis moves DNA into TLR9-containing endosomes/lysosomes. TLR9 recruitment into endosomes was through fusion of endoplasmic reticulum with the endosomal plasma membrane (Latz et al., 2004).

While endosomal localization of TLR9 is well established, the receptor may also be expressed at other cellular sites. For example, gut and tonsil epithelia show clear signs of surface expression (Ewaschuk et al., 2007; Rumio et al., 2004), and in the gut, apical versus basolateral expression appears to even lead to very different results in cell signaling by yet to be understood means of differentiation (Lee et al., 2006). There are also signs of surface expression in human monocytes (Saikh et al., 2004), and possibly on bronchial epithelium and alveolar macrophages in some species (Schneberger et al., 2009; Schneberger et al., 2011). Indeed it appears from cell culture studies that the only restriction to signaling through TLR9 at these sites is the appropriate acidic pH (Hu et al., 2003).

1.6.1. CpG TLR9 Binding

The initial binding of the receptor, though not completely dependent on acidification (pH 5.5 to 6.5 tested), is very weak, and DNA readily dissociates from the receptor (Rutz et al.,

2004). Similarly, binding of DNA was found to be more non-specific at physiological pH. Sequence specificity for the well-established unmethylated CpG motif was returned with acidification to a pH of 6.4 (Hu et al., 2003). Interestingly, in another study, DNA plasmids or oligonucleotides that were non-stimulatory were shown to enhance CpG plasmid DNA binding in a way that didn't result in either remaining bound to the receptor-CpG-DNA complex. There were indications that this DNA may help in the formation of TLR9 dimers. So, even non-stimulatory DNA may enhance the effects of stimulatory DNA on TLR9. Therefore, it is proposed that while there may be some degree of binding with non-CpG DNA, signaling is very sequence specific (Kindrachuk et al., 2007). There is also a threshold TLR9 concentration required for signaling, as it has been shown that cell cultures with varying TLR9 concentrations may be either sensitive or insensitive to CpG oligonucleotides based on receptor concentration (Assaf et al., 2009).

1.7. CpG Structure and Form

The other critical aspect of TLR9 binding is its ligand. TLR9 binds un-methylated DNA with a CpG dimer preceded optimally by two purines and followed by two pyrimidines (Krieg et al., 1995). It must be noted that a cytosine preceding the CpG, or a guanidine immediately following it has been shown to be cis-inhibitory (Stunz et al., 2002, Lenert et al., 2001). The stability of free DNA is very short in circulation. It has been estimated that DNA has a half-life of approximately 10 minutes (Kawabata et al, 1995), yet many studies have verified the necessity of TLR9 in the control of many infections such as *Brucella spp, Leishmania major, Lysteria monocytogonese* and *Streptococcus pneumonia* (Copin et al., 2007; Krieg et al., 1998; K. S. Lee et al., 2007; Lipford et al., 1997). Some of these infections however localize to the alveolar space

such as *Mycobacterium tuberculosis* and *Klebsiella pneumoniae* (Bafica et al., 2005; Bhan et al., 2007), where the stability of such DNA remains unknown.

The use of synthetic ODNs has revealed that changes to the chemical composition of the DNA backbone can significantly alter not only the half-life of the DNA and cellular uptake, but also the type of response generated (Zhao et al., 1993). For example, CpG-B/K, where the entire backbone is thiolated is quite stable and produces responses such as the production of IL-6, IL-12, TNF-α, and B and DC cell maturation. In contrast CpG-A/D, in which only the poly G ends are thiolated induces IFN-α/β production in large quantities in plasmacytoid DC (Hemmi et al., 2003; Klinman, 2004) though monocyte derived DCs have also been stimulated by them (Hoene et al., 2006). Several other modified forms of ODN have been discovered such as CpG-C, which is a hybrid CpG-α/CpG-β ODN (Hartmann et al., 2003; Marshall et al., 2003) and can stimulate all CpG responsive cell types. More recently CpG-P has been described which is phosphothiolated and can form multi-ODN cocatameric structures that exhibit CpG-C like stimulation, but with increased cytokine production, particularly of type-1 interferons (Samulowitz et al., 2010). Further, there is some indication that backbone thiolation may itself be somewhat TLR9-stimulatory (Roberts et al., 2005). Therefore, studies using synthetic ODNs must be taken with some degree of caution as none of the synthetic ODNs are particularly good mimics for natural DNA.

The specifics of the binding of DNA ODN to TLR9 are still not fully determined. For example, data show altered response and binding preferences to different sequences flanking the CpG sequence (Pisetsky & Reich, 1998; Rankin et al., 2001; Yu et al., 2000). In this last case however the species differences may only apply to phosphorothioate-modified ODNs, and not natural phosphodiester ODNs (Roberts et al., 2005). Further, TLR9 signaling is enhanced to

DNA from pathogenic bacteria compared to non-pathogenic species (Ewaschuk et al., 2007; Hoppstadter et al., 2010), though to date no particular sequence or threshold of CpG sequence has been credited with making this distinction. Certainly different bacterial species have differing abilities to stimulate TLR9 (Neujahr et al., 1999).

While much of the work with oligonucleotides has used single stranded DNA, double stranded plasmids have also been effective (Cornelie et al., 2004), but not double stranded oligonucleotides (Rutz et al., 2004). There have been conflicting reports on the ability of these types of DNA to induce TLR9 signaling, though it has been suggested that supercoiling of double stranded DNA may be the critical factor in resolving a number of these conflicting results (Kindrachuk et al., 2007). This suggests that tertiary DNA structure also influences TLR9 signaling.

Indeed, work on required levels of CpG for TLR9 signaling can be difficult to understand. While several of the lower-dose cell cultures and mouse challenge systems have used a stimulatory ODN dose of around 10μg or 10μg/ml CpG-oligonucleotide treatment (Fernandez et al., 2004; J. Li et al., 2004; Roy et al., 2003; Schwartz et al., 1997), studies which elucidated the immunogenicity of the sequence in regards to DNA vaccines were conducted with plasmid DNA, containing changes of only one or two CpG sites in a plasmid (Y. Sato et al., 1996). Such a change in CpG concentration is quite low to total DNA administered, so the sensitivity of the receptor to CpG encountered in different forms can be hard to predict. A more recent finding has been that mitochondrial DNA can activate TLR9 (Q. Zhang et al., 2010). This is to be expected given the different properties of mitochondrial DNA, particularly lack of methylase (Cardon et al., 1994), and does point to TLR9 possibly playing a role in the detection of cellular trauma and necrosis.

Interestingly, there is only one report on differences in immune stimulation by purified environmental DNA samples. Roy et al. (Roy et al., 2003) showed differences in immune response from human PBMCs to DNA from barn dust as compared to house dust. This is quite interesting as it suggests that this DNA, a mixture of DNA from numerous eukaryotic and prokaryotic sources in a variety of sizes can induce detectable immunological changes.

1.8. Intracellular Signaling

The binding of TLR9 is believed to induce dimerization of the receptor, bringing the TIR domains of each receptor monomer into close association, altering their conformation and allowing for recruitment of adapter protein MyD88 to the receptor (Ahmad-Nejad et al., 2002). MyD88 in turn allows for recruitment of IRAK1 and IRAK4 to the complex via the N-terminal death domain followed by sequential phosphorylation and dissociation (Chen, 2005; Janssens & Beyaert, 2003; N. Suzuki et al., 2002). IRAK1 and 4 possess kinase activity, whereas the other forms, IRAK-2 and M don't have the kinase activity (Janssens & Beyaert, 2003; N. Suzuki et al., 2002). These last two may help to reduce signaling in some situations (Janssens & Beyaert, 2003; Kobayashi et al., 2002). IRAK is responsible for subsequent TRAF6 activation (Chen, 2005). This activation allows for TRAF6 to activate transforming growth factor-β-activated protein kinase (TAK1), an association with subunits TAB1, TAB2, and TAB3, which result in TAK1 phosphorylation (Z. J. Chen, 2005). This phosphorylation results in the subsequent phosphorylation and activation of Ikappa-kinases α and β (Shambharkar et al., 2007). These kinases in turn phosphorylate IκB-α and IκB-β resulting in ubiquitination and degradation of these proteins, freeing NF-κB dimers to translocate to the nucleus and bind to their respective

promoter sequences to initiate transcription of genes associated with inflammation and immunity (Hoffmann & Baltimore, 2006).

The TAK1 phosphorylation also leads to phosphorylation of MKK3 and MKK6 that can activate transcription factors JNK and p38 (S. Sato et al., 2005). A note should be made of plasmacytoid dendritic cells that produce large quantities of the transcription factor IRF7 (Coccia et al., 2004; Dai et al., 2004), which translocates to the nucleus upon TLR9 stimulation to induce the strong IFN-α/β production seen in this cell type (Honda et al., 2005; Kawai et al., 2004).

While signaling through the IL-1 pathway is the primary means of signaling from TLR9, there are indications of one or more alternate pathways through which signaling occurs. For example, phosphoinositide 3-kinase (PI3K) pathway activation is seen in neutrophils (Hoarau et al., 2007). One such example shows initiation of a tyrosine-phosphorylation signaling cascade, resulting in changes to the cellular actin cytoskeleton and motility, which was MyD88 independent and chloroquine insensitive (Sanjuan et al., 2006), while another shows a PI3K pathway activation that was only partly inhibited by TLR9 removal (Dragoi et al., 2005).

1.9. TLR9 Cellular Expression

1.9.1. Dendritic Cells

Dendritic cells are perhaps the most studied cells with relation to TLR9 expression and responses to CpG signaling. As these cells play a key role in the identification and presentation of antigens to the specific immune system they express a broad range of receptors to potential innate immune triggers, which act as a second signal. The potential to use CpGs as Th1-biased adjuvants on these professional antigen presenting cells has also played a role in interest in these cells (Takeda et al., 2003).

Expression of TLR9 between dendritic subtypes and between species can be quite varied. In mice it is known that expression of TLR9 occurs in both myeloid as well as plasmacytoid dendritic cells in the liver, while it is restricted to only plasmacytoid cells in the lung (L. Chen et al., 2006). In humans only plasmacytoid dendritic cells express TLR9 (Jarrossay et al., & Lanzavecchia, 2001; Kadowaki et al., 2001). There is evidence, however, of TLR9 expression and response to CpG ODN in human myeloid dendritic cells (Hoene et al., 2006). The expression patterns remain to be determined in other animal species. As these are perhaps two of the best studied populations that exist in the lung, I will concentrate on them alone.

There are many studies on the production of cytokines, chemokines, and induction of cell receptors in dendritic cells exposed to unmethylated DNA. Plasmacytoid dendritic cells (pDC) appear to be particularly responsive to unmethylated DNA, producing large quantities of IFN-α/β in response to CpG or unmodified unmethylated DNA (Hemmi et al., 2003; Klinman, 2004). In addition pDC also produce IL-6, IL-8, TNF-α, IP-10, and IL-12 (Boonstra et al., 2006; Krug et al., 2001). There is also up-regulation of surface receptors CD40, CD80, CD83, CD86, MHCII, CCR7 (Jarrossay et al., 2001; Krug et al., 2001) and down regulation of CXCR3 (Jarrossay et al., 2001). Survival, activation, and maturation also occurred as a result of CpG ODN stimulation (Krug et al., 2001).

Myeloid dendritic cells produce IL-6, 10, 12, 15, and TNF-α (Boonstra et al., 2006; Hoene et al., 2006; Kuwajima et al., 2006; Sparwasser et al., 1998). Interestingly in mice, co-operation between mDC and pDCs to CpG results in mDC IL-12 production somewhat similar to what is seen in pDC in humans (Krug et al., 2001). Mouse mDCs produce IL-15, inducing CD40L surface expression on pDC that then binds CD40 on pDCs to induce mDC to produce IL-12 (Kuwajima et al., 2006). They also produced small amounts of IFN-α, but not IFN-β (21).

There is also increased expression of CD86 and MHCII but not CD80 [in mice] (Sparwasser et al., 1998), indicative of dendritic cell maturation.

1.9.2. Macrophages

Macrophages are an important part of both the innate and adaptive immune system, serving to clear debris and microorganisms as well as provide critical immune signaling to other cells. These cells are thus often specialized to the needs of a particular tissue, and several different sub-populations may work together to tailor responses.

Macrophages are often categorized according to their origin as well as their location. Lung has interstitial macrophages, which exist in the lung parenchyma and alveolar macrophages that reside in the alveolar space. While both populations may secrete a variety of factors, alveolar macrophages appear better at cytokine production, whereas interstitial macrophage are geared more to presentation of antigen, accessory function, (Bedoret et al., 2009; Fathi et al., 2001; Lohmann-Matthes et al., 1994), and possibly tissue remodeling (Ferrari-Lacraz et al., 2001). This said, interstitial macrophages had higher constitutive production of IL-10, IL-1ra and IL-6 (Hoppstadter et al., 2010). Interstitial macrophages suppress immune responses by inhibiting migration of dendritic cell (Bedoret et al 2009). Both populations of macrophages express TLR9, though there has been some dispute about this in human alveolar macrophages (Fernandez et al., 2004; Hoppstadter et al., 2010; Kiemer et al., 2008; K. Suzuki et al., 2005). The cytokine profiles for both types of macrophages, however, are quite different. In alveolar macrophages there was a greater production of TNF-α to *Mycobacterium bovis* bacterial DNA, but interstitial macrophages showed strong induction of IL-6, and IL-10. This last cytokine was absent from the alveolar macrophages challenged (Hoppstadter et al., 2010).

Macrophages in response to CpG express cytokines such as IL-6, 10, 12, TNF-α, IFN-α/β/γ, MIP-1α/β, MIP-2, JE/MCP-1, IP-10, and RANTES (Boonstra et al., 2006; Kiemer et al., 2008; Lipford et al., 1997; Qiao et al., 2005; Takeshita et al., 2000; Yao et al., 2009; Yeo et al., 2003). Functionally CpG also increases MHC-I, CD40, CD80, ICAM-1 and CD16/32 on the cell surface, increase cell cytotoxicity, and survival (Martin-Orozco et al., 1999; Sester et al., 2006). However, the pattern of expression is likely to vary depending on the population of macrophages in question. In addition CpG ODN can synergize with IFN-γ to induce nitric oxide synthase in macrophages (Sweet et al., 1998), and enhances IL-12p40 production (Cowdery et al., 1999). PGE2 is also produced, which can inhibit IFN-γ production (Y. Chen et al., 2001).

A notable change in receptor signaling in response to TLR2, 4, and 9 ligation in alveolar macrophages is inhibition of IL-10R signaling (Fernandez et al., 2004). IL-10 is constitutively expressed in many lung cells presumably to maintain an anti-inflammatory homeostasis. TLR ligation reduces ability of macrophages to respond to IL-10 (Bonfield et al., 1995; Fernandez et al., 2004). This may be a critical point as alveolar macrophages produce a variety of anti-inflammatory cytokines, including IL-10 (Bingisser & Holt, 2001).

It should be noted that within certain species, particularly hoofed mammals, there is a population of specialized macrophages known as pulmonary intravascular macrophages (PIMs) (Staub, 1994). These appear to serve a function similar to vascular macrophages in the liver of other species, being very active in removing particulates from circulation, but also contribute to lung inflammation (Parbhakar et al., 2005; Singh et al., 2004). While no work has been done on the expression of TLR9 in PIMs, it is known that macrophages express TLR9 in at least several species (Burgener & Jungi, 2008; Dar et al., 2010; Griebel et al., 2005; Hashimoto et al., 2005; Shimosato et al., 2005; Tohno et al., 2006; Y. W. Zhang et al., 2008).

1.9.3. Monocytes

Monocytes have been studied significantly less than the cells that differentiate from them. Both human and mouse monocytes express TLR9 (Juarez et al., 2010; Saikh et al., 2004). Human monocytes were shown to express TNF-α and IL-6 in response to bacterial DNA (Juarez et al., 2010) despite earlier work by others that suggested these cells were non-responsive to CpG ODN (Hornung et al., 2002). Beyond this not much has been written on their responses to stimulation through the receptor.

1.9.4. B Cells

B cells were the first cell types to be recognized to respond to unmethylated DNA, as the molecule acts as a strong mitogen on these cells (Krieg et al 1995) and may be sufficient to induce proliferation, inhibit apoptosis, and cytokine secretion (Hanten et al 2008, Krieg and Vollmer 2007, Jiang et al 2007 Malaspina et al 2008, Wang et al 1997). Studies have shown expression of TLR9 on these cells in mice, humans, and some veterinary species (Cognasse et al 2008, Nalubamba et al 2007). The expression levels of TLR9 are also altered depending on the type of B cell in question. For example, naïve human B cells show low expression of a host of TLRs including TLR9 (Bernasconi et al., 2003; Booth et al., 2011). These cells appear to be non-reactive to CpG in humans, unless there is simultaneous B cell receptor engagement, resulting in increased TLR9 expression, a process which could act as a check on generation of self-reactive B cells (Bernasconi et al., 2003). In contrast, memory B cells in humans have much elevated levels of several receptors, including TLR9, and have been shown to differentiate into plasma cells upon TLR ligand stimulation alone (Booth et al., 2011). Location can also play a role, where tonsil B cells express TLRs at heightened levels in naïve, memory, or germinal cell populations

(J. Booth et al., 2011). Contrast this with regulatory B cells of Payer's Patches that appear to not respond to CpG-ODN due to inactivation of the TLR9 signaling pathway (Booth et al., 2010).

CpG-ODN administration to B cells has been known to increase proliferation (Krieg et al., 1995) and induce antibody production, particularly those antibodies associated with Th1 type responses such as $IgG2_{a/b}$ and IgG_3 in mice, and IgG and IgM in humans. They can also induce class switching to these antibody types from other types such as IgE (Hanten et al., 2008; He et al., 2004; Jegerlehner et al., 2007; Lin et al., 2004; N. Liu et al., 2003), though other cytokine mediations are necessary (Krieg, 2002).

B cells also express a variety of cytokines upon CpG-ODN stimulation such as MIP-1α (CCL3), MIP-1β (CCL4), IL-1β, IL-2, IL-2r, IL-10, and IL-6 (Cognasse et al., 2008; Hanten et al., 2008; Lampropoulou et al., 2008; Marshall-Clarke et al., 2003). Cell surface markers are also increased for MHC-I, MHC-II, IL-2R, CD23, CD40, CD58, CD80, CD86, and down regulation of CD32 in one system but not another (Hanten et al., 2008; Martin-Orozco et al., 1999).

1.9.5. T Cells

T cells have been shown to express TLR9 in mice, rats, and humans (Chiffoleau et al., 2007; Gelman et al., 2004; Hammond et al., 2010). Both activated and memory (CD45RO+) CD4+ and CD8+ T cells in humans have been shown to express TLR9 but not naïve T cells (Babu et al., 2006).

What appears common to many of these subtypes is that TLR9 may enhance some responses, but cannot initiate responses in purified T cell populations (Hornung et al., 2002). Upon T cell activation, however, TLR9 has been shown to enhance survival (Gelman et al 2004) and/or proliferation (Chiffoleau et al., 2007). Also, while CD4+NKT cells are responsive to CpG

ODN, this activity appears to be as a secondary enhancement of response to Kupffer cell TLR9 activation (Jiang et al., 2009).

Regulatory T cells (Treg) cells have been shown to play crucial roles in attenuation of immune responses, and prevention of autoimmunity (van Maren et al 2008). Interestingly, while TLR9 expression was low on effectors T cells and natural Treg cells, IL-10 secreting Tregs (inducible Tregs) generated in the presence of vitamin D produced higher TLR9 levels. These adaptive Tregs also produced IL-4, while production of IL-10 and IFN-γ was reduced, abrogating their regulatory function (Urry et al., 2009). Therefore, CpG/TLR9 may work in concert with other molecules to enhance immune responses by interrupting signals that reduce such responses. TLR9 signaling appears able to also block Treg conversion in the gut (Hall et al., 2008).

1.9.6. Natural Killer (NK) Cells

Natural killer cells were shown to express TLR9 in mice and humans (Roda et al., 2005). Natural killer cells are cytotoxic cells that kill by targeting cells deficient in MHC-I receptor and releasing perforins and granzymes that lyse the cell. They also detect antibody-coated cells via the CD16 (human) receptor. In response to CpG stimulation with CD16 ligation they have been shown to produce larger quantities of IFN-γ, MIP-1α, IL-8, macrophage-derived chemokine (MDC), and an increase in cell cytotoxicity (Roda et al., 2005). This may explain why an earlier study showed these cells to be non-responsive to CpG ODN (Hornung et al., 2002), as there is a requirement for additional stimulation.

1.9.7. Neutrophils

As an early responding cell to infections, neutrophils are equipped with a wide range of TLR receptors, including TLR9 (Hayashi et al., 2003). Ligation of TLR9 results in production of IL-6, IL-8 and TNF-α (Jozsef et al., 2006). Up-regulation of CD11b/CD18 on the cell surface was seen (Trevani et al., 2003), but this could be achieved to some degree in TLR9-negative mice as well suggesting a role for other DNA sensing mechanisms (Alvarez et al., 2006). Bacterial DNA prevents apoptosis of these short-lived cells (Jozsef et al., 2004). Interestingly, L-selectin shedding, a marker for neutrophil activation, and phagocytosis were not altered (Hayashi et al., 2003). However, in another study L-selectin shedding (Trevani et al., 2003), phagocytosis and superoxide production in neutrophils were increased (Hayashi et al., 2003; Wheeler et al., 1997). Also, it appears that while the cytokine response relies on the classical TLR to NF-κB pathway, other aspects such as apoptosis were conducted through the MAPK pathway (Hoarau et al., 2007; Jozsef et al., 2004). This expression of TLR9 occurs across a number of species such as human (Hayashi et al., 2003) and mouse/rat (Jozsef et al., 2004).

1.9.8. Eosinophils and Basophils

Eosinophils appear to constitutively express TLR9 (Nagase et al 2003, Mänsson and Cardell 2009). Stimulation of isolated human eosinophils with CpG-ODN caused the up-regulation of CD11b, CD69, down-regulation of L-selectin, and enhanced cell survival. Further, these eosinophils secreted IL-8 and eosinophil-derived neurotoxin, the latter a marker for degranulation. Interestingly, the release of these two mediators was enhanced in allergic patients, suggesting an enhanced effect of CpG in those prone to allergy (Mansson & Cardell, 2009).

While basophil expression of TLR9 has been shown (Fransson et al., 2007), there are no papers published to date on response of these cells to TLR9 stimulation.

1.9.9. Mast Cells

Bone marrow derived mouse mast cells have been shown to express TLR9, but CpG-ODN appeared to have no effect on proliferation or degranulation. There was however an increase in the expression of IL-6 by these cells (Ikeda et al., 2003). In another study of mouse bone marrow and fetal skin derived mast cells a similar failure to induce degranulation with CpG-ODN occurred, but these cells were still induced to express TNF-α, IL-6, RANTES, MIP-1α, and MIP-2 (Matsushima et al., 2004).

1.9.10. Platelets

An interesting observation has been made that human and mouse platelets express a number of TLR receptors, including TLR9 (Cognasse et al., 2005). Given that signaling from TLRs has always been thought to be about activation of transcription factors, this brings up the question of what pathways are used by these receptors and what function do they play in these anucleated cells. TLR9 expression could be increased in human platelets by treatment with thrombin suggesting that expression levels of the receptor are subject to being increased. LPS treatment of these cells showed that TLR4 played a significant role in the induction of TNF-α to LPS in mice (Aslam et al., 2006). While TLR9 was not tested, the possibility exists for a functional role for TLR9 on these cells.

1.9.11. Non-immune cells

While many papers stress the expression of TLR9 on immune cells, it is becoming clearer that TLR9 expression is much broader than initially suspected. This is particularly true of studies along mucosal surfaces, with other cell types being added as time goes on. Epithelial cells of the gut (Schmausser et al., 2004), nose (Fransson et al., 2007), tonsils (Rumio et al., 2004), and the bronchus (Platz et al., 2004) clearly express the receptor. Bronchial epithelial cells in culture, when exposed to bacteria DNA or CpG ODNs expressed IL-6, IL-8, and B-defensin 2 (Platz et al., 2004). Similar in some ways to epithelial cells, conjunctival cells also express TLR9 (Bonini et al., 2005). Other tissues included the monocyte related astrocytes and microglial cells in the brain (Deng et al., 2001; Schluesener et al., 2001).

Vascular endothelium has been shown to express TLR9 across a number of tested species (J. Li et al., 2004). Stimulation of these cells with CpG ODN also showed that vascular endothelium is good at producing IL-8 and ICAM-1 two pro-inflammatory proteins (Li et al., 2004). Similar results have been seen in the nose as far as expression of TLR9 is concerned (Fransson et al., 2007).

1.10. Lung Inflammation

The lung is a complex and vital organ that is faced with a number of unique challenges in order to function properly. It must carry out gas exchange over a large mucosal surface area, while at the same time managing to remove particulates as well as prevent infection and invasion of the body by microorganisms. Inflammation must therefore be activated and subsequently deactivated in a very controlled fashion in order to prevent loss of function either through unrestrained inflammation, or bacterial invasion by insufficient response.

Inflammation is usually divided along the lines of acute versus chronic inflammation. In brief, acute inflammation of short duration is typically rapidly induced without any long term changes to the tissues involved. In contrast, chronic inflammation is often more slowly induced, but leads to persistent inflammation with significant tissue damage and airway remodeling. This persistence can often (but not always) be caused by failure to eliminate the antigenic "irritant", but lasting changes to the tissue as a result of inflammation may also play a role in maintaining this irritation.

1.11. Basics of Lung Inflammation: Shared early features of inflammation

A critical aspect of inflammation is the migration of immune cells to the site of injury or infection. This occurs in both acute as well as chronic inflammation. First, there is signaling from the site of infection. Cells within the area such as DCs, epithelial cells, or macrophages respond to a local insult by sensing "danger signals" (Medzhitov & Janeway, 2000) which can be quite varied, all of which may not yet be known. Some examples include TLR and NOD receptors for microbial molecular motifs (Chen et al 2009, Aharonson-Raz & Singh, 2010). These in turn can cause changes such as the expression of new surface markers as well as cytokine and chemokine production which can signal cells more distal to the site of injury or infection, as well as set up a chemotactic concentration gradient to signal cells to the site.

Another method is to induce migration in mobile cell types such as macrophages and DCs. DCs present a very good example of this. Prior to activation these cells are specialized to detect and gather antigen, expressing a number of pattern recognition receptors such as TLRs, and a strong ability to phagocytose microbes (de Heer et al., 2005; Hornung et al., 2002). Upon contact with an antigen, presumably in the context of a second danger or inflammatory signal of some kind, these cells undergo maturation. In this process pattern recognition receptors are down

regulated and antigen presentation and co-stimulatory molecules such as MHC-II, CD80, and CD86 are up-regulated. The cells also now migrate by up-regulating CCR7 and then following CCL21 to regional lymph nodes to present antigen to cells of the specific immune system such as T-helper cells (Forster et al., 1999; Saeki et al., 1999). As the activation of specific immunity is a prolonged event, such specific immune cells are often not seen in cases of acute inflammation, but forms a critical aspect of chronic inflammatory responses.

Within tissue the next level of change is the induction of changes to the local vasculature. In response to factors such as histamine, bradykinins, vascular endothelial growth factor (VEGF), and platelet-activating factor (PAF) vasodilation occurs, allowing for easier migration of inflammatory cells into tissue (Theoharides et al., 2007). This also results in increased leakage of fluids and the redness that are characteristic of inflammation. At the same time endothelial cells are induced to increase the expression of several cell surface receptors and ligands of the selectin and integrin families to attract appropriate cell populations based on the type of surface protein induced. For example, P-selectin is expressed on endothelial cell surfaces in response to acute inflammation signals such as histamine to attract monocytes and neutrophils whereas E-selectin is induced by IL-1 or TNF-α (Springer, 1994). These signals allow for the attachment, rolling, and final migration out of circulation (Carlos & Harlan, 1994; Lawrence & Springer, 1991; Springer, 1994). At the same time, lymphocytes express different selectins and integrins such as Mac-1 or LFA-1 that can bind intercellular adhesion molecules (ICAM) (Springer, 1990). A precisely coordinated sequence of such interactions is required for proper migration of neutrophils. Selectins first mediate tethering and rolling, but chemoattractant signaling is required for firm attachment to ICAM followed by migration out of circulation (Lawrence & Springer, 1991; Springer, 1994). These chemokines also impart directional signaling to guide

different populations of immune cells to the site of inflammation, as well as signal cells at or near the site to produce other mediators (Carlos & Harlan, 1994; Hechtman et al., 1991; Huber et al., 1991; Springer, 1994).

The first cells that migrate to the site of inflammation make up members of the innate immune system, but migration to the site continues throughout infection. Neutrophils often comprise the greatest number of cells initially (Tsushima et al., 2009). Neutrophils produce numerous cytokines, including IL-6 and IL-6 soluble receptor leading to increased movement of macrophages to the area with subsequent decreased neutrophil migration (Bellingan et al., 1996; Hurst et al., 2001). If inflammation is not resolved and a more chronic inflammation occurs there can be additional migration of B and T cells to the area. Alveolar macrophages, already resident in the alveoli are already at the site of many lung infections and secrete a range of pro-inflammatory cytokines including IL-1, 6, 8, and TNF-α (Ware & Matthay, 2000).

Resolution of inflammation is likewise a controlled and regulated process that extends beyond simple removal of chemotactic and inflammatory signals. Neutrophil accumulation is resolved through apoptosis of these cells (Abraham, 2003; Matute-Bello & Martin, 2003), while alveolar macrophage clear debris, proteins, and apoptotic neutrophils (Ware & Matthay, 2000). Edema and fluid in the alveoli is resolved through active transport of fluid into the lung interstitium by water channels such as aquaporins, and proteins by diffusion between cells (Dobbs et al., 1998; Matthay et al., 1996; Tsushima et al., 2009). Fluid is then carried away by the lung lymphatic system (Brigham et al., 1974; Ohkuda et al., 1978). Damage of the alveolar epithelium is repaired by proliferation of Type-II cells, with differentiation into Type-I cells (Adamson & Bowden, 1974).

1.12. Acute Lung Injury (ALI)

Acute lung injury is perhaps the most common type of lung inflammation for most people. A microbial infection, or presence of bacterial components such as LPS cause an inflammatory response, which subsequently resolves itself after the antigen is removed. This resolution typically proceeds with removal of excess cells, debris and fluids and cellular repair of nearby tissues, resulting in few if any permanent changes to the tissue.

To build on the previously mentioned basic description of inflammation, cases of ALI are seen to produce large quantities of macrophage migration inhibitory factor (MIF) in lavage samples. This cytokine is known to induce IL-8 and TNF-α production from resident macrophages, which have been shown to lead to neutrophil chemotaxis (Donnelly et al., 1997). It also has a role in the up-regulation of receptors such as TLR4 (Roger et al., 2001) and improves survival of macrophages (Goodman et al., 2003). MIF production is in keeping with the early signs of ALI, which includes diffuse neutrophilic infiltration of the lung, haemorrhage, and edema (Wheeler & Bernard, 2007). IL-1β appears to also be critical to the disease and found in the BAL as well (Goodman et al., 1996).

Recruitment of neutrophils is a common feature yet there has been some controversy about whether the number of neutrophils in the lung is sufficient for activation (Goodman et al., 2003). However, most studies have shown more reactive neutrophils in acute respiratory distress syndrome (ARDS) patients. The release of cytokines such as IL-1β, TNF-α and IL-8 strongly suggest activated neutrophil involvement is a critical part of this inflammation (Abraham, 2003). Interestingly, apoptosis of these same neutrophils can induce macrophages to inhibit their production of a number of pro-inflammatory cytokines (Fadok et al., 1998; Huynh et al., 2002). B and T cells are typically not a feature of ARDS (Goodman et al., 2003). The action of these

immune cells results in increased local oxidant stress and protease action, resulting in reduced production and breakdown of surfactant, inhibiting gas exchange. Local production of elastases can further damage alveolar capillary and epithelial cells (Wheeler & Bernard, 2007).

Containment of inflammatory processes appears to occur in conjunction with inflammation, as levels of antagonists such as IL-1ra or anti-inflammatory cytokines such as IL-10 are produced (Bogdan et al., 1991; Fiorentino et al., 1989; Fiorentino et al., 1991; Goodman et al., 1996). The latter in particular is known to inhibit macrophage cytokine production (Fiorentino et al., 1989; Fiorentino et al., 1991). This can lead to anti-inflammatory conditions over time (Goodman et al., 2003).

It is of note that patients can have different outcomes to ALI. For some there is simple resolution, whereas others may have fibrosing alveolitis that seems to start early in inflammation, and is characterized by mesenchymal cells appearing in the alveoli. This leads to fibrosis and angiogenesis, characteristic of more prolonged or chronic lung disorders (Ware & Matthay, 2000). Interestingly this outcome has been induced with adenoviral gene delivery of IL-1β gene into the lung, suggesting that this cytokine may be responsible for this fibrosis (Kolb et al., 2001). This was further confirmed in that those with lower IL-1ra, an IL-1β antagonist showed this persistent ARDS (Goodman et al., 1996).

1.13. Asthma, an Example of Chronic Inflammation

One of the most common types of inflammation of the lung is asthma. It is estimated there are 300 million people around the world with asthma, and 10% of the North American population (Braman, 2006). Airway obstruction is caused by constriction of the smooth muscle cells surrounding the bronchi, and diagnosis is done by inducing irritation of the bronchial

epithelial cells with an agent such as methacholine, and measuring drop in the forced expiratory volume at different concentrations of irritant (AARC clinical practice guideline. bronchial provocation. american association for respiratory care.1992).

While asthma is typically associated with a specific allergen(s) called atopic or extrinsic asthma, not all cases of asthma have a recognizable allergen. These non-atopic or intrinsic cases proceed similar to asthma, but may be induced by physical triggers such as cold air and exercise (Humbert et al., 1999; Wenzel, 2006) though there are some that suggest an infection may be responsible in some or most of these cases (Barnes, 2009). Likewise, a number of people may have allergies but not asthma, so the specifics of asthma formation are as yet not easy to discern. However, most work on asthma does assume a specific response to an environmental allergen. One key feature of asthma is the induction and accumulation of inflammatory helper T cells of the antibody mediated, or Th2 subtype in the lung. These cells in turn secrete cytokines such as IL-4, 5, 9 and 13, instructing B cells to produce IgE antibodies, which in turn bind to FcεRI on mast cells attracted by the same cytokines (Barnes, 2008; Cyphert et al., 2009; Gould et al., 2000; Kay, 2006; Robinson et al., 1992). Upon binding allergen, which results in IgE cross-linking, these mast cells release a number of pro-inflammatory mediators such as histamine, leukotrienes, prostaglandin D_2, IL-4, IL-5, IL-13, and TSLP (Barnes, 2008; Galli et al., 2005; Ying et al., 2005) that induce inflammation and bronchoconstriction, the hallmarks of the disease (Cyphert et al., 2009). Mast cells may be found in smooth muscle, a situation linked to airway hyperresponsiveness, or also recruited to the mucosal surface by stem-cell factor (Brightling et al., 2002; Reber et al., 2006).

The other cell type associated with inflammation is the bronchial epithelial cell. In promotion of inflammation they produce a number of factors including CCL11 (eotaxin-1) which

induces eosinophil migration (Gonzalo et al., 1996), and thymic stromal lymphopoietin (TSLP) (Allakhverdi et al., 2007). This last factor has been shown to play a role in myeloid DC maturation, and inducing release of CCL17, and CCL22, which both bind CCR4 that is selectively expressed on Th2 cells (Liu, 2006). TSLP can also directly activate DCs to prime T cells to produce Th2 cytokines (Ito et al., 2005; Wang et al., 2006). The epithelial layer acts as a barrier with the cells connected by tight junctions and zonula occludens proteins (Hammad & Lambrecht, 2008). It may also act as a barrier to allergen entry. Indeed, a number of more common and clinically relevant allergens such as *Aspergillus* species and ragweed pollen have cysteine and serine protease activities that can disrupt cell junction proteins (Runswick et al., 2007; Tai et al., 2006).

Basolateral to the epithelial barrier are mucosal DCs that extend processes between the cell junctions in order to sample antigens (Chieppa et al., 2006; Jahnsen et al., 2006). The epithelial cell barrier limits access to other DC subtypes. In many cases it is believed that insufficient stimulation of DCs results in abortive or insufficient stimulatory DCs, which can induce tolerance (Akbari et al., 2002; de Heer et al., 2004), a situation that can be overcome by addition of additional stimuli such as LPS (Eisenbarth et al., 2002; Piggott et al., 2005). Given that epithelial cells are well armed with TLR and PAR receptors (Kauffman, 2006) these may provide secondary signals for full activation of DCs.

Control or avoidance of asthma has been hard to deduce. It has been shown that naturally occurring populations of macrophages such as interstitial and alveolar macrophages (AM) may play a role. AMs though critical to inflammatory responses and clearance of many pathogens from the alveoli are often not mentioned in regards to asthma. However, there are some indications that they may exert anti-inflammatory effects. This is through several mechanisms.

First, they have been shown to inhibit antigen presentation by resident DCs (Holt et al., 1993). Second, AMs in standard OVA airway hyperresponsiveness challenge systems appear to preferentially produce IFN-γ, IL-12, and IL-18, and inhibit IL-4 and IL-5 (Ho et al., 2002; Plummeridge et al., 2000; Thepen et al., 1991), polarizing responses towards a Th1-type response, inhibiting Th2 responses. Many such factors in the right context however can be pro-inflammatory (Peters-Golden, 2004). They also produce anti-inflammatory cytokines IL-10 and TGF-β (Kay, 2003). Another interesting feature is that AMs produce prostiglandin-E2, TGF-β, and platelet-activating factor upon phagocytosis of apoptotic cells (Fadok et al., 1998), but pro-inflammatory cytokines in response to bacteria that are ingested. In the case of asthma, where epithelial cells are readily lost and more susceptible to apoptosis (Barnes, 2008; Jeffery, 2001), it is possible that AMs provide negative feedback to asthmatic symptoms. There is even some evidence to suggest that they can tolerize CD4+ T cells in an antigen-specific manner (Blumenthal et al., 2001).

Others have shown a role for the development of Tregs (CD4+ FoxP3+) in limiting responses and maintaining lung homeostasis (Holt et al., 2008). Upon stimulation they secrete cytokines known to play roles in limiting inflammation such as IL-10 and TGF-β (Li et al., 2006; O'Garra et al., 2008). They also express CTLA4 and PD-1 that can inhibit responses through direct contact with other cells (Vignali et al., 2008). Tregs may also compete with other T cells for growth factors (Barthlott et al., 2003).

Indeed repeated low dose exposure to inhaled allergen induced tolerance that could be transferred to naïve mice through Treg adoptive transfer (Ostroukhova et al., 2004).
Atopic individuals may be less susceptible to Treg control though as *in vitro* studies have shown CD4+CD25- cells from atopic patients were inhibited less by these cells. Further, they may play

a role more in prevention of atopy as non-atopic volunteers depleted of Tregs exhibited Th2 responses to allergens similar to those that were atopic (Ling et al., 2004). Certainly they seem to limit airway hyperresponsiveness (Lewkowich et al., 2005). Another thought is that DCs exposed to antigen without sufficient co-stimulation drive the production of Tregs, as non-atopic OVA challenge still drives T cell proliferation (Holt et al., 1981). It is likely that antigen presenting cells such as DC are important in this generation, as DCs that produced IL-10 in response to an allergen were able to induce Tregs, and DC transfer could re-create this in naïve mice (Akbari et al., 2002).

Persistent changes are a hallmark of chronic inflammation. These are mainly associated with the bronchial epithelium and surrounding tissues. Given the more fragile nature of asthmatic epithelial cells there is evidence of epithelial growth and dedifferentiation (Jeffery, 2001), mucous/goblet cell metaplasia and changes to submucosal glands leading in increased mucus plugging of airways (Aikawa et al., 1992; Shimura et al., 1996), and sloughing of epithelial cells in asthmatic sputum is common (Beasley et al., 1989; Jeffery et al., 1989; Laitinen et al., 1985). This may induce further responses such as platelet aggregation and fibrin deposition (Jeffery et al., 1989). These changes may only exacerbate symptoms as increased epithelial loss is associated with greater hyperresponsiveness (Jeffery et al., 1989). This persistent damage may result in a level of persistent secretion of cytokines and growth factors associated with angiogenesis (Orsida et al., 1999; Vrugt et al., 2000), repair, and chemotaxis, affecting nearby cells including fibroblasts, leading to other tissue changes and altering mucus and immune cell composition (Jeffery, 2001). Mediators of epithelial repair such as CD44, epidermal growth factor receptor, and TGF-β can be found at the site, however, these mediators may in turn reduce e-cadherin making the cellular barrier more permeable to antigen (Boxall et al., 2006; Knight &

Holgate, 2003; Puddicombe et al., 2000; Silverman et al., 2004). Molecular study of the bronchial epithelium has shown persistent changes in cytoplasmic and nuclear levels of NF-κB dimers in these cells between challenges (Hart et al., 1998), suggesting some persistent activation of these cells. NF-κB is a transcription factor that is strongly associated with induction of a wide array of pro-inflammatory genes (Ghosh et al., 1998), suggesting asthmatic epithelial cells are primed towards inflammation between challenges. This was confirmed in studies of NF-κB activation in OVA challenged mice (Poynter et al., 2002).

Another change characteristic of asthma is a thickening of the reticular basement membrane (Jeffery, 2001). This change occurs fairly early in asthma, and appears unaffected by duration or severity of symptoms (Jeffery, 2001; Payne et al., 2003). This thickening is often called subepithelial fibrosis, and the membrane may see increases in reticulin (Laitinen et al., 1997). Collage deposition also accompanies the thickening of the basement membrane (Minshall et al., 1997).

In addition there is also an increase in smooth muscle mass (Jeffery, 2001). This increase may be due to muscle hyperplasia (Heard & Hossain, 1973) and hypertrophy (Ebina et al., 1990; Ebina, Takahashi et al., 1993). Theories also exist for fibroblast transformation to myofibroblasts (Jeffery, 2001) and smooth muscle change to fibromyocytes (Gizycki et al., 1997). Others have reported a qualitative increase in smooth muscle coverage of the bronchi (Bai, 1990; Benayoun et al., 2003; Dunnill et al., 1969). While increase in smooth muscle component of the airways and cytokine stimulation may account for some of the bronchoconstriction seen in asthma, there are other contributors as well. Changes in rigidity of the airway, through increased collagen deposition, edema, or basement membrane thickening with opposed muscle constriction that

shortens the airways also occur, redirecting this tension towards airway constriction (Jeffery, 2001).

The end result is an inflammation that is based largely around a Th2 antibody-mediated response that induces IgE, and production of pro-inflammatory mediators. This results in episodic bronchoconstriction, but also in long term irreversible changes to several tissues such as smooth muscle, epithelial cells, and the epithelial basement membrane. Some of these changes, particularly to the epithelial cell layer may result in persisting changes that can ensure some level of immune response to tissue damage or leakage of antigens across the epithelial layer.

1.14. Chronic Obstructive Pulmonary Disease (COPD)

To briefly contrast with this we should also consider COPD. COPD has at times been considered another form of asthma (Barnes, 2008) but has been shown to have several distinct features. Increase severity of each disorder has a greater overlap of immune response (Barnes, 2008; Jeffery, 2001). COPD appears to originate from chronic exposure to lung irritants, the most studied being tobacco smoke (Barnes, 2008). There is however question about the antigen, a feature which also sets it apart from atopic asthma. Suggestions have been made for bacterial or viral antigens, but a recent model system suggests the possibility of induced autoimmunity to elastase, which could explain some of the observed symptoms of emphysema (Barnes, 2008; S. H. Lee et al., 2007).

Similar to asthma there is obstruction of breathing due to inflammation and remodeling of the bronchus (Barnes, 2000; Jeffery, 2000). In COPD inflammation also extends to a greater degree into small airways and lung parenchyma (Barnes, 2008; Jeffery, 2001) and airway hyperreactivity is absent (Barnes, 2000). While asthma can be characterized with episodic attacks of airway hypersensitivity in response to exposures to relevant environmental

antigens/stimuli COPD symptoms are persistent, exacerbated by general chemical irritants as opposed to a known specific antigen (Barnes, 2008).

While COPD and asthma both are chronic diseases, the type of inflammation between the two is significantly different. There is no indication of mast cell activation in COPD and infiltrates are often dominated by neutrophils in the airway lumen, not the eosinophils found in asthma, and this correlates with severity of disease (Hogg et al., 2004; Keatings, et al., 1996). There is also increased macrophage infiltration compared to asthma, and these cells may be responsible for attracting neutrophils (Barnes, 2004). Interestingly, alveolar macrophages from smokers appear to produce reduced levels of inflammatory cytokines, suggesting they may be less reactive than those from normal lungs (Dandrea et al., 1997).

The most striking difference to asthma is that COPD CD4+ T cells exhibit a Th1 or cell-mediated immune response phenotype (Barnes, 2008; Grumelli et al., 2004). This means that the cytokine milieu will be quite different to asthma, favoring production of TNF-α, IL-12, RANTES, and IFN-γ (Barnes, 2008; Costa et al., 2008), and a cell-mediated response. This is evident in the increases of neutrophils, macrophages, and CD8+ T cells (AKA cytotoxic T cells) in COPD patients, the last of which can out-number CD4+ T cells (Barnes, 2008; Saetta et al., 1998).

Unlike in asthma there is no increase of the reticular basement membrane in the bronchi. Epithelial cell shedding is also absent, though squamous and goblet cell metaplasia still occur, as well as submucosal gland hypertrophy, with goblet metaplasia in the peripheral airways. Likewise no angiogenesis is evident. Smooth muscle enlargement still occurs, but only in distal airways (Beasley et al., 1989; Dunnill et al., 1969; Jeffery et al., 1989; Jeffery, 2001; Kuwano et al., 1993; Laitinen et al., 1985; Saetta et al., 2000). Most changes are seen the small airways and

lung parenchyma. Fibrosis can occur around these small airways (Hogg, 2004). A key feature of COPD is destruction of alveolar walls due to apoptosis of type-1 epithelial cells and protease-degradation of connective tissue leading to emphysema (Majo et al., 2001; Taraseviciene-Stewart et al., 2006).

This highlights that the lung is well-armed to respond to a number of infectious as well as environmental insults. These responses may not always be appropriate however, such as in cases of chronic inflammation like asthma and COPD.

CHAPTER 2: HYPOTHESIS AND OBJECTIVES

2.1. Hypotheses

1. TLR9 expression in whole lung tissue is similar across different species.
2. TLR9 modulates lung inflammation induced following exposure to poultry barn air.

2.2. Objectives

1. Characterize TLR9 expression in whole lung in cattle, pigs, dogs, horses, mice, and humans.
2. Determine if TLR9 expression is altered in cases of lung inflammation, such as LPS (horses), *Mannheimia hemolytica* infection (cattle), asthma (humans), or barn air exposure (mice).
3. Determine if removal of pulmonary intravascular macrophages in horse lung alters levels of lung TLR9 expression.
4. Determine the contribution of TLR9 to lung inflammation due to poultry barn air exposure in mice.

2.3. Rationale

Several studies have clearly shown an association between endotoxin exposure and lung dysfunction in workers in high-intensity barn operations (Donham et al., 2000, Dosman et al., 2006). Endotoxin is a component of bacterial cells walls which binds TLR4, and induces an inflammatory response (Medzhitov et al., 1997).

The microbial content of barn facilities is diverse, and many organisms express inflammatory molecules other than endotoxin (Just et al., 2009). Toll-like receptor-9 (TLR9) detects unmethylated DNA motifs that are present in all bacteria, some viruses, and moulds (Merchant & Ross, 2002). To date very few studies have used environmental DNA (Roy et al., 2003), and to our knowledge none have attempted to look at the contribution of TLR9 in an environmental exposure model.

Before studying such exposure, a clear idea of the extent of the expression of TLR9 in the lung is necessary. While there have been many cell culture and cell isolates studied, little work has been done to image TLR9 in whole lung. In the case of veterinary species even less is known. As a number of species are subject to organic dusts, imaging TLR9 in their lungs may provide some clues to lung problems they encounter, and provide a basis for cross-species comparisons, as it has been long established that there are species-specific TLR9 ligand preferences (Rankin et al., 2001).

CHAPTER 3: EXPRESSION OF TOLL-LIKE RECEPTOR 9 IN NORMAL LUNGS OF PIGS, DOGS, AND CATTLE[*]

3.1. Abstract

Toll-like receptors are important components of the innate immune system. Compared to other TLRs such as TLR4, there is less data on the expression and function of TLR9, which binds to bacterial DNA. Currently no data exists on the cell-specific protein expression of TLR9 in lungs of cattle, dog and pigs. Given that a number of immune cells present in the lungs of mice and humans can express TLR9, and the similarity of the protein sequence we surmised that the same would be true for other species, and that they should be detectable using existing antibodies. Using light and electron microscopic techniques we show expression of TLR9 in airway epithelium, vascular endothelium, and intravascular monocytes in all three species. TLR9 was also localized in pulmonary intravascular macrophages of cattle and pigs.

3.2. Introduction

Toll-like receptors are highly-conserved cell receptors that form a critical component of the innate immune system (Rankin et al., 2001; Roach et al., 2005). These receptors are vital in the detection and subsequent containment, and eventual clearance of a number of diseases (Bhan et al., 2008; Rutz et al., 2004), and are being studied for their impact on the induction of Th1 versus Th2 responses of the specific immune system (Dorn & Kippenberger, 2008; Kline et al., 1998). Further, as bacterial products may be problematic beyond the scope of infections, significant work is now underway into the impact of TLR agonists such as lipopolysaccharide (LPS) and

[*] Portions from International Journal of Experimental Pathology (Schneberger et al 2011)

their effect on such conditions as COPD and lung dysfunction (Frieri, 2005; Senthilselvan et al., 2009). Some of these receptors, such as TLR9, are being tested as possible targets for oligonucleotide adjuvants which holds promise for the development of new classes of immune modulators (Kline et al., 1998; Rankin et al., 2001; Rankin et al., 2002).

TLR9 is a cell membrane receptor that detects non-methylated CpG motifs. The CpG sequences are typically methylated and genetically repressed in vertebrates, but can be found unmethylated in viruses, bacteria, and moulds (Hemmi et al., 2000; Krieg et al., 1995; Kuramoto et al., 1992; Ramirez-Ortiz et al., 2008). TLR9 is predominantly localized in the lysosomes, where the low pH promotes specific TLR9 binding to unmethylated DNA (Latz et al., 2004; Macfarlane & Manzel, 1998; Rutz et al., 2004). Several groups however have shown cell surface expression of TLR9 (Eaton-Bassiri et al., 2004; Ewaschuk et al., 2007; Hu et al., 2003).

The cell-specific pattern of TLR9 expression varies between species. The most notable example of this has been plasmacytoid dendritic cells in human lungs but not in mouse lungs express TLR9 and this difference may be important in species-specific immune response (Banchereau et al., 2000; Chen et al., 2006; Demedts et al., 2006). In contrast TLR9 is expressed in pulmonary intravascular macrophages which are present in the horse but not in humans and mice, (Aharonson-Raz & Singh, 2010; Schneberger et al., 2009). These differences in TLR9 expression macrophages could result in significantly different outcomes to stimulatory DNA exposure and point to a need to develop better understanding of TLR9 expression in each of the species.

Domestic animal species such as cattle, dog and pigs suffer from many bacterial lung diseases. In cattle, *Mannheimia hemolytica* causes significant morbidity and mortality, which translates into nearly $1 billion economic loss to cattle industry in the USA alone (Morsey et al.,

1999). Lung inflammation associated with *Actinobacillus pleuropneumoniae* can be endemic in pigs and cause significant financial losses (Chiers et al., 2002). Finally, *Bordetella bronchiseptica* infections and subsequent lung disease is commonly seen in dogs (Goodnow, 1980). While little is known of the interactions of TLR9 with any of these pathogens, we know that the expression of TLR4 can be altered in lungs of cattle with *M. hemolytica* (Singh et al., 2004). The expression of TLR9 in horse lungs was increased following treatment with E. coli lipopolysaccharide (Schneberger et al., 2009). Given that the LPS from these bacteria can potentially alter TLR9 expression and that TLR9 signaling may impact the TLR4 response (Hong et al., 2004; Yeo et al., 2003), it is important to study the expression of TLR9 in normal and inflamed lungs.

Our objectives therefore were to examine expression of TLR9 in whole lungs of cattle, pigs, and sheep, both at the tissue as well as subcellular levels. With cattle we further looked to see if there were differences in expression due to immune stimulation in cattle infected with *M. hemolytica*.

There are very limited data on the expression of TLR9 in cattle, pig and dog in general and virtually none in the lungs of these species. TLR9 mRNA is expressed in many tissues such as the thymus, lymph nodes, spleen and lung to name a few within the pig (Shimosato et al., 2005). TLR9 protein expression was detected in pig gut (Shimosato et al., 2005; Tohno et al., 2006) by western blot and PCR. In the dog, to date detection of TLR9 has been limited to leukocytes and lymph nodes (Burgener & Jungi, 2008; Hashimoto et al., 2005). However, expression within multiple tissues has been shown to be the case in humans, mice (Eaton-Bassiri et al., 2004; Ewaschuk et al., 2007), pigs (Shimosato et al. 2005), and horses (Zhang et al., 2008), suggesting expression may be broader than indicated in these studies. We therefore set out to determine the

expression of TLR9 in whole lung tissue sections in pigs, cattle, and dogs, and to also examine the expression at the subcellular level. We examined cell-specific protein expression of TLR9 in intact normal lungs of cattle, pig and dog, and inflamed lungs from cattle with immunohistology and immuno-electron microscopy. The data show expression of TLR9 within the bronchial epithelium, vascular endothelium, alveolar macrophages, and cells within the alveolar septa in all species, and pulmonary intravascular macrophages (PIMs) of the pig and cow.

3.3. Materials and Methods

3.3.1. Animals

Animals used for this experiment have been previously described (Wassef et al. 2004). Briefly, untreated dogs, pigs, and calves (n=2) and mouse lungs were taken and tissue was preserved and mounted in paraffin or LR White for light or EM immunohistochemical staining respectively (Parbhakar et al., 2004). Frozen lung tissue was also used for western blot protein extraction. Two mouse spleens (C57BL/6) were kindly provided to us by Dr. Sylvia van Den Hurk to use for mouse protein extracts.

3.3.2. Western Blotting

Dog, pig, calf and mouse lung samples were lysed in 200µl of freshly prepared lysis buffer (150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50mM Tris (pH 8.0), 5mM ethylenediaminetetraacetic acid (EDTA), and protease inhibitor cocktail (100µl/10ml; Sigma-Aldrich). Proteins were separated on a 10% polyacrylamide gel and transferred on to Hybond-ECL nitrocellulose membrane (GE Bioscience, Germany). Membranes were blocked with 5% skim milk in phosphate buffer saline (PBS ; pH 7.4) and incubated with TLR9 antibody (IMG-305A, Imgenex, San Diego, CA, USA) for 2 hours

at room temperature before washing and incubation for 1 hour with goat anti-mouse HRP (P0447, Dako, Ontario, Canada). Blots were developed using the EC Western Blotting Detection kit (GE Bioscience, UK) and exposed to Hyperfilm ECL (GE Bioscience, UK) film.

3.3.3. Immunohistology and Immuno-electron Microscopy

Immunohistology was done with mouse anti-human TLR9 antibody on dog, pig, and calf lung tissue. Briefly, after de-paraffinization, re-hydration, tissue peroxidase quenching (0.5% hydrogen peroxide in methanol), and antigen unmasking with pepsin (2mg/ml 0.01N hydrochloric acid), the tissue sections were blocked with 1% BSA to block non-specific binding for 1 hour. Sections were treated with TLR9 antibody (1:50 dilution) and incubated overnight at 4^oC. The next day horseradish peroxidase-conjugated goat anti-mouse antibody was added at 1:75 dilution to sections for 1hr at 37^oC (P0447, Dako, Ontario, Canada). Color was developed using a color developing kit (Vector Laboratories, Ontario, Canada). Slides were counterstained with methyl green (Vector Laboratories) prior to mounting. A control was similarly run with omission of the primary antibody or the secondary antibody.

For immuno-electronmicroscopy 100nm lung sections were placed on nickel grids and floated in blocking buffer for 30min prior to 1hr incubation with TLR9 antibody at 1:5 dilution. Sections were rinsed 3 times in Tris-buffered saline for 5 minutes before addition and incubation with 20nm gold-conjugated anti-mouse secondary antibody (1:100 dilution goat anti-mouse IgG EMGAM20, Fitzgerald Industries International, Concord, MA, USA) for 1hr. A control with omission of the primary antibody was also run.

3.4. Results

3.4.1. Sequence Alignment and Western Blotting

As the number of species for which commercial TLR9 antibodies exist is limited, we first had to compare the amino acid sequence of peptides against the human epitope the mouse anti-human TLR9 antibody was raised to TLR9 amino acid sequence from cattle, dog and pig. Information from the manufacturer stated that the peptide used to raise the anti-human TLR9 antibody was a KLH-conjugated synthetic peptide to amino acid 268-284 of TLR9 isoform A (GenBank accession No. AAF78037.1). This sequence was aligned against a similarly filed TLR9 sequence for mouse (GenBank accession No. NP 112455), dog (GenBank accession No. NP 001002998), domestic pig (GenBank accession No. NP 999123), and bovine (GenBank accession No. CAD52054), using Clustal-W. The results show 53%, 71% (dog and pig), and 82% homology, respectively, with mouse, dog and pig, and cattle (Figure 3.1). Western blotting of lung protein extracts showed a band of approximately 110-115kDa, which corresponds to TLR9 band detected in lung extracts from mouse (Figure 3.1).

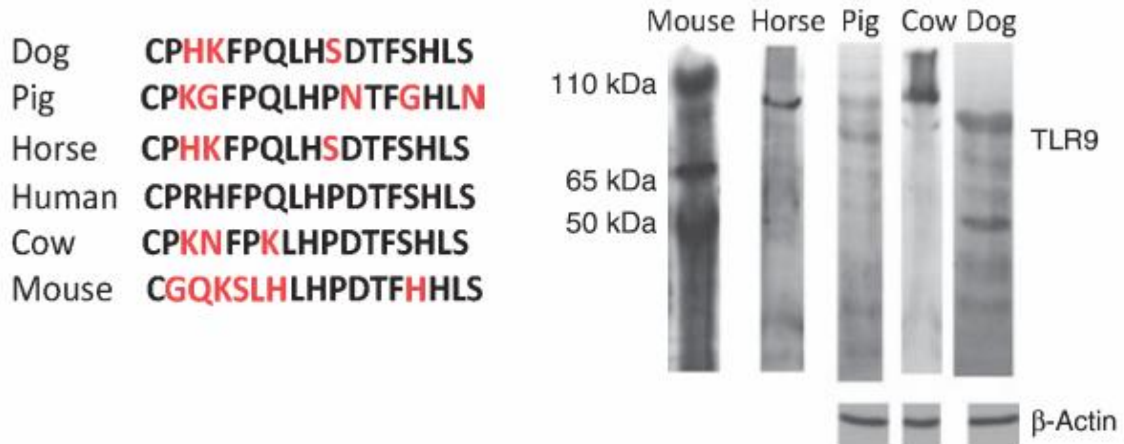

Figure 3.1: Western blot of TLR9 from several species.

Comparison of amino acid sequences corresponding to that detected by the antibody used. Human (GenBank assention # AAF78037.1) was compared to mouse (GenBank assention # AAU04981.1), canine (GenBank assention # NP_001002998), pig (GenBank assention # AAP43691), and equine TLR 9 (GenBank assention # ABD36388) at amino acids 268-284. 10 out of 17 mouse (58%), 12 out of 17 pig (71%), and 14 out of 17 equine, and canine (82%) amino acids were shown to match. Protein extracts were isolated from mouse spleen and horse, dog, cow, and pig lung tissue and run on a 10% polyacry-lamide gel followed by staining with human anti-mouse TLR9 antibody which detected a 110kD band in mouse and horse tissues.

3.4.2 Light and Electron Microscopic Immunocytochemistry

Lung sections from calf (Figure 3.2a) pig and dog (data not shown) stained with only secondary antibody lacked staining while von Willebrand Factor antibody reacted with vascular endothelium in pig (Figure 3.2b), cattle and dog (data not shown).

TLR9 staining of cells within the alveolar septa was readily apparent in calf (Figure 3.2a, 3.2b), dog (Figure 3.2c, 3.2d) and pig (Figure 3.2e, 3.2f), although staining appeared to be more intense in lung sections from the pig and dog compared to calf. Immuno-electron microscopy resolved TLR9 staining to be in pulmonary intravascular macrophages in the septum of calves (Figure 3.3), and alveolar macrophage in all (Figure 3.4). TLR9 was localized in the cytoplasm and nuclei of various cells in the lung. TLR9 antibody also showed reaction with vascular endothelium in the lung (Figure 3.5a-c) but the expression was not uniform across species.

Bronchiolar epithelium from all the species showed TLR9 expression (Figure 3.6a-c). Dog bronchiolar epithelium showed pronounced apical staining compared to the other species (Figure 3.6b). Immuno-EM confirmed the TLR9 staining on the surface membrane and the cytoplasm airway epithelial cells (Figure 3.6d).

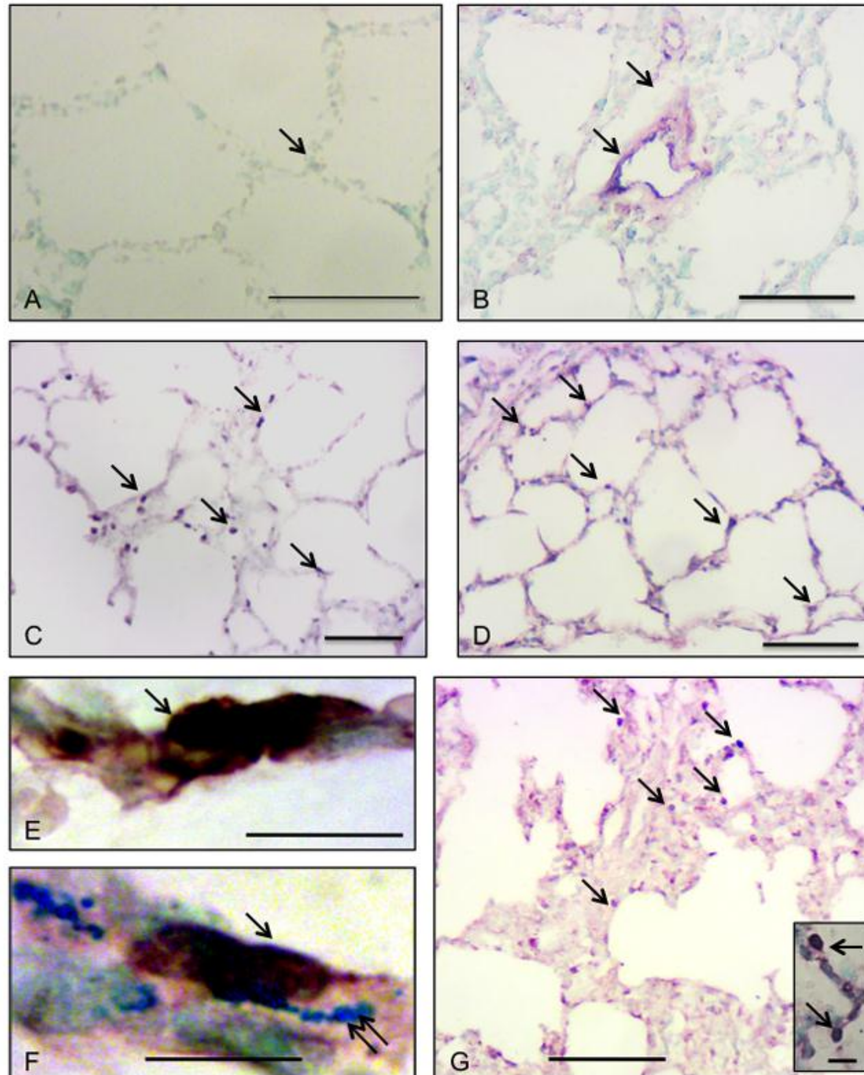

Figure 3.2 TLR9 immunohistochemistry.

Sections stained with only a secondary antibody (A, calf) lacks staining (arrow) in the alveolar septa whereas those stained with vWF antibody (B, pig) shows staining in endothelium (single arrows) but not in bronchiolar epithelium (double arrows). Lung sections from normal pig (C) and normal calf (D) show TLR9 staining in alveolar septa (arrows). High magnification views show large mononuclear phagocytes in the alveolar septum of pig (E) and the calf (F). Calf lung also has shows Monastral blue (F; double arrows) which was injected before euthanasia and is phagocytosed by macrophages. Inflamed lung section (G and inset) has increased TLR9 staining (arrows) compared to the normal calf lung (D). Original magnification A-C,E,J: 100X, D,F,H: 400X, D,G,I,K: 1000X. Bars are 0.1mm for 100X and 0.01mm for 400X and 100X.

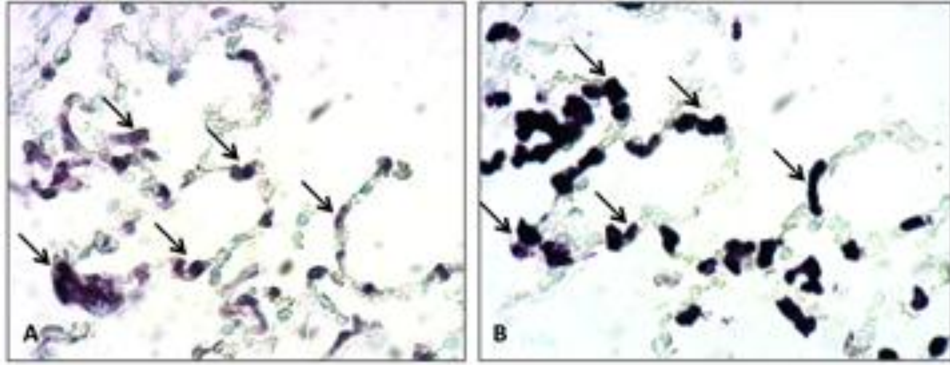

Figure 3.3: TLR9 staining in alveolar septal macrophages.

TLR9 staining (A) observed in alveolar septa of dog lung. Serial sections were stained with macrophage specific antibody (B) to show TLR9 staining occurs in macrophage within the septa. Arrows denote staining in the same cell with both antibodies. Original magnification 400X. Bars 0.01mm.

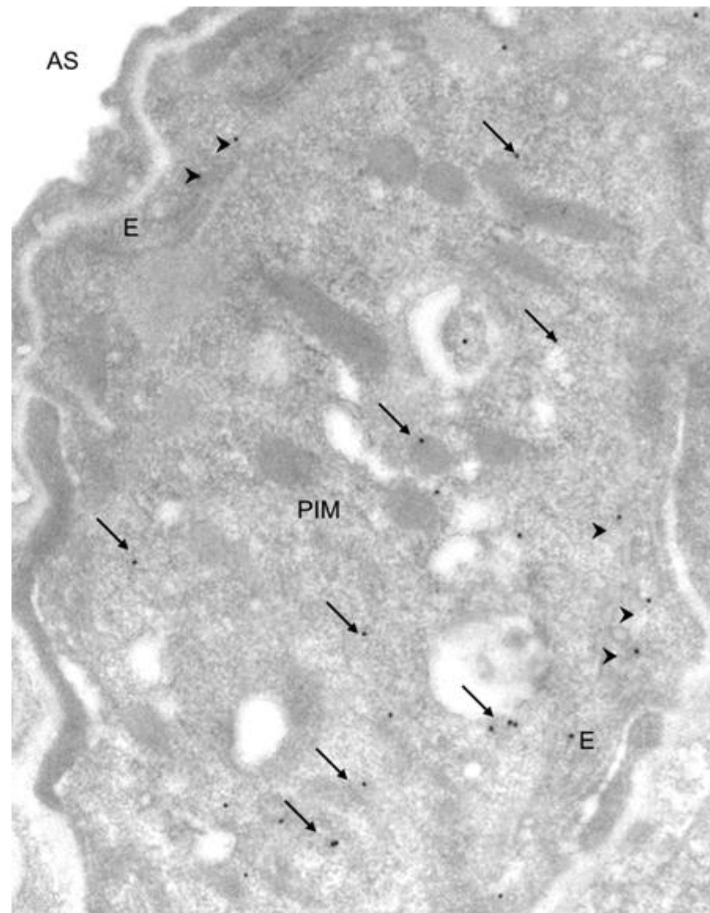

Figure 3.4: TLR9 staining in a calf pulmonary intravascular macrophage.

TLR9 staining (arrows) observed in a pulmonary intravascular macrophage (PIM). Endothelium (E) also shows TLR9 staining (arrowheads). AS: Alveolar Space; Original magnification 15200X.

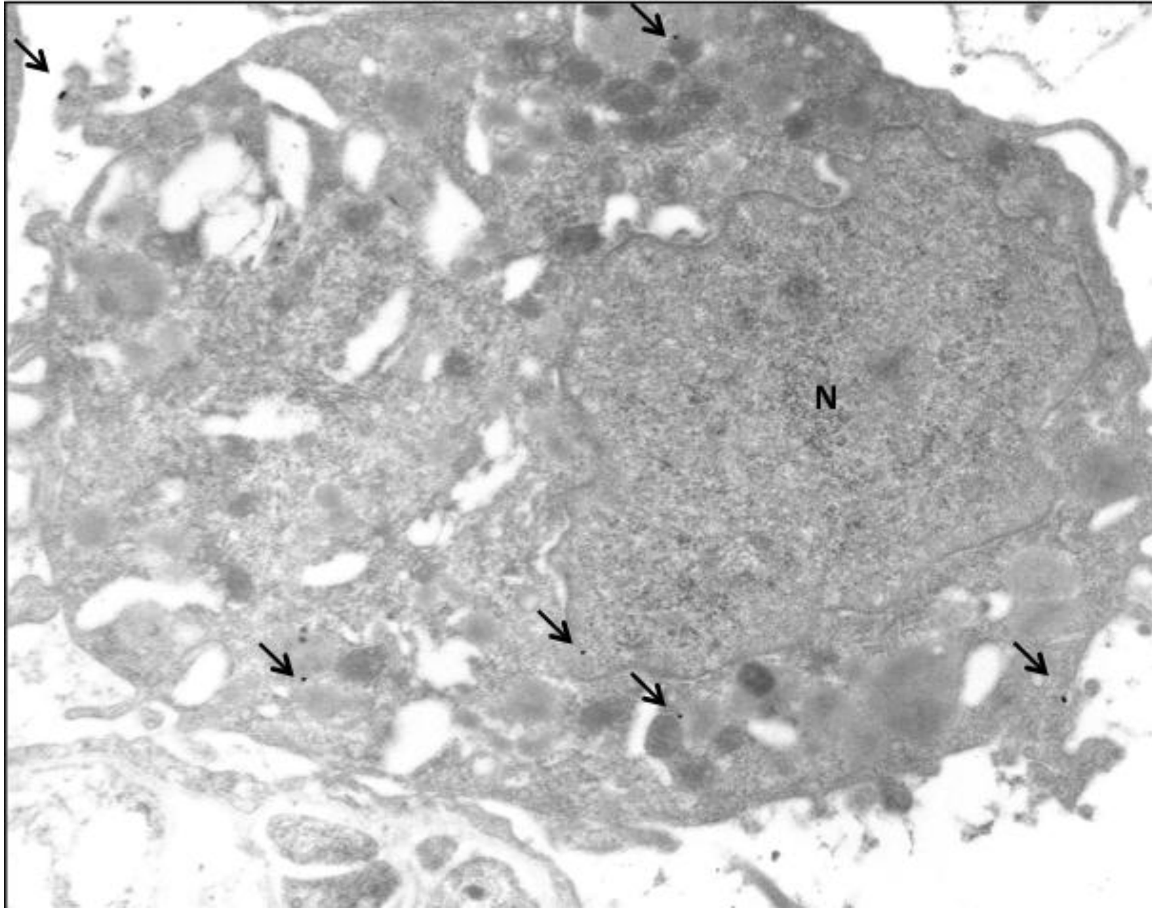

Figure 3.5: TLR9 staining in a dog alveolar macrophage.

TLR9 staining (arrows) observed in an alveolar macrophage. Original magnification 10000X.

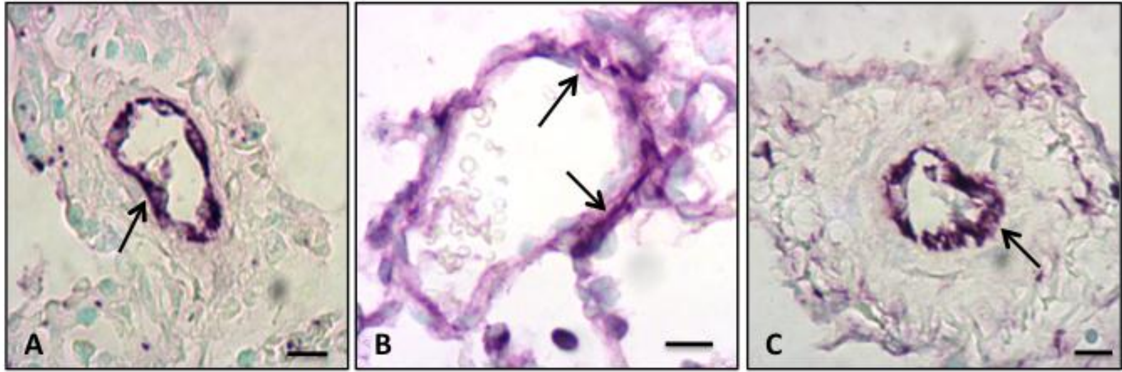

Figure 3.6: TLR9 staining in vascular endothelium.

TLR9 staining (arrows) observed in vascular endothelium of calf (A), dog (B), and pig (C) lung vascular endothelium. Original magnification A-C: 400X. Bars 0.01mm.

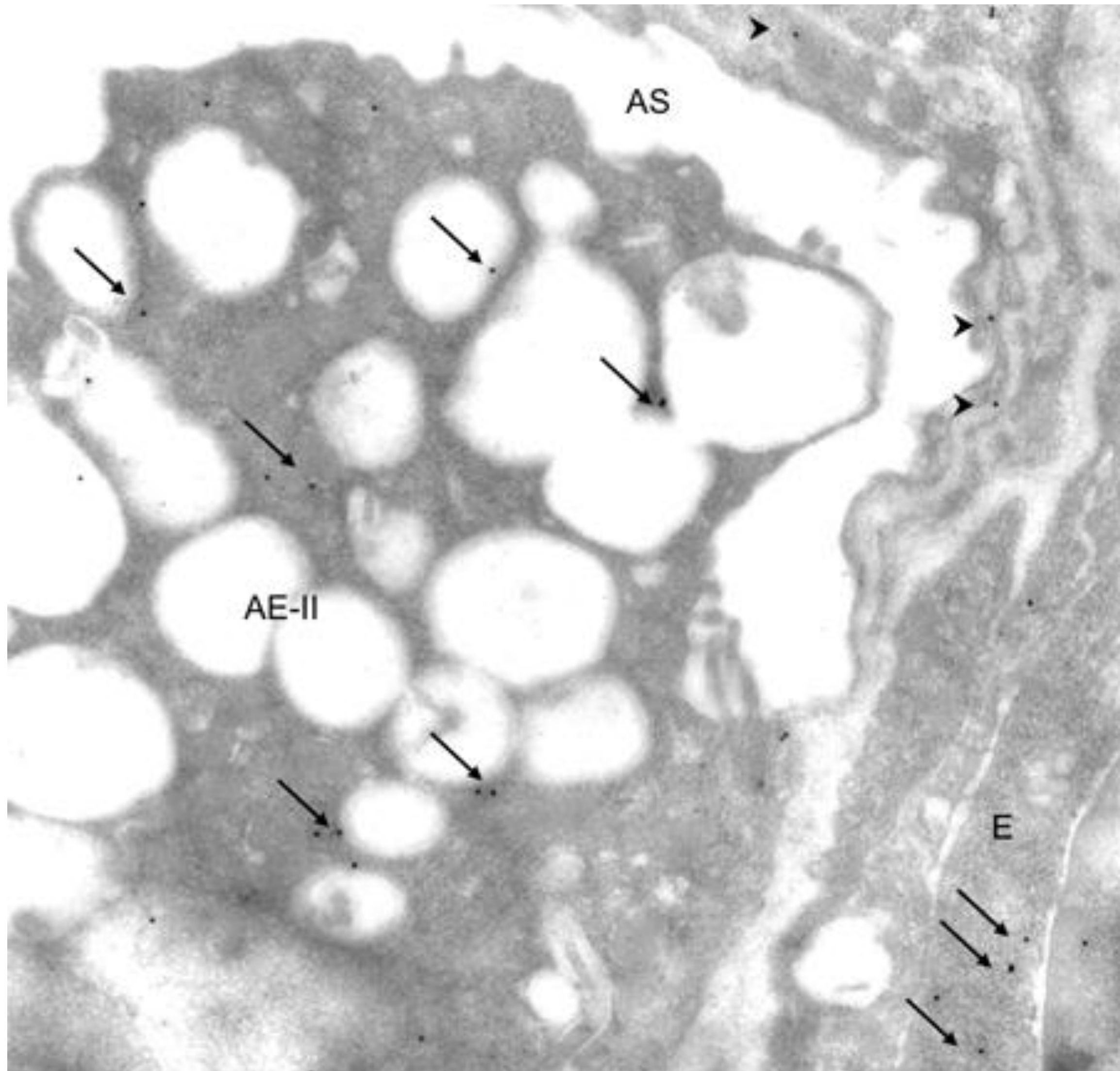

Figure 3.7: TLR9 staining in alveolar epithelium.

TLR9 staining (arrows) is observed in type II (AE-II; arrows), type I (arrowheads) epithelial cells in a calf lung. Endothelium (E) also shows staining (arrows) for TLR9. AS: Alveolar space. Original magnification 13000X.

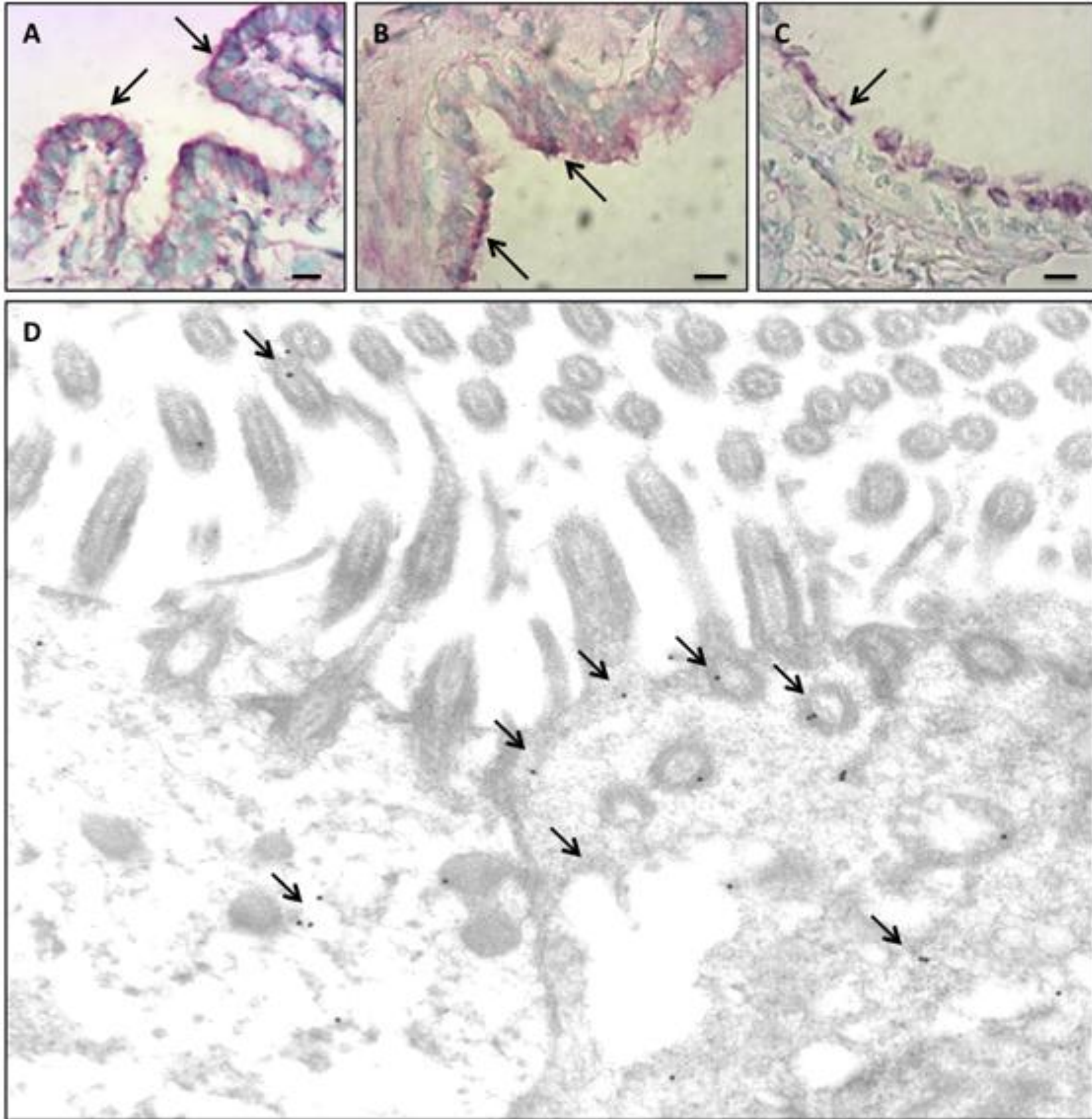

Figure 3.8: TLR9 staining in a bronchiolar epithelium.

TLR9 staining (arrows) observed in vascular epithelium of calf (A, D), dog (B), and pig (C). Staining was seen to be much lighter and more restricted to the bronchiolar surface within the dog. Immuno-gold micrograph (D) shows TLR9 is seen in the cytoplasm, cell surface and also cilia of the airway epithelium in a calf lung. Original magnification A-C: 400X, D: 13000X. Bars 0.01mm.

3.5. Discussion

We report data on TLR9 protein expression from normal intact lungs of cattle, pig and dog, and inflamed lungs from cattle. To our knowledge, these are the first cell specific *in situ* data on the expression of TLR9 lungs of these species. We show that TLR9 is expressed in airway epithelium, vascular endothelium and macrophages in the lungs of cattle, pig and dog. In addition, PIMs of cattle show TLR9. We also report characterization of a mouse TLR9 antibody for use in cattle, pig and dog. Because bacterial infections remain major causes of morbidity, mortality and economic losses in domestic animal species, these data on the cell-specific expression of TLR9 will provide a better understanding of cell specific responses to bacterial components in these species.

Disease, mortality and economic losses caused by bacterial infection are major problems in many veterinary species. As has been shown TLRs can play a role in proper bacterial clearance (Bafica et al., 2005) as well inflammation (Singh et al., 2004), so the presence and localization of these receptors may significantly impact how different species respond to such infections. Furthermore, since distinct cell types may play dominant and differing roles in lung inflammation, it is important to know cell-specific expression of TLR9. So far, most of the data on TLR9 expression has come from isolated and cultured cells (Demedts et al., 2006; Jozsef et al., 2004; Platz et al., 2004; Schwartz et al., 1997; Yeo et al., 2003). Recently, we reported TLR9 expression in normal and inflamed horse lungs and showed that endotoxin treatment significantly increased TLR9 (Schneberger et al., 2009).

First, western blotting was done to confirm that the commercial IMG-305a mouse anti-human TLR9 antibody detects a band of approximately 110kD which corresponds to the known molecular weight of TLR9. The sequence of the peptide against which the human TLR9

antibody was raised also showed 53% to 82% homology with the amino acid sequence of TLR9 in cattle, pig, dog and mouse. This same antibody was used by us to detect TLR9 in horse lung (Schneberger et al., 2009), and by others in dog (Burgener & Jungi, 2008).

Light and electron microscopic immunocytochemistry showed TLR9 staining in alveolar septa of all three species. The role of septal vasculature in the lung in the engagement of blood-borne bacteria and their products such as endotoxins and engendering of subsequent inflammatory response is well established (Andonegui et al., 2003). The localization of TLR9 in lung capillary endothelium will enable these cells to induce responses to circulating CpG in bacterial DNA (Li et al., 2004). Prior to entry of bacteria and their products into the blood, inhalation is the most common route of entry of microbes such as *M. hemolytica* into the lungs of animals. Therefore, presence of innate immune receptors such as TLRs is critical in evoking an anti-microbial defense response. In this context, the mucosal TLR9 will assume an important role in protection of lungs. Similar to the epithelium in the airways, the mucosal barrier of the gut and oral cavity expresses TLR9 (Eaton-Bassiri et al., 2004; Ewaschuk et al., 2007; Shimosato et al., 2005). Therefore, airway epithelial and vascular endothelial TLR9 in cattle, pig and lung may have important roles in protection and may serve as targets for CpG-based immune modulation (Kline et al., 1998).

Macrophages play a central role in orchestration of immune responses. Alveolar macrophages phagocytose inhaled bacteria and other particulate matter to keep the alveolar epithelium clean. Although alveolar macrophages also express TLRs, their responses may be anti or pro-inflammatory dependant on a variety of factors. Some animal species such as horse, cattle, and goat contain a unique population of resident vascular macrophages called pulmonary intravascular macrophages, which express TLR4 and generally play pro-inflammatory roles in

lung inflammation (Aharonson-Raz & Singh, 2010; Singh et al., 2004). From our work in horses we have determined that these cells express TLR9 and that gadolinium chloride depletion of them abrogates all increases in TLR9 expression in the lung in response to LPS (Schneberger et al., 2009). This is predicted to be due to a combination of reduction of TLR9-positive cell migration as well as depletion of TLR9-positive PIMs. We would expect a similar outcome in cattle as both have significant PIM populations within their lungs, and show expression of TLR4 and TLR9 within these cells (Singh et al., 2004; Wassef et al., 2004).

CHAPTER 4: EXPRESSION OF TOLL-LIKE RECEPTOR 9 IN HORSE LUNGS*

4.1. Abstract

Toll-like receptor 9 has been found to be the main receptor used for responding to bacterial DNA in a wide variety of species. Recent work has shown that this receptor is further expressed in a diverse set of cells within the lung. However, much of this data has been centered on human and mouse cell culture lines or primary cultures and very little is known of TLR9 expression in intact lung especially that of the horse. We hypothesized that expression would be similar across other mammalian species. Here we show that TLR9 is expressed in the lungs of horses in a wide variety of cells. In particular we note expression in pulmonary intravascular macrophages (PIMs), alveolar macrophages, bronchial epithelial cells, and type-II cells amongst others. The data also show *E. coli* lipopolysaccharide increased expression of TLR9 mRNA in lungs as well as protein expression in airway epithelium, vascular endothelium and inflammatory cells in blood vessels. Immunogold electron microscopy localized TLR9 in nuclei, cytoplasm and plasma membrane of various lung cells. We conclude that TLR9 is expressed in lung cells including PIMs and that LPS treatment increases TLR9 expression.

4.2. Introduction

In recent years bacterial DNA has been deduced to be an important non-specific immune activating molecule. While most work has centered around use of bacterial DNA as a immune system adjuvant to vaccines (Rankin et al., 2002; Zimmermann et al., 2008) it has also been

shown to be critical in containment of infections such as *Mycobacterium tuberculosis* (Bafica et al., 2005; Rutz et al., 2004), *Propionibacterium acnes* (Kalis et al., 2005), and *Legionella pneumonia* (Bhan et al., 2008). Detection of this bacterial DNA is mediated through TLR9 (Hemmi et al., 2000).

TLR9 is a member of the Toll-like receptor family which has been described in a wide-variety of animal species (Rankin et al., 2001; Roach et al., 2005). The receptor binds a particular C-G DNA sequence which is methylated in vertebrate DNA but not in bacteria or viruses (Hemmi et al., 2000; Krieg et al., 1995; Krieg, 2002; Kuramoto et al., 1992). Ligation and signaling through the receptor results in expression of NF-κB and consequent expression of a range of cytokines, many associated with what could be considered TH1-like responses (Krieg, 2002). Much work has been done to develop CpG oligonucleotides for vaccines, adjuvants, and therapy for conditions such as asthma or endotoxemia (Kline et al., 1998; Rankin et al., 2001; Schwartz et al., 1999). Only recently has the receptor been examined as a possible factor in environmental bacterial exposure (Roy et al., 2003). The role of TLR9 in lung inflammation is not clear as the receptor has been implicated in lung inflammation (Knuefermann et al., 2007; Schwartz et al., 1997), prevention of inflammation (Parilla et al., 2006), and modifying inflammatory responses to LPS (Schwartz et al., 1999) and asthma (Kline et al., 1998).

Innate receptors such as TLR9 can determine the role of specific cells in engendering immune response in an organ and are promoted as novel targets for drugs and vaccines (Barrat et al., 2007; Dorn & Kippenberger, 2008; Kitagaki et al., 2006; Yasuda et al., 2008). Therefore, it is important to have a precise and detailed description of expression of TLRs in normal organs. Much of the data on TLR9 expression has been obtained in mouse and human systems and cell

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* Portions from The Anatomical Record (Schneberger et al 2009)

cultures from the same. The data from these experiments shows that TLR9 is expressed in a wide variety of cells such as B-cells (Krieg et al., 1995) eosinophils (Wong et al., 2007), neutrophils (Jozsef et al., 2004; O'Mahony et al., 2008; Schwartz et al., 1997), dendritic cells (Demedts et al., 2006), macrophage (O'Mahony et al., 2008; Yeo et al., 2003), bronchial epithelial cells (Platz et al., 2004), and type-I and type-II lung cells (Li et al., 2004), amongst others. In fact, it appears that many cells of the lung express TLR9 to some degree, though for alveolar macrophage the evidence is mixed (Fernandez et al., 2004; Suzuki et al., 2005). Although the receptor is highly conserved (Rankin et al., 2001; Roach et al., 2005), there are a number of differences between species with regards to optimal CpG signaling (Demedts et al., 2006; Rankin et al., 2001) and expression. One of the most notable of these is expression of TLR9 on plasmacytoid but not myeloid dendritic cells in humans, whereas mouse lung dendritic cells do not express the receptor at all (Chen et al., 2006; Demedts et al., 2006). Therefore it is important to determine the expression characteristics of TLR9 in each species.

To our knowledge there are no data on the expression of TLR9 in the horse lung other than a basic comparison of mRNA in tissue versus other organs (Zhang et al., 2008). Given the susceptibility of horses to LPS-induced cardiopulmonary distress and lung inflammation (Morris, 1991) it is apparent that they are sensitive to bacterial infection of the lung, and have already been shown to express TLR2 and TLR4 (Singh Suri et al., 2006). The horse also has an unique population of pulmonary intravascular macrophages (PIMs) which have significant expression of TLR4 (Singh Suri et al., 2006). Therefore we set out to examine the expression of TLR9 in whole horse lung sections to survey which cells express TLR9, and to also look at its subcellular expression. Further, we wanted to look at if the expression levels of TLR9 were altered upon challenge with LPS or after depletion of the pulmonary intravascular macrophage to see if this

unique population of macrophages contributed to potential TLR9-mediated responses. Now, we report TLR9 mRNA and protein expression in lungs of normal and LPS-treated horses.

4.3. Materials and Methods

4.3.1. Animals

Animals used for this experiment have been previously described (Singh Suri et al., 2006). Briefly, horses were treated either with saline, saline with *Escherichia coli* lipopolysaccharide (LPS) or pretreated with gadolinium chloride before LPS treatment or gadolinium chloride alone.

At the end of this time horses were euthanized and the lungs were processed for light and electron microscopy as described elsewhere (Parbhakar et al., 2004).

Two mouse spleens (C57BL/6) were kindly provided to us by Dr. Sylvia vanDen Hurk to use for mouse protein extracts.

4.3.2. Western Blotting

Horse lung and mouse spleen samples were lysed in 200µl of freshly prepared lysis buffer (150mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris (pH 8.0), 5 mM ethylenediaminetetraacetic acid (EDTA), and protease inhibitor cocktail (100µl/10ml; Sigma-Aldrich). Proteins were separated on a 10% polyacrylamide gel and transferred on to Hybond-ECL nitrocellulose membrane (GE Bioscience, Germany). Membranes were blocked with 5% skim milk in phosphate buffer saline (PBS ; pH 7.4) and incubated with TLR9 antibody (IMG-305A, Imgenex, San Diego, CA, USA) for 2 hours at room temperature before washing and incubation for 1 hour with goat anti-mouse HRP

(P0447, Dako, Ontario, Canada). Blots were developed using the EC Western Blotting Detection kit (GE Bioscience, UK) and exposed to Hyperfilm ECL (GE Bioscience, UK) film.

4.3.3. Immunohistology and Immuno-electron Microscopy

Immunohistology was done with mouse anti-human TLR9 antibody on both LPS and saline treated horse lung tissue. Briefly, after de-paraffinization, re-hydration, tissue peroxidase quenching (0.5% hydrogen peroxide in methanol), and antigen unmasking with pepsin (2mg/ml 0.01N hydrochloric acid), the tissue sections were blocked with 1% BSA to block non-specific binding for 1 hour. Sections were treated with TLR9 antibody (1:50 dilution) and incubated overnight at 4^oC. The next day horseradish peroxidase-conjugated goat anti-mouse antibody was added at 1:75 dilution to sections for 1hr at 37^oC (P0447, Dako, Ontario, Canada). Color was developed using a color developing kit (Vector Laboratories, Ontario, Canada). Slides were counterstained with methyl green (Vector Laboratories) prior to mounting. A control was similarly run with omission of the primary antibody or the secondary antibody.

For immuno-electronmicroscopy thin lung sections were placed on nickel grids and floated in blocking buffer for 30min prior to 1hr incubation with TLR9 antibody at 1:10 dilution. Sections were rinsed 3 times in Tris-buffered saline for 5 minutes before addition and incubation with 20nm gold-conjugated anti-mouse secondary antibody (1:100 dilution goat anti-mouse IgG EMGAM20, Fitzgerald Industries International, Concord, MA, USA) for 1hr. A control with omission of the primary antibody was also run.

4.3.4. Real Time Reverse-transcriptase Polymerase Chain Reaction

Quantitative real time reverse-transcriptase polymerase chain reaction (RtPCR) was performed on purified horse lung mRNA from animals treated with LPS, gadolinium chloride, gadolinium chloride plus LPS, or left untreated as described earlier. RNA was purified using the RNease mini kit followed by RNase-free DNase (Qiagen, Mississauga, ON, Canada). RNA integrity was confirmed by agarose gel electrophoresis and quantified Nano drop spectrophotometery (Thermo Fisher Scientific, Ottawan ON). The mRNA was reverse transcribed using the QuantiTect reverse transcription kit (Qiagen) with a mixture of universal oligo dT and random primers as per manufacturer's instructions. The cDNA generated by this methods was used for RtPCR analysis of the expression of TLR9 (GenBank Accession No. DQ157779) using QuantiFast SYBR Green PCR kit (Qiagen, Canada). The glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH; GenBank Accession No. AF157626) was used as the reference housekeeping gene. The reactions were performed using the primer pairs; 5'-ATTACCTGGCCTTCTTCAATTG-3' and 5'-CTGCCATTGCTCAGAGCCTTC-3' for TLR9, and 5'-TCACCATCTTCCAGGAG-3' and 5'-GTCTTCTGGGTGGCAG-3' for GAPDH. The negative control consisted of all the components of the reaction mixture excepting RNA. Real-Time PCR analysis was performed using the MX3005P LightCycler (Stratagene, La Jolla, CA, USA) as per manufacturer's instruction. The cDNA was denatured at 5 minutes at 95°C, followed by amplification of target DNA through 45 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds and elongation at 60°C for 45 seconds. Relative expression levels were calculated after correction for expression of GAPDH using MxPro software.

4.3.5. Statistical Analysis

All values presented were given as the mean ± standard deviation. We performed a one-way ANOVA to determine significance between control, gadolinium chloride, LPS, and gadolinium chloride plus LPS treated lung tissue mRNA levels.

4.4. Results

4.4.1. Sequence Alignment and Western Blot

Information from the manufacturer stated that the peptide used to raise the mouse anti-human TLR9 antibody was a KLH-conjugated synthetic peptide to amino acid 268-284 of TLR9 isoform A (GenBank assention No. AAF78037.1). This sequence was aligned against a similarly filed TLR9 sequence for mouse and equine TLR9 (GenBank assention No.AAU04981.1 and ABD36388 respectively) using Clustal-W. These results show 58% and 82% homology across the region for mouse and equine TLR9 respectively. (Fig. 4.1). Western blotting of horse lung protein extracts showed a band of approximately 110kDa weight which corresponds to that of extracts from mouse spleen (Fig. 4.1) and results as provided by the manufacturer. Mouse spleen showed a secondary band around 60kDa similar to that shown by the manufacturer.

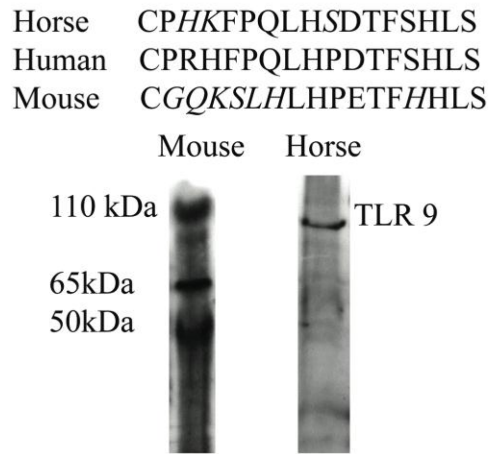

Figure 4.1. Western blot of TLR9 from mouse and horse tissue.

Comparison of amino acid sequences corresponding to that detected by the antibody used. Human (GenBank assention #AAF78037.1) was compared to mouse (GenBank assention # AAU04981.1) and equine TLR 9 (GenBank assention # ABD36388) at amino acids 268-284. 10 out of 14 mouse (58%) and 14 out of 17 (82%) amino acids were shown to match. Protein extracts were isolated from mouse spleen and horse lung tissue and run on a 10% polyacrylamide gel followed by staining with human anti-mouse TLR9 antibody which detected a 110kD band in mouse and horse tissues.

4.4.2. Light and Electron Microscopic Immunocytochemistry

Lung sections stained with only secondary antibody lacked staining (Fig. 4.2A) while von Willebrand Factor antibody stained vascular endothelium but not bronchiolar epithelium (Fig. 4.2B). Lung sections from control (Fig. 4.2C) and LPS-treated (Fig. 4.2D) horses showed TLR9 staining in cells in alveolar septa and the staining was more intense in LPS-treated horses. These septal cells were large mononuclear cells (Fig. 4.2E) and were confirmed to be TLR9 expressing pulmonary intravascular macrophages (PIMs) with electron microscopy (Fig. 4.3). PIMs showed TLR9 labeling in their cytoplasm and nucleus. Alveolar septal endothelial cells (Fig. 4.3), neutrophils in lung capillaries and type-II epithelial cells of alveolar septa (Fig. 4.4) also showed TLR9 expression.

TLR9 staining was observed in vascular endothelium in lung sections from control (Fig. 4.5A) and LPS-treated (Fig. 4.5B) horses. One of the dramatic finding was accumulation of TLR9-positive inflammatory cells in pulmonary blood vessels of LPS-treated horses (Fig. 4.5B). Immuno-electron microscopy confirmed TLR9 staining of lung vascular endothelium as well as presence of TLR9 in the nucleus of endothelial cells (Fig. 4.5C).

Airway epithelium in control horses (Fig. 4.6A) showed light staining for TLR9 compared to the intense reaction in the airway epithelium of LPS-treated horses (Fig. 4.6B). Immuno-electron microscopy localized TLR9 staining to the surface, cytoplasm and nucleus of airway epithelial cells (Fig. 4.6C). Alveolar macrophages showed TLR9 expression in their cytoplasm and nucleus (Fig. 4.7).

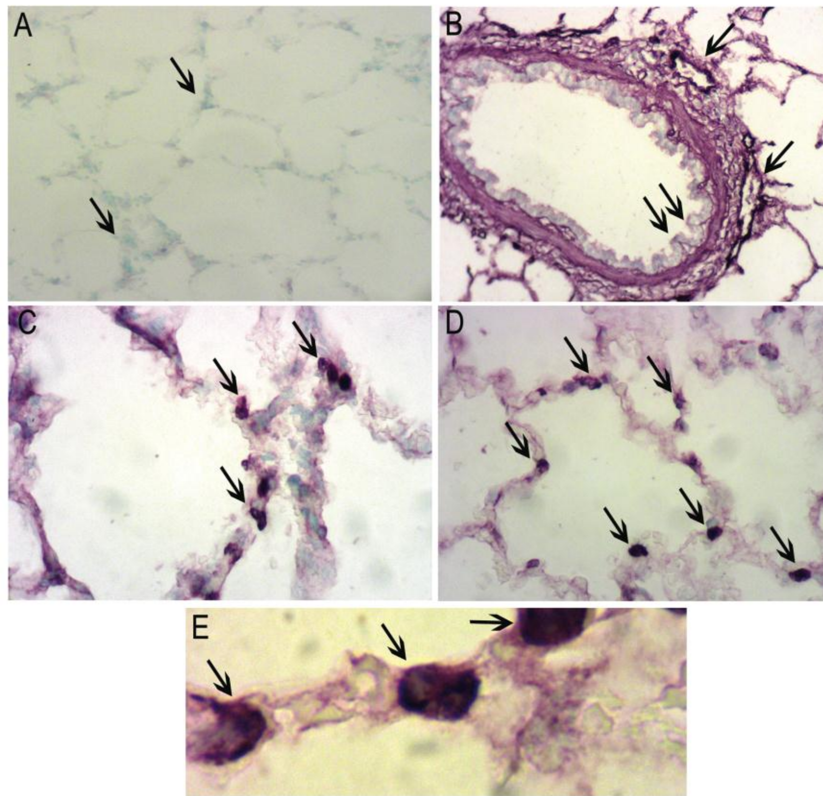

Figure 4.2. TLR9 immunohistochemistry.

Lung section from a control horse stained with only a secondary antibody (A) lacks staining (arrows) in alveolar septa whereas the one stained with vWF antibody (B) shows staining in endothelium (single arrows) but in bronchiolar epithelium (double arrows). Lung sections from control (Figure C) and an LPS-treated (D) horses shows TLR9 staining (arrows). High magnification view (E) shows staining in large macrophage-like (arrows) in alveolar septa of LPS-treated horse. Original magnification A,B: 100X, C-D: 400X, E: 1000X.

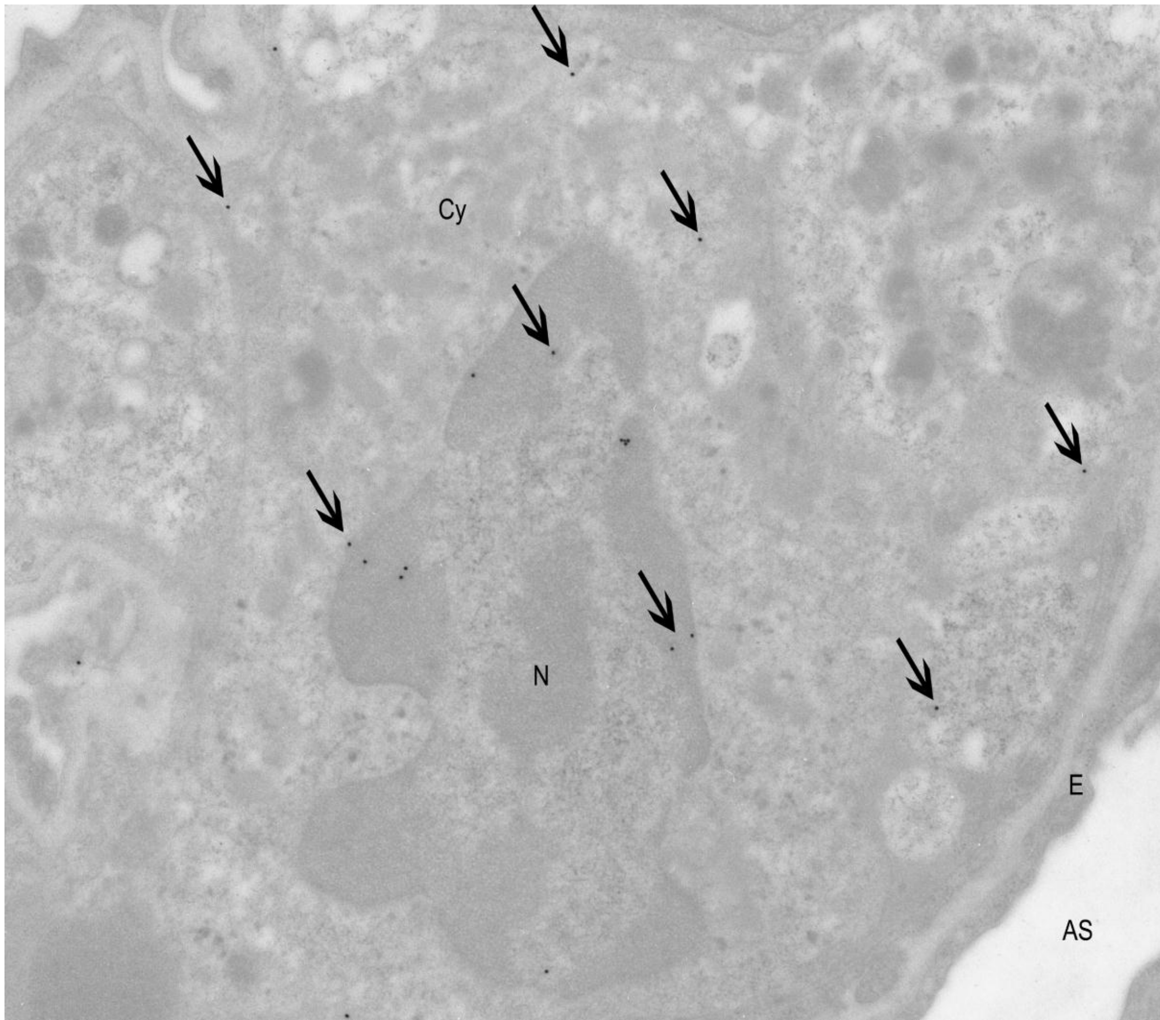

Figure 4.3. TLR9 staining in a pulmonary intravascular macrophage.

TLR9 staining (arrows) observed in the cytoplasm (Cy) and nucleus (N) of a PIM. E: Epithelium; AS: Alveolar Space. Original magnification A-C: 10000X; D 7500X.

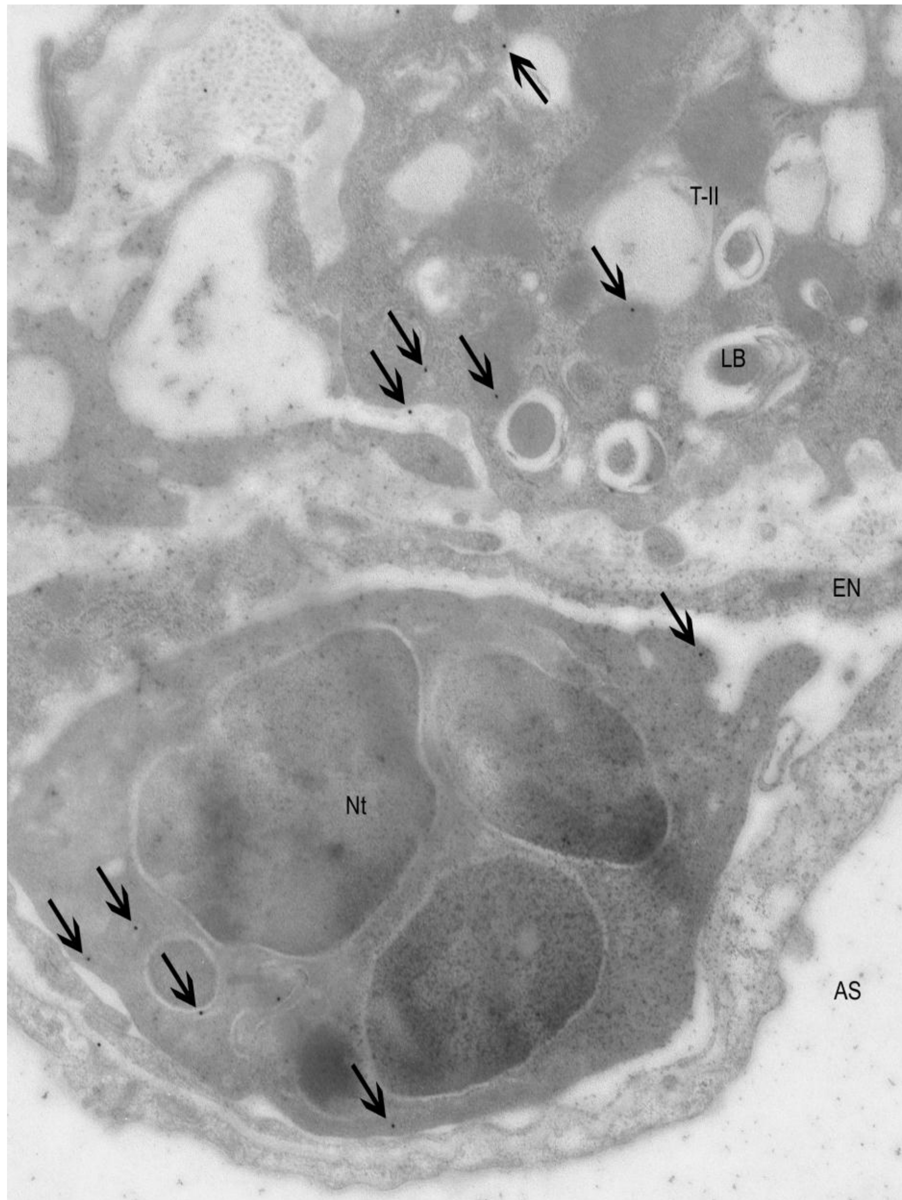

Figure 4.4: TLR9 staining in a neutrophil and type II alveolar epithelial cell.

TLR9 staining (arrows) is seen in cytoplasm but not in lamellar bodies (LB) of type-II alveolar epithelial cell (T-II). Neutrophil (Nt) in alveolar capillary is also positive for TLR9 (arrows). AS: alveolar space; En: endothelium. Original magnification 10000X.

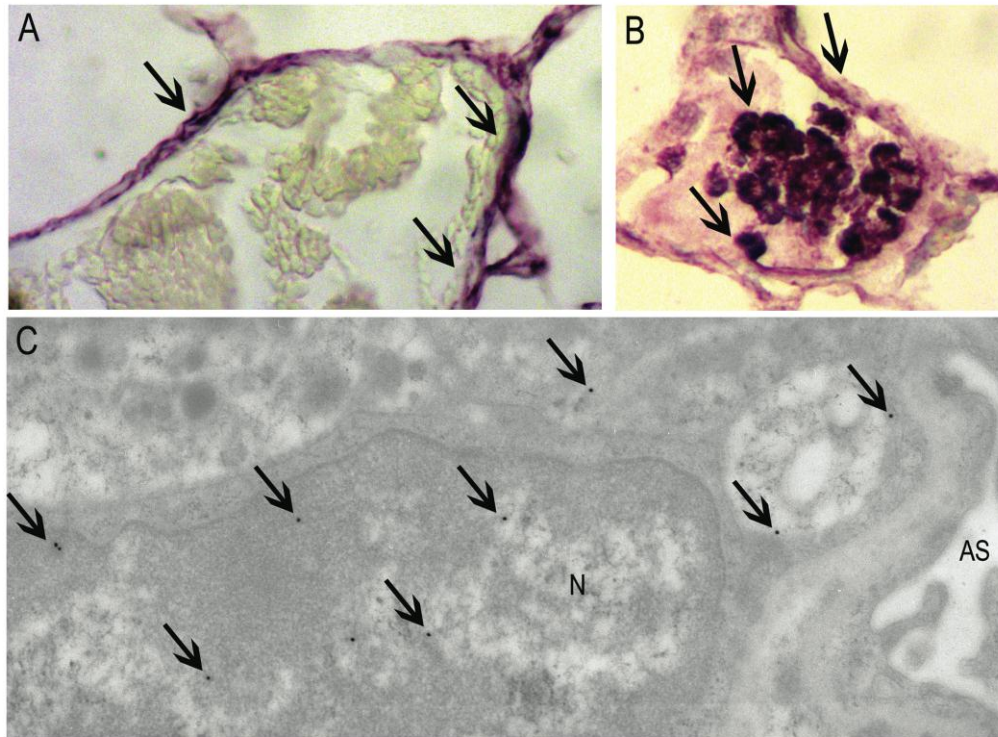

Figure 4.5: TLR9 vascular immunostaining.

TLR9 staining was vascular endothelium in lungs of control (A) and LPS-treated horses (B). Immuno-electron microscopy (C) showed TLR9 staining in nucleus (N) of capillary endothelial cells in the lung. . Note accumulation of inflammatory cells (double arrows) positive for TLR9 in the lumen of blood vessel in lung section from the LPS-treated horse (B). Original magnification A-B: 400X, C: 10000X.

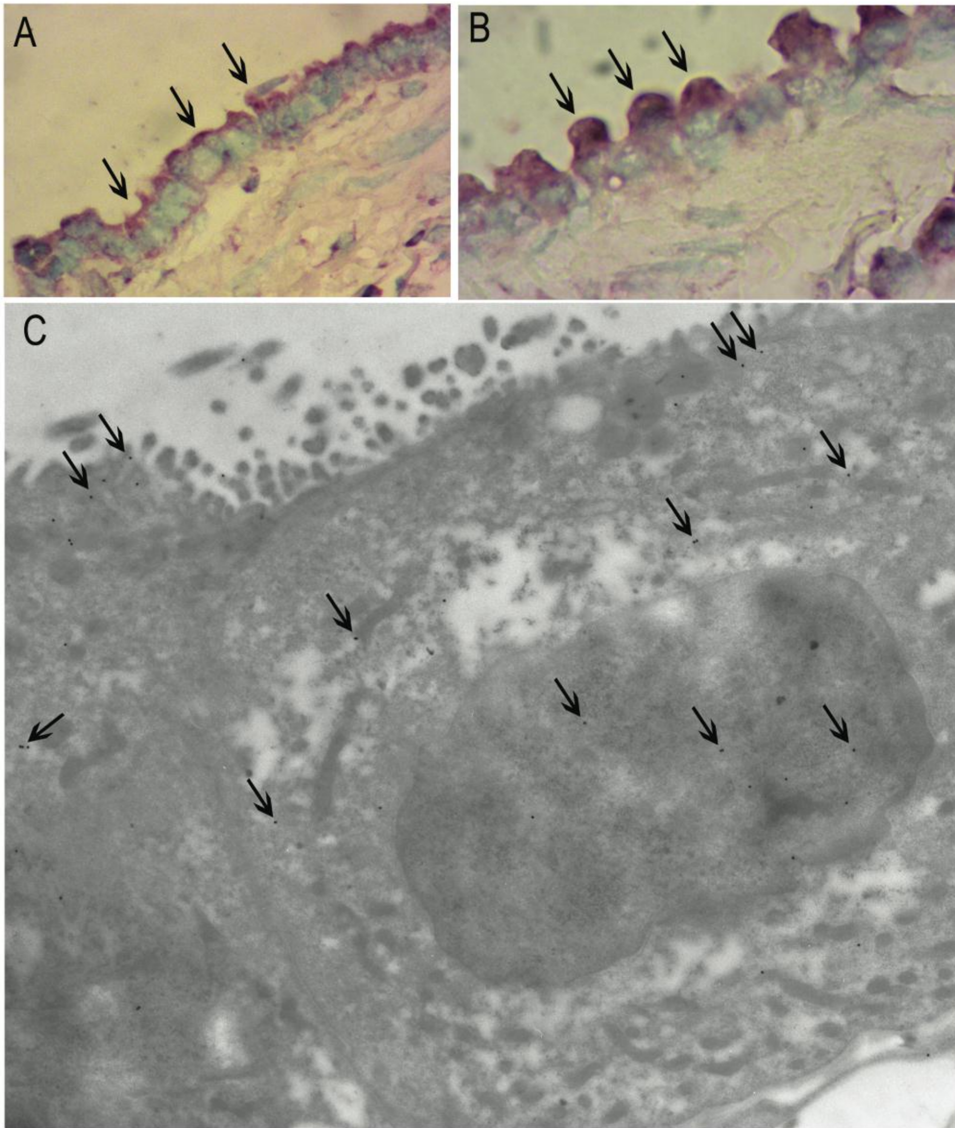

Figure 4.6: TLR9 staining in an airway epithelium.

TLR9 staining (arrows) is seen on bronchiolar epithelium in lung section from control (A) and LPS-treated (B) horses. Staining is more intense in lung section from LPS-treated horse. Immuno-EM shows TLR9 labeling on the surface (double arrows) as well as in cytoplasm and nucleus (single arrows) of a bronchiolar epithelial cell in a lung section from LPS-treated horse. Original magnification 10000X.

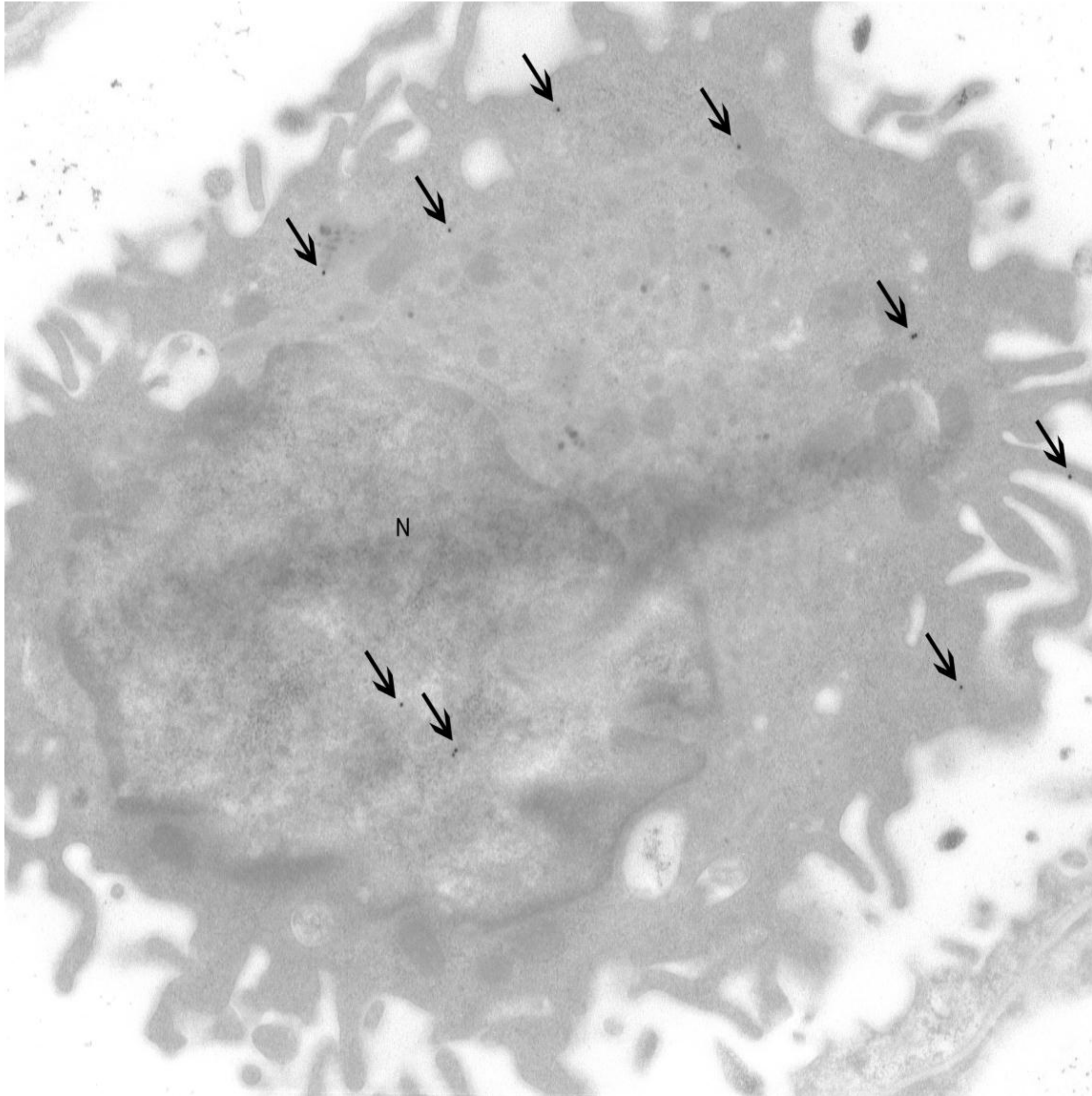

Figure 4.7. TLR9 staining in an alveolar macrophage.

TLR9 staining (arrows) is seen in cytoplasm and nucleus (N) of an alveolar macrophage. Original magnification 10000X.

4.4.3. Real Time Reverse-transcriptase Polymerase Chain Reaction

Our results showed that LPS treatment increased TLR9 mRNA expression in the lung compared to that observed in normal horses without ($p<0.001$) or with treatment with gadolinium chloride ($p=0.007$). However, pre-treatment with gadolinium chloride before LPS treatment reduced TLR9 mRNA expression compared to LPS-treated horses ($p<0.001$) and brought the expression to a level below that of the control group (Fig. 4.8).

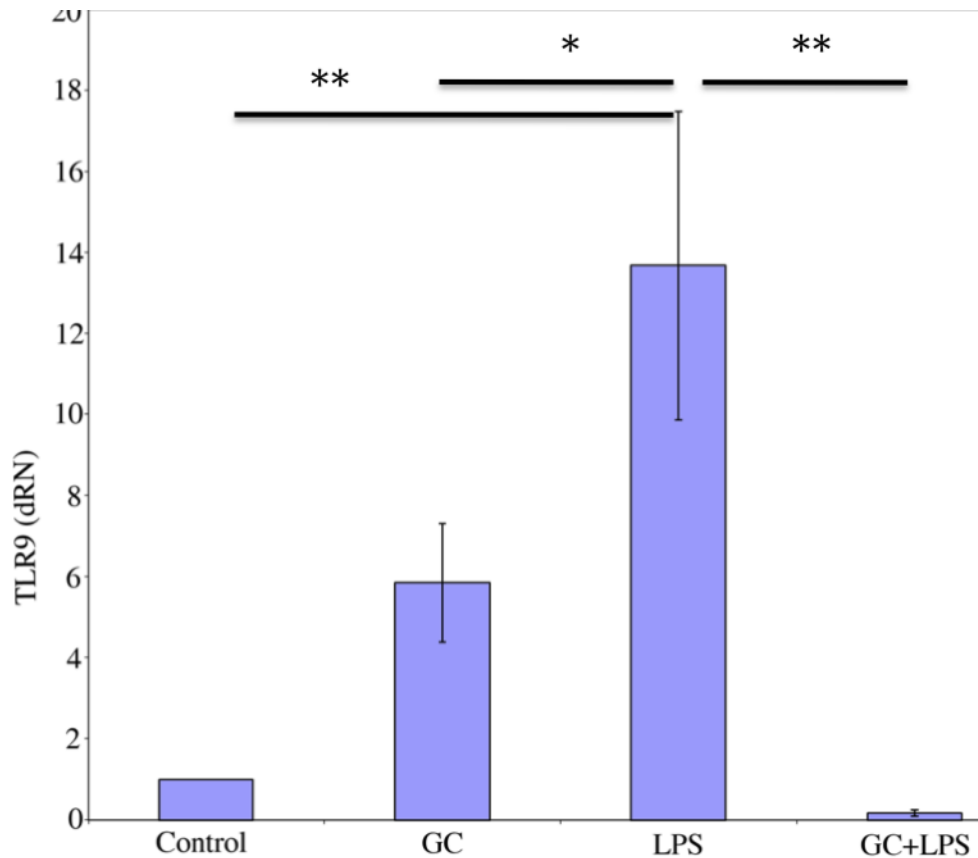

Figure 4.8: TLR9 mRNA of LPS and gadolinium chloride treated horse lungs.

mRNA was isolated from horses treated with saline (Control), gadolinium chloride (GC), LPS (LPS), or gadolinium chloride plus LPS (GC+LPS) (n=3, all groups) . Results are standardized to saline treated animals and given as fold increase over saline mRNA levels. All results are given as averages ± standard deviation. Statistical analysis was done using one-way ANOVA to determine significance (*= $p \leq 0.05$, **= $p < 0.001$).

4.5. Discussion

We previously reported expression of TLR4 and TLR2 in horse lungs to understand mechanisms of their higher sensitivity to bacterial lung diseases (Singh Suri et al., 2006). Lung infections caused by any virus or bacterium however will be expected to introduce non-methylated DNA into the lung, making the identification of TLR9 in the lung an important factor in our understanding of early non-specific immunity to various challenges in the horse. To our knowledge, we report the first *in situ* data on the protein expression of TLR9 in the normal and LPS-treated horse lung. These data have added broader significance because much of the data available for the more studied mouse and human systems has been derived from cell lines and primary cell cultures and there is a paucity of *in situ* cell-specific data on TLR9 expression.

First, we selected and characterized an antibody to recognize the equine TLR9. TLR9 is highly conserved between species and the epitope to which the antibody has been raised was found to be only 58% homologous between human and mouse and 82% homologous between human and equine, with matches of 14 out of 17 amino acids using a Clustal-W comparison of the human and horse amino acid sequences. Since beginning our study, Zhang and colleagues have published their findings confirming that the mouse anti-human antibody clone employed in our work does cross-react with equine TLR9 (Zhang et al., 2008), and can recognize TLR9 on cells such as macrophages and neutrophils, which is consistent with our findings. Western blots of protein extracts from mouse spleen show that the antibody binds to a protein of approximately 110kDa, which corresponds to the size of the TLR9 receptor. Protein extracts from horse lung showed a band slightly lower than that from mouse spleen (about 113kDa), well within the range expected for this protein depending on the species and tissue used (Tran et al., 2007). These data show that anti-human antibody used in our experiment cross reacts with the equine TLR9.

Light and electron microscopic immunocytochemistry showed strong expression of TLR9 in PIMs, alveolar macrophages, neutrophils, bronchial epithelial cells, and alveolar epithelial cells. It appeared that type-I epithelial cells showed low level of TLR9 protein expression. These first results from horse lung are similar to those seen in mouse and human cells including neutrophils (Jozsef et al., 2004; O'Mahony et al., 2008; Schwartz et al., 1997), macrophage (O'Mahony et al., 2008; Yeo et al., 2003), monocytes (O'Mahony et al., 2008), epithelial cells (Li et al., 2004) and columnar epithelial cells (Platz et al., 2004). Our use of immuno-EM to study TLR9 expression in intact lung resulted in localization of TLR9 in type-II epithelial cells and alveolar macrophages of the horse. There has been a controversy regarding TLR9 expression in alveolar macrophages in mice (Fernandez et al., 2004; Suzuki et al., 2005). The reasons for these differences are not apparent but could be an outcome of exposure to environmental dust and endotoxin present in stables (Berndt et al., 2007). While TLR9 expression in alveolar macrophages may point to a greater susceptibility or ability of horses to respond to bacterial DNA, the function of TLR9 in type-II alveolar epithelial cells is open to speculation.

One of the interesting findings was TLR9 staining in PIMs and robust contributions of PIMs to total TLR9 expression in the lung. PIMs are present in septal capillaries of horses, ruminants and pigs (Berndt et al., 2007; Chitko-McKown et al., 1991; Parbhakar et al., 2004; Parbhakar et al., 2005; Singh Suri et al., 2006; Singh et al., 2004; Staub, 1994). Equine PIMs express TLR4 and TLR2, endocytose LPS and express proinflammatory cytokines (Singh Suri et al., 2006). In fact, PIM depletion results in reduced amounts of TLR4 and TLR2 mRNA as well as inhibition of lung inflammation in horses upon challenge with endotoxin (Parbhakar et al., 2004). The expression of TLR9 in PIMs is unique and highly intriguing. First, TLR9 is generally

observed in epithelial cells such as those in the airways and the intestine (Ewaschuk et al., 2007; Schwartz et al., 1997). Second, PIMs are a population of fixed macrophages in lung capillaries which express TLR4 to handle bacterial endotoxins (Singh Suri et al., 2006). The presence of TLR9 in the same cells will enable them to respond to bacterial DNA released upon their degradation in lysosomes. This scenario creates tantalizing possibilities of targeting PIMs with CpG adjuvants or inhibitors to reduce, enhance, or modulate immune responses of these cells, and overall lung response. Although it is generally believed that humans do not have PIMs but these assumptions are based on only a couple of studies and require further investigations in lungs of normal or those suffering from respiratory diseases such as asthma or acute respiratory distress syndrome (Staub, 1994). Nevertheless, the biology of TLR9 in horse lungs needs further investigation.

The immuno-EM also helped in precise subcellular localization of TLR9 in various lung cells. Work by others in mice has shown that TLR9 in the cytoplasm, on the endoplasmic reticulum, and associated with lysosomes, where it is believed to interact with the receptor's ligand (Latz et al., 2004; Macfarlane & Manzel, 1998; Parbhakar et al., 2005). More recent work however has shown cell surface expression of TLR9, especially in gut epithelial cells (Ewaschuk et al., 2007) and some human peripheral mononuclear cells (Eaton-Bassiri et al., 2004). We thus confirm our immunohistologic results showing TLR9 can be found in the cytoplasm of these cells, and show the receptor in the nucleus as well as the cell surface. Expression within the cytoplasm is in keeping with reports by others (Latz et al., 2004; Zhang et al., 2008). TLR9 association with the lysosome is often highlighted due to the requirement for acidification of the DNA (pH 6.5) in order to activate TLR9 signaling (Parbhakar et al., 2005; Rutz et al., 2004), and to increase receptor binding and specificity for non-methylated DNA (Rutz et al., 2004). However, robust

presence of TLR9 in the cytoplasm (lysosomes) may be an outcome of membrane endocytosis. Further, under some conditions airway mucus pH may become more acidic, allowing for these conditions to be met on the cell surface similar to the observations made in cell cultures (Hu, Sun, & Zhou, 2003). Expression of TLR9 within the cell has been shown to localize to the endoplasmic reticulum, lysosomes, and cell surface (Eaton-Bassiri et al., 2004; Ewaschuk et al., 2007; Latz et al., 2004) but to our knowledge these are the first results documenting nuclear expression of TLR9.

We examined TLR9 mRNA expression with RtPCR on the lungs of gadolinium chloride and LPS treated horses to determine if the expression of TLR9 is susceptible to PIM depletion (gadolinium chloride) or vascular treatment with LPS. We have previously reported significant reduction in PIM numbers with gadolinium chloride treatment (Parbhakar et al., 2004). As expected, LPS treatment resulted in a significant increase in the level of expressed mRNA as well as TLR9 in airway epithelium, vascular endothelium and inflammatory cells. Our results are consistent with the findings of other investigators showing that TLR4 engagement by LPS is capable of inducing TLR9 expression in mice (An et al., 2002). We believe that increased TLR9 mRNA in LPS-treated horse lungs may be partially due to recruitment of inflammatory cells into inflamed lungs as well as increased expression in resident lung cells. Zhang and colleagues also explained low expression of TLR9 mRNA in horse lungs compared to lymphoid organs as a result of more leukocytes in lymphoid organs compared to the lung (Zhang et al., 2008). Although gadolinium chloride treatment of normal horses did not affect TLR9 mRNA expression, pretreatment with this chemical blocked LPS-induced increase in TLR9 mRNA expression and resulted in TLR9 mRNA expression similar to observed in normal horses. Considering that TLR9 is expressed in many cells including PIMs in the lungs, it is novel that

PIMs alone are major contributors to the amount of TLR9 mRNA present in the lungs. We have previously shown that depletion of PIMs with gadolinium chloride causes significant reduction in the amount of TLR4 as well as recruitment of neutrophils in LPS-treated horses to underscore robust contributions of PIMs to TLR4 expression (Parbhakar et al., 2005; Singh Suri et al., 2006). Because we are reporting TLR9 in neutrophils in horse, it is possible that reduced recruitment of these cells in PIM-depleted LPS-treated horses would have contributed to reduced TLR9 mRNA expression. Taken together, these data show that LPS treatment increases TLR9 expression and that PIMs are major contributors to TLR9 expression in the lung.

In conclusion we show TLR9 expression in the lungs of horses that would enable them to deal with and respond to bacterial DNA. In particular we show that PIMs may be important cells in the response of horses to inhaled bacteria and bacterial products, and that indeed the expression of TLR9 is linked in part to LPS. Other groups have shown complex links in other animal species between severity and character of lung inflammation and co-ordinated TLR4 and TLR9 induction (Fernandez et al., 2004; Hemmi et al., 2000; Yeo et al., 2003). Therefore elucidating the role of TLR9 in horse lung inflammation will be vital to our understanding of how the lung responds to bacterial challenge.

CHAPTER 5: EXPRESSION OF TLR9 IN MOUSE AND HUMAN LUNGS

5.1. Abstract

Toll-like receptors recognize conserved molecular motifs of microorganisms, and constitute an important part of the innate immune system. Numerous studies have shown the importance of these receptors in establishing effective immune responses to a broad range of infections. TLRs have also been implicated in disorders such as COPD. TLR9, responsible for detection of unmethylated DNA, is expressed in a wide range of immune cells in mice, humans, as well as other species. However, much of this work has centered on cell cultures, and isolated cell populations. We hypothesized that expression pattern for TLR9 in human and mouse lungs would be similar to that seen in the lungs of other species tested so far. We determined the *in situ* expression of TLR9 in whole mouse and human lungs. We used immunohistochemical, *in situ* hybridization (ISH), and immunoelectron microscopy to localize TLR9 in bronchial epithelium, vascular endothelium, alveolar septal cells and alveolar macrophages in both species. We further show that in cases of asthma in humans there is an apparent influx of TLR9-positive cells to the lung. We conclude that both immune and non-immune cells in the lung express TLR9 which may contribute to pulmonary immune responses.

5.2 Introduction

The innate immune system plays important role in protecting mucosal surfaces. The TLRs are one of the most studied members of the innate immune system. The TLRs expressed on endothelium, epithelium and immune cells regulate the induction of inflammation and protection

against pathogens. In tissues such as the lung TLR4 has been implicated in reduced airway response in farm workers (Senthilselvan et al., 2009), and in the clearance of numerous pathogens (Bhan et al., 2008; Kalis et al., 2005; Rutz et al., 2004). While TLR4 in relation to endotoxin-induced inflammation has been studied the most, other receptors such as TLR2 and TLR9 are also potentially important molecules. Interestingly, the effects of such receptors on lung inflammation have not been fully understood. For example, TLR9 has been shown in various studies to induce (Knuefermann et al., 2007; Schwartz et al., 1997), prevent (Parilla et al., 2006), or modify (Schwartz et al., 1999) lung inflammatory responses.

TLR9 is a membrane-bound receptor found primarily associated with endosomes (Rutz et al., 2004). It binds non-methylated CpG sequences of DNA of a given structure (Krieg, 2002). The effects of non-methylated DNA were first observed in mice (Kuramoto et al., 1992), and TLR9 as the receptor responsible for much of the immune response to this ligand was first elucidated in mice as well (Hemmi et al., 2000). Since then much work has been done to determine the effects of non-methylated CpG oligonucleotides on TLR9 as well as its expression and localization.

Experiments into localization of the TLR9 have focused primarily on cultured isolated immune cells. These studies established TLR9 expression in B cells (Hornung et al., 2002; Krieg et al., 1995), neutrophils (Jozsef et al., 2004; Schwartz et al., 1997), and eosinophils (Wong et al., 2007). The information on the expression of TLR9 even in immune cells is far from complete as indicated by the complexity of its expression in monocytes and macrophages. While mice show expression of TLR9 in macrophages (An et al., 2002), the expression in humans was observed only in professional antigen presenting cells such as dendritic cells (Hoene et al., 2006; Kadowaki et al., 2001; O'Mahony et al., 2008) in the lung. Interestingly, TLR9 was absent in lung but not splenic dendritic cells of mice (Chen et al., 2006). Alveolar macrophages are vital

in the clearance of a variety of pathogens as well as inflammatory debris from the alveolar space (Bowden, 1984). These cells typically express a larger panel of innate immune receptors (Schneberger et al., 2011). There is controversy over the expression of TLR9 in mouse and human alveolar macrophages (Fernandez et al., 2004; Suzuki et al., 2005).

Much of the data on TLR9 expression in the lung has been derived from either cell lines or isolated and cultured cell populations. Intriguingly, to our knowledge, with the exception of one study on neoplastic lungs (Droemann et al., 2005) there are no data on TLR9 expression in intact lungs of mouse or human. The study of receptor expression in intact lungs is important because of the role of intercellular communication and spatial arrangement of receptor expression in inflammatory responses. Further, we can observe expression of a particular receptor on various lung cells in a single view. Therefore, we set out to examine the expression and subcellular localization of TLR9 in the mouse and human lung with the use of immunohistochemistry, immunoelectron microscopy, and ISH. We further expanded this work to compare TLR9 expression in normal human lung versus that of asthmatic lung. We show TLR9 mRNA and protein expression in various cells of the lung.

5.3. Materials and Methods

5.3.1. Lung Tissues

Six to eight week old C3HeB/FeJ mice (n=5) were purchased from Jackson Laboratories, (Bar Harbor, ME) and housed at the Animal Care Unit of the Western College of Veterinary Medicine for one week prior to their euthanasia. The animals were used following approval by the institution in accordance with the Canadian Council on Animal Care guidelines. Lungs from euthanized animals were processed and embedded in paraffin as described previously (Parbhakar et al., 2004).

Paraffin-embedded human lung samples from patients with (n=5) and without asthma (n=3) were obtained following consent from the archives of the Department of Pathology in the College of Medicine at the University of Saskatchewan. The sections were prepared and placed on coated glass slides.

5.3.2. Immunohistology

Lung sections were stained with mouse anti-human TLR9 antibody (IMG-305a, Imgenex, San Diego, CA) following protocols described previously (Schneberger et al., 2009). Briefly, after de-paraffinization, re-hydration, tissue peroxidase quenching (0.5% hydrogen peroxide in methanol), and antigen unmasking with pepsin (2mg/ml 0.01N hydrochloric acid), the tissue sections were blocked with 1% bovine serum albumin to block non-specific binding for 1 hour. Sections were treated with TLR9 antibody (1:50 dilution) and incubated at 4°C for 16 hours followed by incubation with horseradish peroxidase-conjugated goat anti-mouse antibody (1:75) for 1hr at room temperature (P0447, Dako, Ontario, Canada). Color was developed using a color development kit (Vector Laboratories, Ontario, Canada). Slides were counterstained with methyl green (Vector Laboratories) prior to mounting. Immunohistochemical controls included omission of the primary antibody or the secondary antibody or staining with anti-von Willebrand factor antibody.

5.3.3. Immuno-electron Microscopy

For immuno-electronmicroscopy 100nm lung sections were placed on nickel grids and floated in blocking buffer for 30min prior to 1hr incubation with TLR9 antibody at 1:50 dilution. Sections were rinsed 3 times in Tris-buffered saline for 5 minutes each before addition and incubation with 20nm gold-conjugated anti-mouse secondary antibody (1:100; Fitzgerald

Industries International, Concord, MA, USA) for 1hr. A control with omission of the primary antibody was also run.

5.3.4. In-situ Hybridization

5.3.4.1. Probe generation

Human TLR9 sequence obtained from Invivogen (pUNO1-hTLR9a) was cut with HindIII and SmaI and ligated with T4 DNA ligase (NEB, Pikering ON Canada) into pSPT18 (Roche, Laval QC, Canada). This plasmid was then transfected and expanded in HIT-DH5α competent cells (Real Biotech Corp., Taipei, Taiwan) as per manufacturer's specifications. Mouse TLR9 in vector pCR II-TOPO (Letiembre et al 2007) was kindly provided by Dr. Serge Rivest from Laval University, Quebec City. Plasmids were purified using GelElute DNA purification kit (Sigma, Oakville, ON, Canada). Purified human plasmid insert was confirmed by cutting with HindIII and SmaI and looking for insert fragment on ethydium bromide acrylamide gel. DIG-labeled probes for TLR9 were generated using the Roche DIG RNA Labeling Kit (Roche, Laval QC, Canada) according to manufacturer's instructions. Probe concentration was determined using the Roche DIG Luminescent Detection Kit (Roche, Laval QC, Canada) protocol.

5.3.4.2. Tissue Probing

Fresh sections were prepared and allowed to adhere to the slides for 2 hours at 60^oC followed by de-paraffinization and re-hydration described in (Parbhakar et al., 2004). Sections were treated thereafter as per manufacturer's protocol (Biochain, Hayward CA). Briefly, tissue was fixed with 4% paraformaldehyde, washed with DEPC-H2O prior to incubation with pre-hybridization solution for 4 hours at 50^oC in a humidified chamber. The sections were incubated with 2ng/ml DIG-labeled probe for 16hr at 45^oC followed by washes through reducing

concentrations of saline-sodium citrate buffer before blocking with 1X block buffer in phosphate buffered saline for 1hr. Then, the sections were treated with AP-conjugated anti-DIG antibody in 1X blocking buffer (1:500) for 4hr at room temperature, washed with 1X alkaline phosphatase buffer before incubation for 4 hours with BCIP-NBT. Control sections were fixed and treated without addition of probe.

5.3.5. Cell field Count

TLR9-positivly stained cells were counted in randomly selected fields of asthma/COPD lungs (n=4) and those from patients with no identified lung problems (N=4). Five fields were counted at 400X magnification.

5.3.6. Statistical Analysis

All values presented were given as the mean +/- standard deviation. P values less than 0.05 were considered significant. We performed a one-way Mann-Whitney test to determine significance between normal and asthmatic/COPD lungs.

5.4. Results

5.4.1. Mouse Lung

First, we determined TLR9 protein expression and distribution with immunohistochemistry. Lung sections stained without primary (Figure 5.1A) or secondary antibody (data not shown) lacked staining while VWF antibody stained vascular endothelium but not the airway epithelium (Figure 5.1B). Lungs showed TLR9 protein in bronchiolar epithelium (Figure 5.1C, 5.1D), vascular endothelium (Figure 5.1C, 5.1E) and cells in the alveolar space and the septa (Figure 5.1C, 5.1F).

TLR9 mRNA was detected in mouse lungs with in-situ hybridization. Staining without probe showed no background staining (Figure 5.2B). While negative control lung sections lacked any staining, TLR9 mRNA was localized in vascular endothelium (Figure 5.2C), airway epithelium (Figure 5.2A, 5.2E), alveolar macrophages and sepal cells (Figure 5.2A, 5.2D).

We confirmed the identity of the cells and the subcellular localization of TLR9 with immuno-gold electron microscopy. TLR9 staining was observed in the alveolar macrophages (Figure 5.3), neutrophils (Figure 5.4), and type-2 endothelial cells (Figure 5.5). While nuclear and cytoplasmic staining for TLR9 was noticed in alveolar macrophages and type-2 cells, only cytoplasmic staining was present in neutrophils.

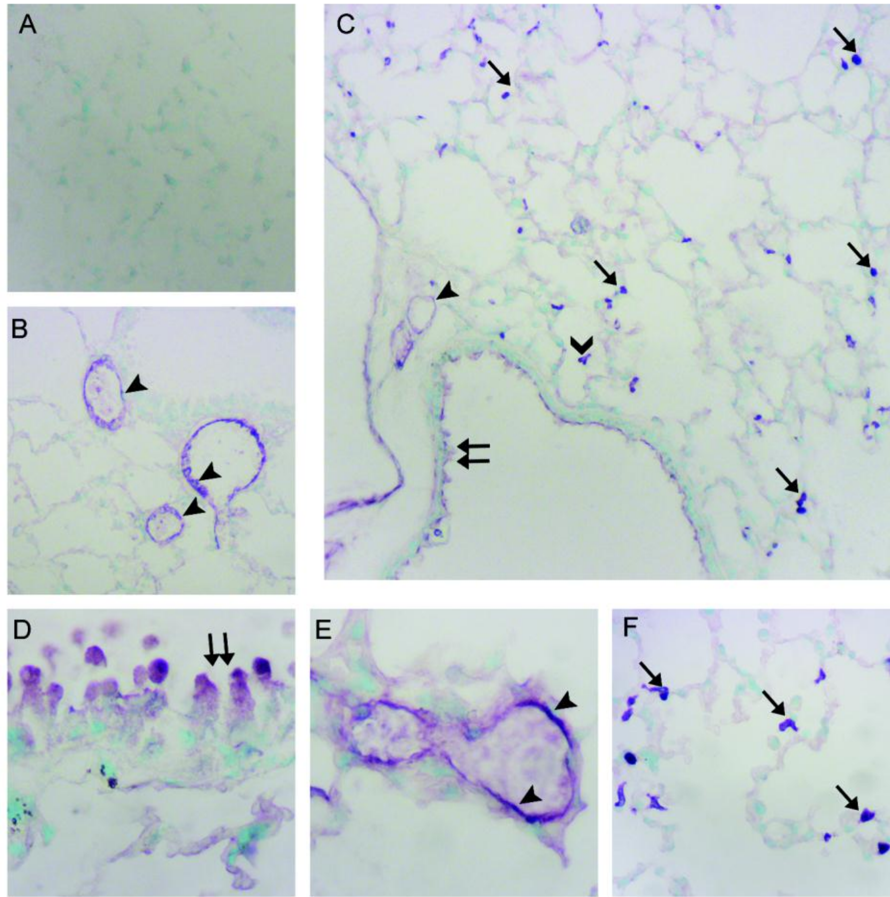

Figure 5.1. TLR9 Immunohistochemistry on Mouse Lungs

Lung sections from mice stained with only a secondary antibody (A) lack staining in alveolar septa, whereas those stained with vWF antibody (B) shows staining in endothelium (arrowhead) alone. Lung sections stained using TLR9 antibody show staining in several tissues (arrows) (C). High magnification shows staining in bronchial epithelium (double arrow) (D), vascular endothelium (arrowhead) (E), and septal (arrow) as well as alveolar macrophages (chevron) (F). Original magnification A,B,C 100X; E,F 400X; D 1000X.

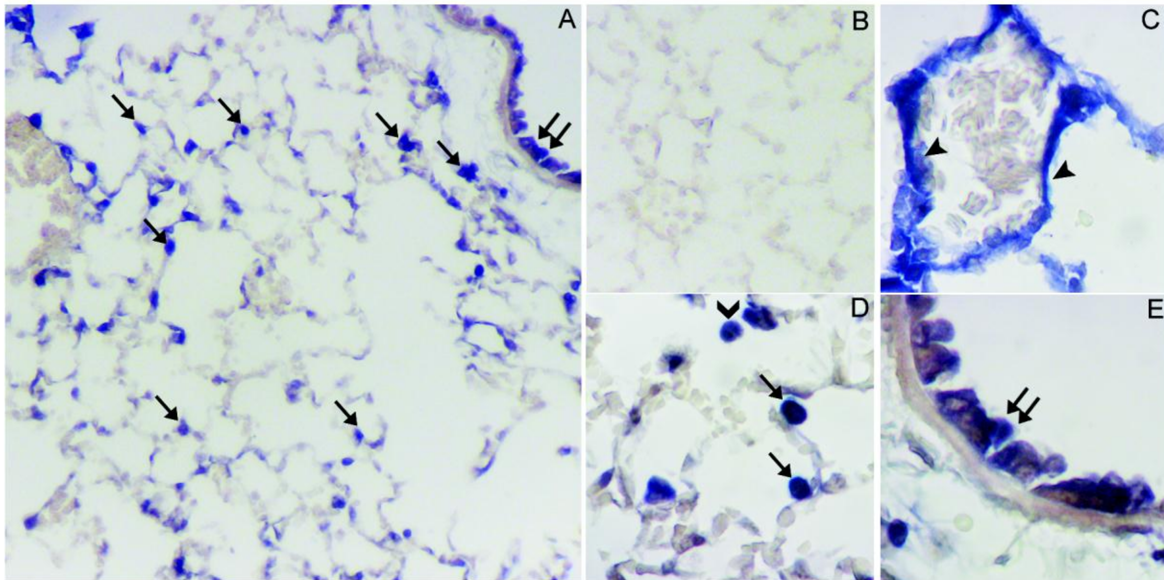

Figure 5.2. TLR9 In-Situ Hybridization on Mouse Lungs

Lung sections stained using TLR9 DIG-labeled RNA show staining (arrows) in several cell types (A). Lung sections from mice stained without a specific probe (B) lacks staining in any tissue. High magnification shows staining in vascular endothelium (arrowhead) (C), septal (arrow) as well as alveolar macrophages (chevron) (D), and bronchial epithelium (double arrow) (E). Original magnification A 100X; B,C,D,E 400X.

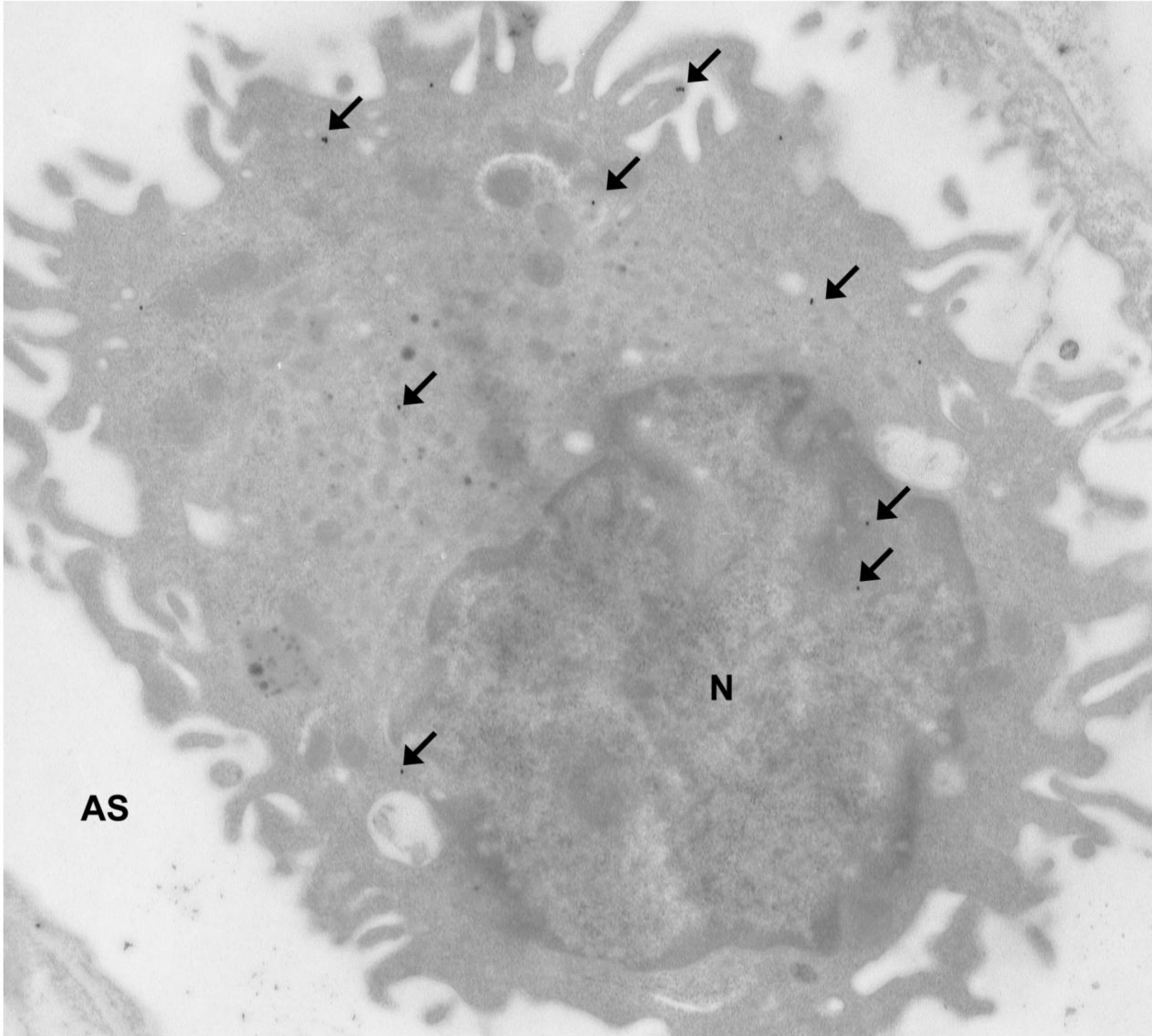

Figure 5.3. TLR9 staining in mouse alveolar macrophage.

TLR9 staining (arrows) observed in mouse alveolar macrophage. N: Nucleus; AS: Alveolar Space. Original magnification 10000X.

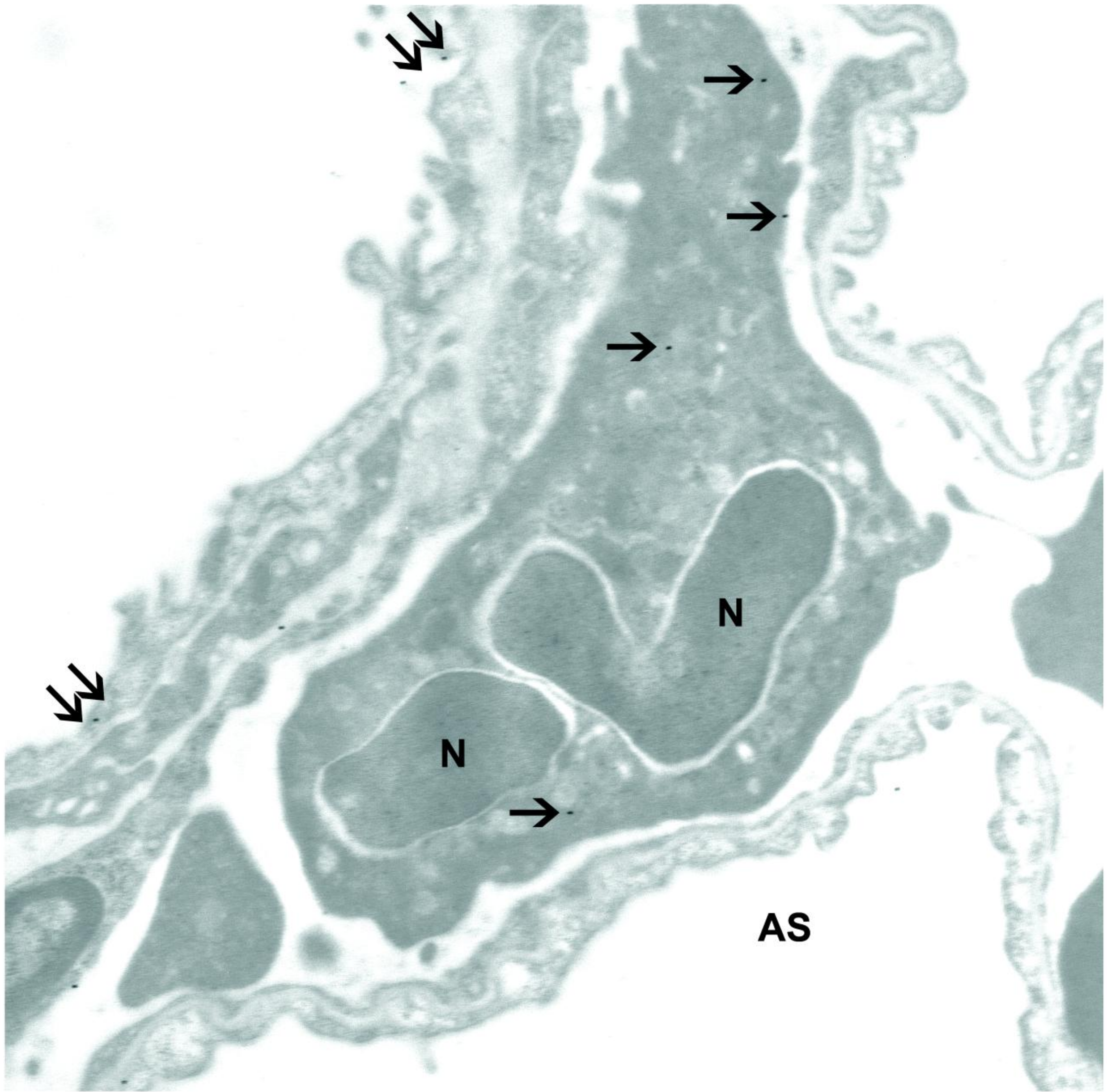

Figure 5.4. TLR9 staining in mouse neutrophil.

TLR9 staining (arrows) observed in a mouse lung neutrophil and alveolar septa (double arrows). N: Nucleus; AS: Alveolar Space. Original magnification 10000X.

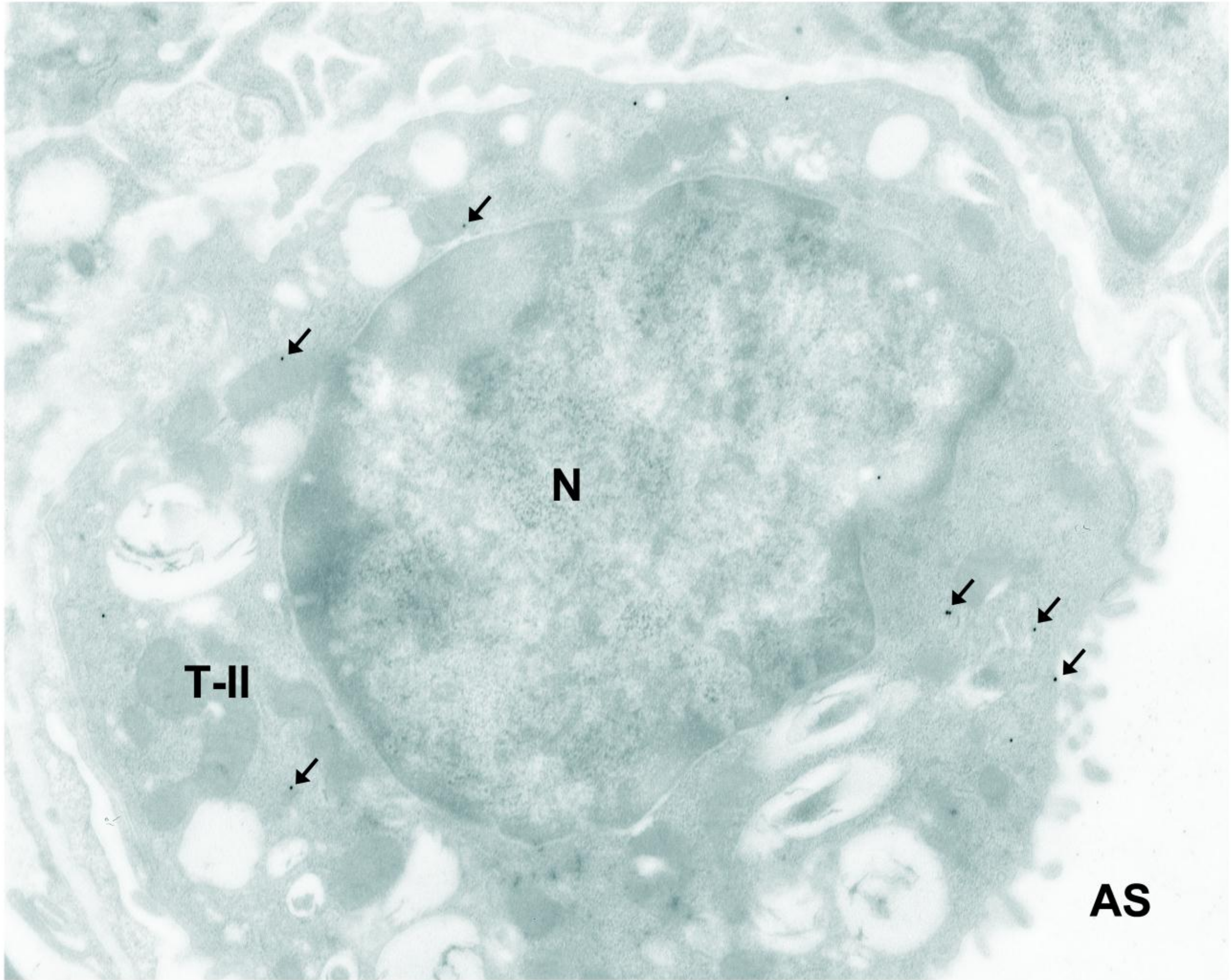

Figure 5.5. TLR9 staining in mouse type-II cell.

TLR9 staining (arrows) observed in a mouse lung type-II cell. N: Nucleus; AS: Alveolar Space. Original magnification 10000X.

5.4.2. Human Lung

Immunohistochemistry of lungs without primary (Figure 5.6A) or secondary antibody (data not shown) lacked staining whereas vWF stained vascular endothelium (Figure 5.6B). TLR9 antibody reacted with alveolar septal cells and alveolar macrophages in healthy (Figure 5.6C) and asthmatic (Figure 5.6D) lungs. The number of TLR9 positive septal cells was increased in the asthmatic lungs (Figure 5.6I). Lungs showed TLR9 protein expression in vascular endothelium (Figure 5.6E), bronchiolar epithelium (Figure 5.6F), alveolar septal cells (Figure 5.6G), and alveolar macrophages (Figure 5.6H).

In-situ hybridization showed TLR9 mRNA in lungs from healthy (Figure 5.7A) and asthmatic (Figure 5.7B) humans. Asthmatic lungs appeared to show more intense staining for TLR9 mRNA in the resident as well as recruited cells (Figure 5.7A-B). The negative control lacked positive reaction (Figure 5.7C). Large and small lung vessels showed TLR9 mRNA expression in their endothelium (Figure 5.7D-E). Large septal cells most likely macrophages and alveolar macrophages were also positive for TLR9 mRNA (Figure 5.7F).

Human lungs subjected to immuno-electronmicroscopy delineated TLR9 expression in cytoplasm, nucleus and the plasma membrane of alveolar macrophages (Figure 5.8). Type-II alveolar epithelial cells showed TLR9 expression in their cytoplasm (Figure 5.9).

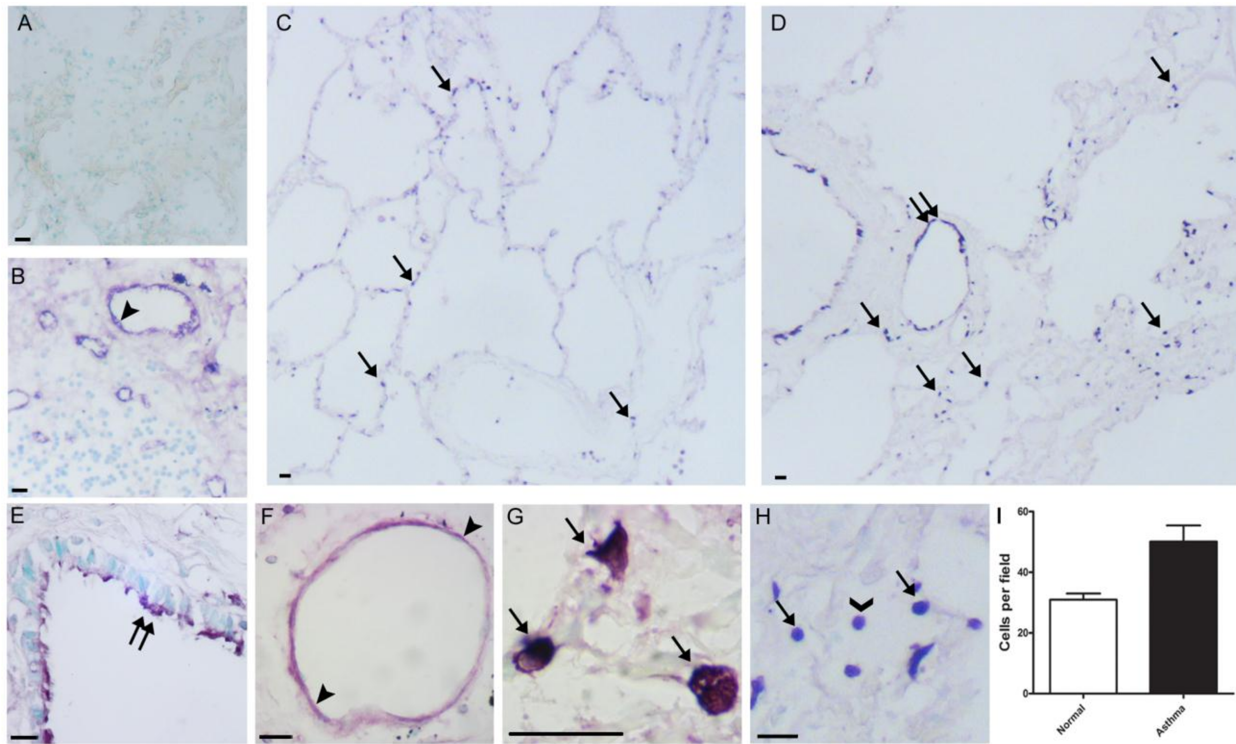

Figure 5.6. Human Lung TLR9 Immunohistochemistry.

Lung sections from human patients stained with only a secondary antibody (A) lacks staining in alveolar septa, whereas the those stained with vWF antibody (B) shows staining in endothelium (arrowhead) alone. Lung sections stained using TLR9 antibody show staining (arrows) in several cell types in both normal (C) as well as asthmatic patients (D). High magnification shows staining in bronchial epithelium (double arrow) (E), vascular endothelium (arrowhead) (F), and septal (G) as well as alveolar macrophages (chevron) (H). Original magnification A,C,D 40X; B 100X; E,F,H 400X; G 1000X. Bars represent 0.2mm. Field counts of control and Asthma/COPD patients (I) showed a greater density of TLR9 positive cells in those with Asthma/COPD ($p<0.01$).

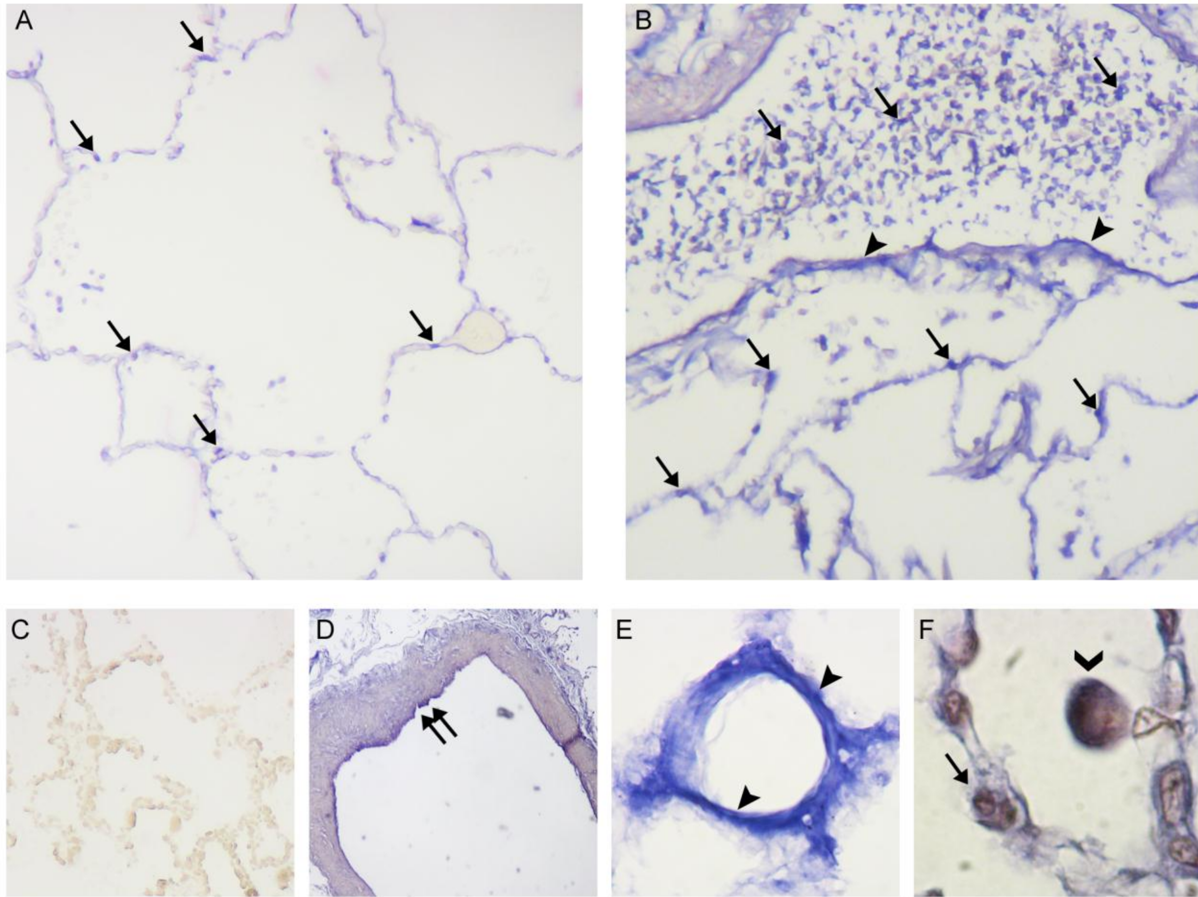

Figure 5.7. Human Lung TLR9 In-Situ Hybridization.

Lung sections from normal (A) or asthmatic (B) human patients stained using TLR9 DIG-labeled RNA probe show staining (arrows) in several cell types with a clear influx of TLR9 positive cells in the bronchus of asthmatics. Staining without a RNA probe shows lack of staining in any tissue (C). High magnification shows staining in bronchial epithelium (double arrow) (E), vascular endothelium (arrowhead) (F), and septal (arrow) as well as alveolar (chevron) macrophages (G). Original magnification A,B,D 40X; C 100X; E,F 400X.

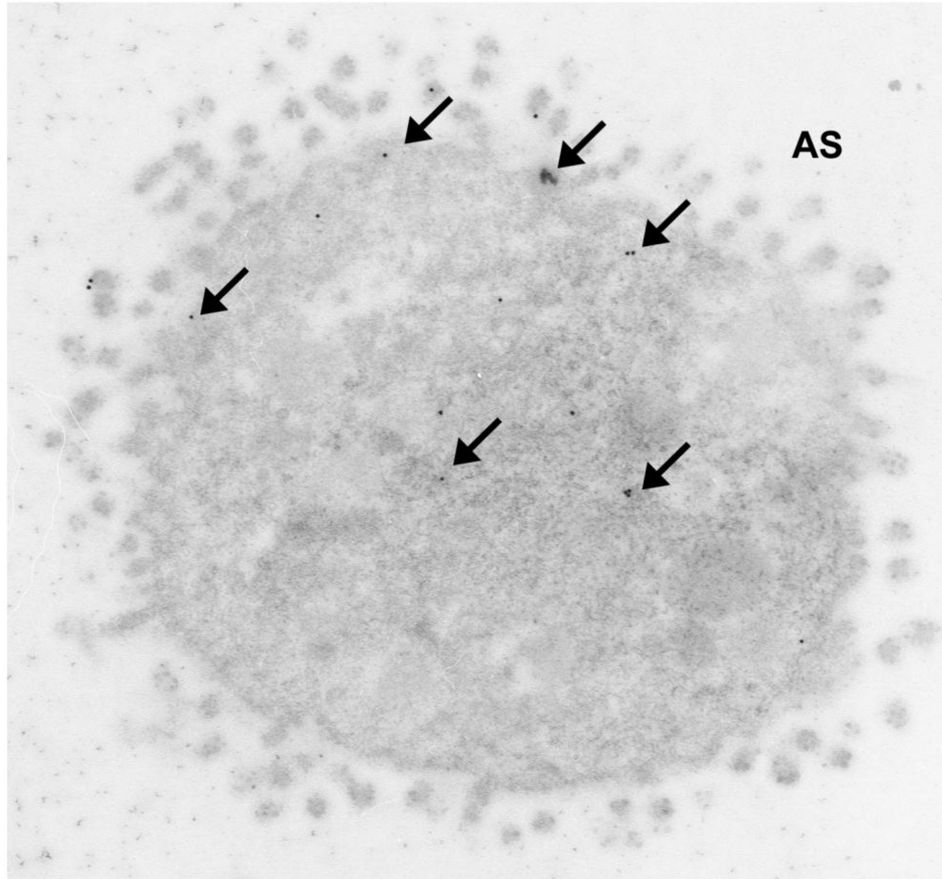

Figure 5.8. TLR9 staining in human alveolar macrophage.

TLR9 staining (arrows) observed in a human alveolar macrophage. N: Nucleus; AS: Alveolar Space. Original magnification 13000X.

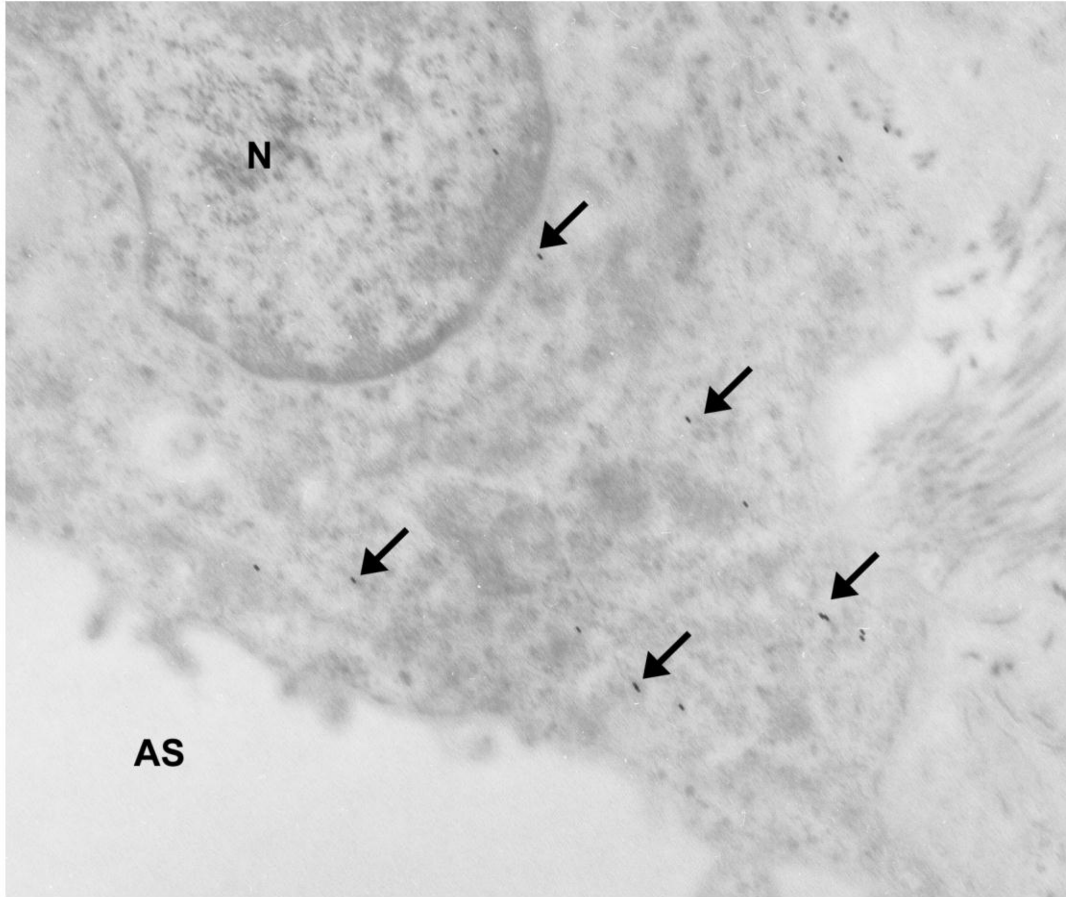

Figure 5.9. TLR9 staining in human type-II cell.

TLR9 staining (arrows) observed in a human lung type-II cell. N: Nucleus; AS: Alveolar Space. Original magnification 13000X.

5.5. Discussion

We provide data on *in situ* expression of TLR9 protein and mRNA in intact lungs of mice and humans through the use of multiple morphological methods. These data are important because of very limited information available on TLR9 expression in intact lungs and the potential role of TLR9 in various respiratory diseases. The lung is comprised of many types of resident and transiting cells that make specific contributions to physiological responses in the lung. Cell responses are largely governed by the type and amount of expression of immune receptors such as TLR9, and inter-cellular communications. Therefore, it is very important to understand precise cell-specific expression of receptors such as TLR9 in intact organs. The data reported here show TLR9 mRNA and protein expression in vascular endothelium, bronchiolar epithelium, alveolar septal cells, and alveolar macrophages in lungs of both mice and humans.

We used a combination of methods to detect TLR9 mRNA and protein expression in mouse and human lungs. The reason for studying both human and mouse lungs is that mice are the most commonly used animal model to study human lung physiology but the basal expression of proteins may not be similar between the two. Because the mRNA may not always be translated into its protein, we studied TLR9 protein and mRNA expression *in situ* in intact lungs of both species. Lastly, we employed immuno-gold electron microscopy to clarify the sub-cellular expression of TLR9 protein.

Innate immune receptors such as TLR9 recognize microbial motifs and their expression on epithelia and alveolar macrophage forms the first line of defense in the lung. Interestingly, there is controversy regarding the expression of TLR9 in monocytes and macrophages. The data from isolated cells show that expression of TLR 9 appears to be primarily restricted to certain human dendritic cells particularly plasmacytoid dendritic cells and alveolar macrophages (Hoene et al., 2006; Hornung et al., 2002; Kadowaki et al., 2001; O'Mahony et al., 2008), whereas in mice,

macrophages and alveolar macrophages express the receptor, but dendritic cells do not. This pattern is more restricted in mouse lung than in other tissues as these dendritic cells do express TLR9 in the mouse spleen (Chen et al., 2006). Further, some species such as cattle and horses have a highly phagocytic lung macrophage population known as pulmonary intravascular macrophages, which bind to and reside in lung capillaries (Staub, 1994). These cells have such a strong expression of TLR9, that depletion of these cells can greatly reduce overall TLR9 mRNA in lungs of these animals (Schneberger et al., 2009; Schneberger et al., 2011). There has been some dispute over expression of TLR9 in alveolar macrophages within both human as well as mouse lungs. While some found functional inhibition of IL-10R in alveolar macrophages stimulated with CpG-DNA (Fernandez et al., 2004), others found no mRNA expression in lavaged mouse alveolar macrophages (Suzuki et al., 2005). However Kiemer and colleagues (Kiemer et al., 2008) showed that expression of TLR9 mRNA and protein does in fact occur in these cells *in vitro*. Our data are the first to confirm TLR9 protein and mRNA in alveolar macrophages in intact lung.

This information on TLR9 expression in alveolar macrophages is of particular use as the location of a receptor within the lung can greatly alter its ability to detect its ligand. For example, particulate size has a clear role in where inhaled debris is likely to deposit (Bowden, 1984). Thus, expression of TLR9 in the bronchial epithelium allows for sensing unmethylated DNA on particles much larger than would be encountered in the alveolar space. Additionally, as the half life of naked DNA in circulation is only a matter of minutes (Kawabata et al., 1995), local tissue receptor interaction may be more important than DNA reaching circulating cells.

There have been studies that proposed TLR9 expression in a number of lung cell types. Platz and colleagues (Platz et al., 2004) showed expression in lung epithelial cell line CALU-3, and

induction of cytokines in response to CpG stimulation. While ISH and immunohistochemical staining was not as good for these cells, we do show immune-electronmicroscopic evidence for expression in type-2 alveolar epithelial cells (Figure 5.5 and 5.9), consistent with their findings. Li and colleagues (Li et al., 2004) showed that not only do pulmonary endothelial cells express TLR9, but in cultures stimulated with CpG DNA resulted in expression of proinflammatory proteins such as IL-8 and ICAM-1.

In patients diagnosed with asthma we see a large influx of cells into the lung (Fig 5.6d), many of which show expression of TLR9. While we did not seek to identify these specific cells, work by others has suggested that these cells could include myeloid dendritic cells (Schaumann et al., 2008), eosinophils (Ilmarinen et al., 2009), and neutrophils (Jozsef et al., 2004) all of which express TLR9 and may account for the positive staining we see of cells in the vascular space. The influx of inflammatory cells into an organ would increase the total amount of TLR9 protein and result in more robust inflammatory signaling in the organ.

It should be noted however that Assaf and colleagues (Assaf et al., 2009) found in tumor cell lines that the expression of TLR9 is no guarantee of signaling. Indeed, there appears to be a threshold amount of receptor required for effective signaling upon encounter of ligand. While our study does not address this issue directly we do note that staining intensity both for protein and mRNA in the stated tissues was of an intensity similar to that found in macrophages, suggesting that they may have sufficient expression of TLR9 for immune signaling upon ligation.

CHAPTER 6: ROLE OF TLR9 IN EXPOSURE OF MICE TO CHICKEN BARN AIR

6.1. Abstract

Exposure to animal barn air is an occupational hazard that causes lung dysfunction in barn workers. Respiratory symptoms experienced by workers are typically associated with endotoxin, but within these environments gram negative bacteria constitute only a portion of the total microbial population. In contrast, unmethylated DNA can be found in all bacteria, some viruses, and mould. We hypothesized that in such environments TLR9 contributes to the overall immune response seen in the lung. Therefore we investigated the role of TLR9, which binds unmethylated DNA, in barn air induced inflammation. Using a mouse model, wild-type and TLR9$^{-/-}$ mice were exposed to chicken barn air for 1, 5, or 20 days. Examination of blood serum and bronchiolar lavage against a panel of six TLR9-induced cytokines (IL-1β, IL-6, IL-10, IL-12, TNF-α, and IFN-γ) showed no significant differences after a single day exposure. TNF-α and IFN-γ levels in TLR9-/- mice were reduced after exposure at 5 days in lavage, and TNF-α was also reduced in serum at this point. After 20 days of exposure IFN-γ was significantly reduced in lavage of TLR9$^{-/-}$ mice. Neutrophil accumulation in the lung as measured by myeloperoxidase was also reduced at 20 days of exposure in TLR9-/- mice, as was total lavage cell counts. However, Masson's staining revealed no apparent histological differences between any of the treatment groups. Taken together our data show TLR9 contribute to lung inflammation induced following exposure to chicken barn air potentially through binding of unmethylated DNA.

6.2. Introduction

Workers in high-intensity livestock operations have been recognized to be at risk for a number of chronic respiratory problems including bronchitis, rhinitis, chronic cough and phlegm, occupational asthma, and organic dust toxic syndrome (ODTS) to name a few (Donham et al., 2000; Kirychuk et al., 2003; Kirychuk et al., 2006; Radon et al., 2001; Whyte, 1993). Workers in such facilities are exposed to a wide variety of agents such as ammonia, hydrogen sulfide, dust particles, and LPS (Just et al., 2009; Kirychuk et al., 2006). Single exposures to such facilities have been shown to elevate a number of pro-inflammatory cytokines in nasal lavages and serum of animal models (Senthilselvan et al., 1997; Charavaryamath et al., 2008; Rylander, 2002; Smit et al., 2009).

Endotoxin has been targeted as a critical component responsible for many of the lung problems seen to exposure to barn air (Charavaryamath et al., 2008; Donham et al., 2000; Thelin et al., 1984; Zejda et al., 1994). More recent work however suggests that within chicken barns endotoxin-producing gram-negative bacteria make up only a small number of the bacterial species within the barn, and that these bacteria may be in the minority as far as total numbers are concerned (Just et al., 2009; Whyte, 1993). In contrast, all bacteria and some viruses and mold contain unmethylated DNA in their genomes, which has been shown to induce a variety of cytokines through binding the TLR9 receptor. Many of these cytokines are similar to those induced by endotoxin, which binds the related TLR4 receptor (Klinman, 2006; Medzhitov et al., 1997). Previous work had shown that DNA from barn dust extracts could induce IL-10 and IL-12p40 (Roy et al., 2003). This system however uses purified DNA given to peripheral blood mononuclear cells *in vitro*, which may not be the best reflection of barn conditions, where significant production of cytokines may occur in the lung from a variety of cell types. Further, such a system doesn't account for effects of numerous other components in the air that may

synergize or counter responses through TLR9. Therefore, we exposed TLR9-/- and wild-type mice to chicken barn air for 1, 5, or 20 days to determine the contribution of TLR-9 to lung inflammation. We examined cytokines, immune cells, and histology in the lungs of normal and exposed wild-type and TLR-9 deficient mice. Our data show that TLR9 plays a role in lung inflammation induced following exposure to chicken barn air.

6.3. Materials and Methods

6.3.1. Animals

The experimental protocols were approved by the University of Saskatchewan Committee on Animal Care Assurance and all experiments conducted according to guidelines of the Canadian Council on Animal Care. Breeding pairs of TLR9-deficient mice (C57BL/6 background) were a gift from Dr. Heather Davis and obtained from Taconic. Mice were raised at the Western College of Veterinary Medicine Animal Care Unit. C57BL/6 mice where obtained from the Animal Resource Centre at the University of Saskatchewan.

6.3.2. Experimental Exposure

Mice were transported in sealed cages with vents and driven to a cage-based chicken barn in the morning and placed on a shelf approximately 1.8 meters (6 feet) off of ground. Mice were kept in barn for 8 hours and then returned to animal care facilities at the University of Saskatchewan where they were transferred out of their cages for the evening. Mice were taken to the barn for 1, 5, or 20 days. The 20 day exposure animals were rested for 2 days after each cycle of 5 exposures to more closely approximate a 5 day work week exposure. Parallel control groups of mice that were transported but not exposed to barn air were transported with exposed mice in separate cages.

6.3.3. Tissue, Blood, and Lavage Collection

At the end of exposure time mice were euthanized (100mg/kg ketamine + 20mg/kg xylazine, intraperitonial injection), and blood was collected by cardiac puncture with 0.01ml heparin (Sandoz, Boucherville, QC, Canada). BAL fluid was collected by flushing lungs with 3ml of cold HEPES buffer and centrifuged at 400Xg, and stored at -80°C for later use, while the cell pellet was resuspended in 100µl HEPES and counted with a haemocytometer (Hausser Scientific, Horsham, PA). Cells were resuspended to 800µl and cytospun onto microscope slides, and dried overnight before staining.

Lung sections were divided in two and one half snap-frozen in liquid nitrogen and stored at -80°C. The other lung was fixed in 4% paraformaldehyde overnight before dehydration and polymerization and embedding in white resin (London resin company, USA).

6.3.4. Immunohistochemistry

Immunohistology was done on lung tissue, cut on a microtome to a thickness 5 microns. Briefly, after de-paraffinization, rehydration, tissue peroxidase quenching (0.5% hydrogen peroxide in methanol), and antigen unmasking with pepsin (2 mg/mL 0.01 N hydrochloric acid), the tissue sections were blocked with 1% BSA to block nonspecific binding for 1 hour. Sections were treated with F4/80 (1:75 dilution) and incubated overnight at 4°C. The next day horseradish peroxidase-conjugated goat anti-rabbit antibody (ab6845, Abcam) was added at 1:100 dilution for 1 hour at 37°C. Color was developed using a color developing kit (Vector Laboratories, Ontario, Canada). Slides were counterstained with methyl green (Vector Laboratories) before mounting. A control was similarly run with omission of the primary antibody or the secondary antibody. We counted F4/80 positive cells in the alveolar septa of lungs to quantify number of

septal macrophages. The counts were made in three fields in 3-4 mice randomly selected from each group in the study at 100X magnification.

6.3.5. Histochemical Staining

Cytospins of BAL cell were stained using a Hemacolor kit (EMD Chemicals, Mississauga, ON, Canada) according to manufacturer's protocol. Lung tissue sections were stained using Masson's trichrome stain. Tissue was deparaffinized and placed in Bouin's fixitive overnight prior to staining. Slides were dehydrated and mounted after staining.

6.3.6. Cytokine Bio-Plex Enzyme Linked Immunosorbent Assay

Cytokines IL-1β, IL-6, IL-10, IL-12, IFN-γ, and TNF-α were measured using bead-conjugated antibodies and recombinant standards with the Bio-Plex multiplex ELISA assay system (Bio-Rad, Mississauga ON, Canada). Assay was carried out as per manufacturer's instructions for magnetic bead ELISA. Lavage fluid and serum were centrifuged prior to use as described earlier.

6.3.7. Protein and RNA Extraction

Frozen lung tissue was homogenized in microcentrifuge tubes using a pestle (Bel-art, Pequannock, NJ, USA). Protein and RNA was extracted using an AllPrep DNA/RNA/Protein purification kit (Qiagen, Mississauga, ON, Canada) as per manufacturer's instructions. Protein and RNA fractions were saved, quantified, and stored at -80°C until used.

6.3.8. Myeloperoxidase assay

Briefly, protein samples were placed on a 96-well plate at several concentrations in phosphate citrate buffer (0.2M $Na_2HPO_4 – 7H_2O$, 0.1M citric acid, pH 5.0) in duplicate along with a

recombinant standard. TMB substrate was added to all wells and developed for 2 minutes at room temperature before reaction was stopped with 1M H_2SO_4 and read at a450.

6.4. Results

6.4.1 Histological Examination

Lung tissue sections were stained with Masson's stain to determine if there were any apparent histological changes (Figure 6.1). No changes were apparent across times, exposure times, or species.

6.4.2. Neutrophil myeloperoxidase

Myeloperoxidase levels were similar across all groups, with no significant differences between exposed and non-exposed animals at 1 and 5 days of exposure to barn air (Figure 6.2). However, as shown in Figure 6.2, at 20 days there is a significant reduction in myeloperoxidase ($p \leq 0.05$) in the lungs of TLR9-deficient animals compared to similarly exposed normal mice.

6.4.3. Septal macrophage cell counts

Tissue sections were cut of lungs from all mice and stained for macrophages using F4/80 antibody to determine numbers of septal macrophages at various exposures. Single exposure to barn air induced a significant decrease in both wild-type and knockout mice compared to the unexposed mice ($p \leq 0.01$ and 0.05 respectively) (Figure 6.3). The numbers of F4/80 septal macrophages were higher after 5 exposures compared to single exposure mice ($p \leq 0.05$). At 20 days exposure, the numbers of septal macrophages once again dropped significantly ($p \leq 0.05$) for the WT mice compared to the knockout mice, resulting in a significant difference in septal macrophages between WT and TLR9 knockout exposed mice ($p \leq 0.01$).

6.4.4. BAL Cell Counts

After a single day exposure there were no significant changes to BAL cell counts to barn air either between TLR9-/- and WT animals, or between exposed animals and their transport controls. There was however a significant decrease in number of cells in the BAL of both WT and knockout mice at 5 days exposure compared to similar 1 day exposed animals ($p \leq 0.05$ between TLR9-/- 1 vs 5 days exposed and $p \leq 0.01$ between WT 1 vs 5 days exposed) (Figure 6.4). BAL cell numbers increased once more when examined in 20 days exposed animals compared to 5 day exposed animals, however, at this time the number of cells in the TLR9-/- 20 day exposed BAL fluid were significantly lower than similarly exposed WT mice ($p \leq 0.05$). Cell staining and counting of cell types showed a predominance of alveolar macrophage in all BAL fluids from all exposure conditions and times with no significant differences between any treatment groups. Therefore, the changes in BAL cell counts are a reflection primarily of changes in numbers of alveolar macrophages.

6.4.5. Expression and Quantification of Cytokines in Lung BAL and Serum

A panel of cytokines (IL-1β, IL-6, IL-10, IL-12, IFN-γ, and TNF-α) were examined in blood serum and BAL. Of these cytokines significant differences were detected in IFN-γ and TNF-α in serum (Figure 6.5) and BAL (Figure 6.6). We do note however that no IL-12 was detected in any samples, indicative of a problem with this particular antibody.

At 1 day exposure, while there were no significant differences in lavage cytokines, there was a reduction in serum TNF-α in the TLR9-/- animals compared to normal wild type (WT) animals ($p \leq 0.05$).

At 5 days of exposure to barn air TLR9-/- animals now showed continued significant reduction in serum TNF-α ($p \leq 0.05$) compared to similarly exposed WT animals, but now BAL also showed significant reductions of both TNF-α and IFN-γ in these same animals. ($p \leq 0.06$).

After 20 days of exposure to barn air, while serum cytokines levels were no longer significantly different between WT and TLR9-/- exposed mice, BAL IFN-γ remained reduced in the knockout mice ($p \leq 0.05$).

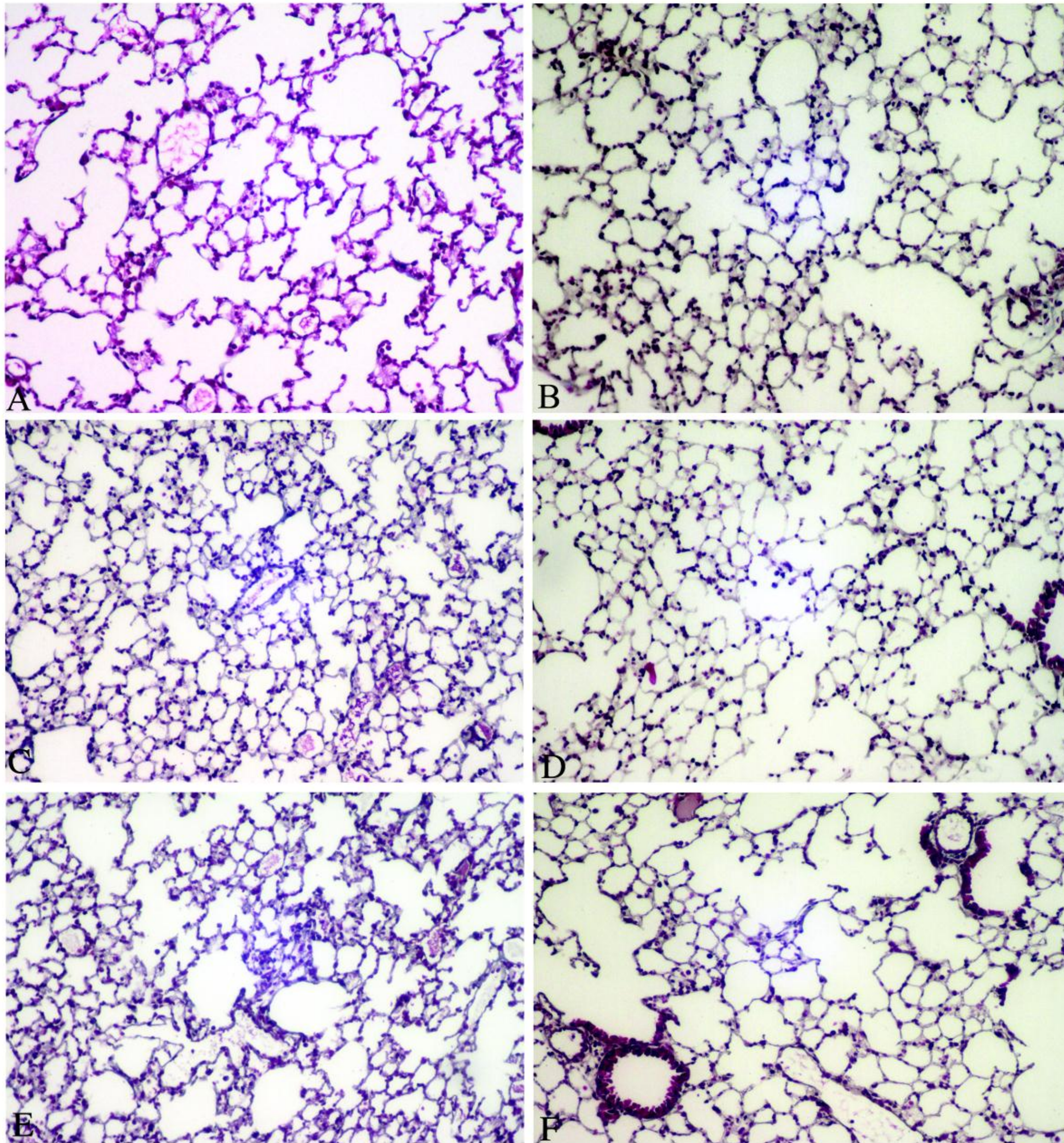

Figure 6.1. Lung histology: Masson's Trichrome staining

Tissue sections from animals (n=3 per group) were mounted and stained with Masson's trichrome and examined. No significant changes were noted between barn exposed WT and TLR9- animals at 1 day (A and B respectively) or 20 days (C and D respectively). 20 day exposure controls for WT (E) and TLR9- (F) were similarly indistinguishable. Original magnification 40X.

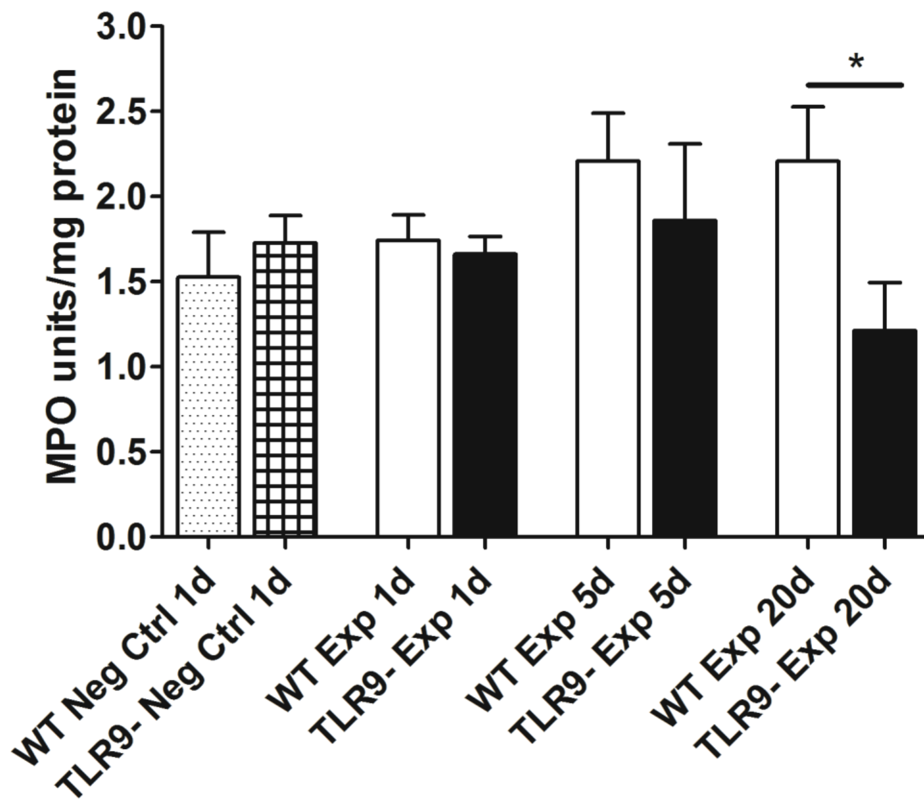

Figure 6.2. Myeloperoxidase activity assay for lung neutrophil quantitation.

TMB substrate was added to protein extracts of whole lung tissue from exposed and unexposed WT and TLR9-deficient (TLR9-) mice and read at a450 after 2 minutes. Activity was assessed by comparison to a standard curve. (*= $p \leq 0.05$).

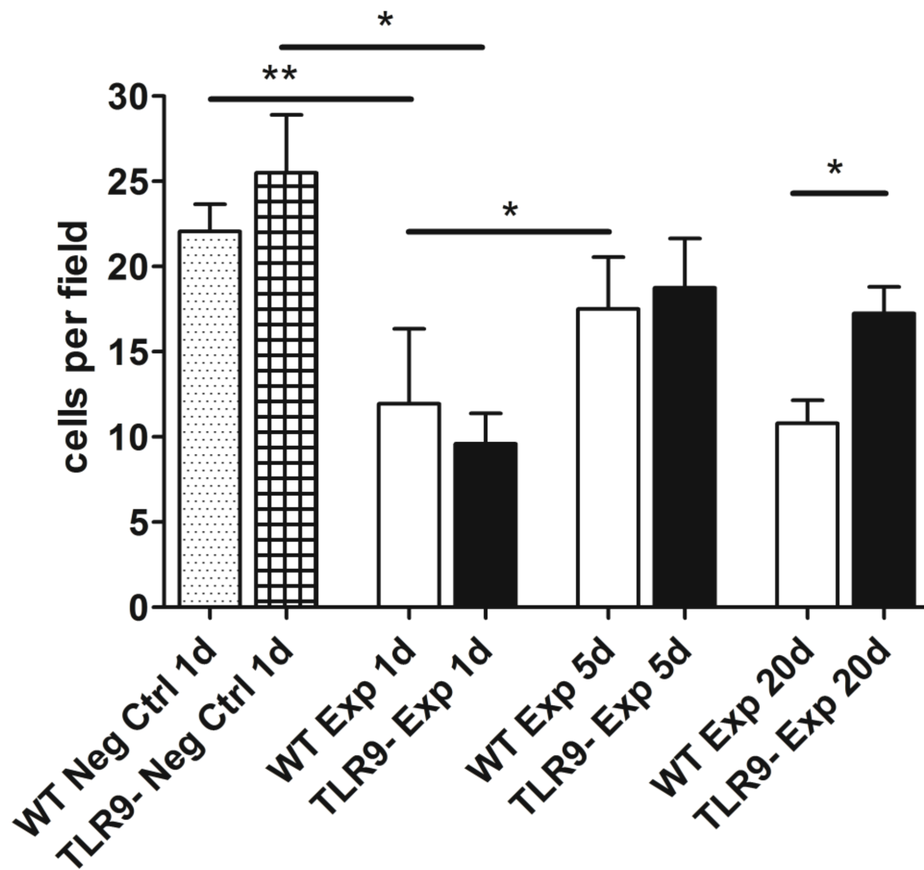

Figure 6.3. Septal macrophage staining and quantification.

Immunohistochemistry with F4/80 antibody was done on lung sections from exposed and unexposed WT and TLR9-deficient (TLR9-) mice. Blinded counts of macrophages were done on 5 400X magnification fields on 2 separate lung sections from each animal and averages of these counts used. (*= $p \leq 0.05$ **= $p \leq 0.01$).

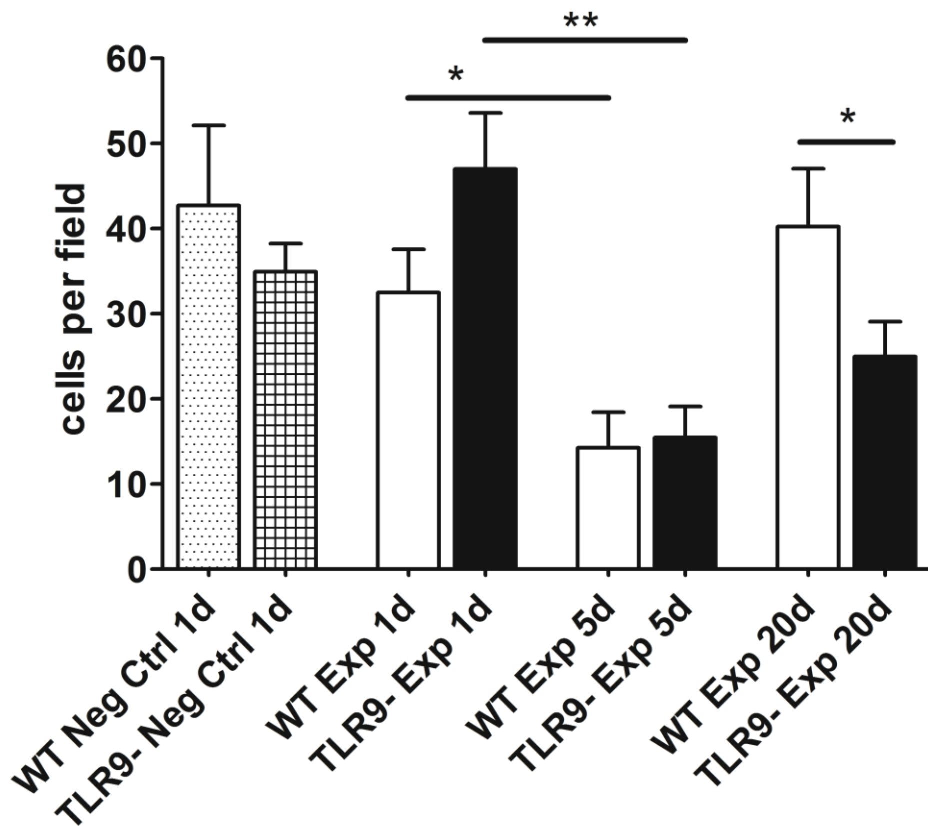

Figure 6.4. BAL cell counts.

BAL from exposed and unexposed WT and TLR9-deficient (TLR9-) mice was spun briefly at 400Xg, supernatant removed, and cells resuspended to 100µl for counting with hemocytometer (shown). (*= $p \leq 0.05$, **=$p \leq 0.01$).

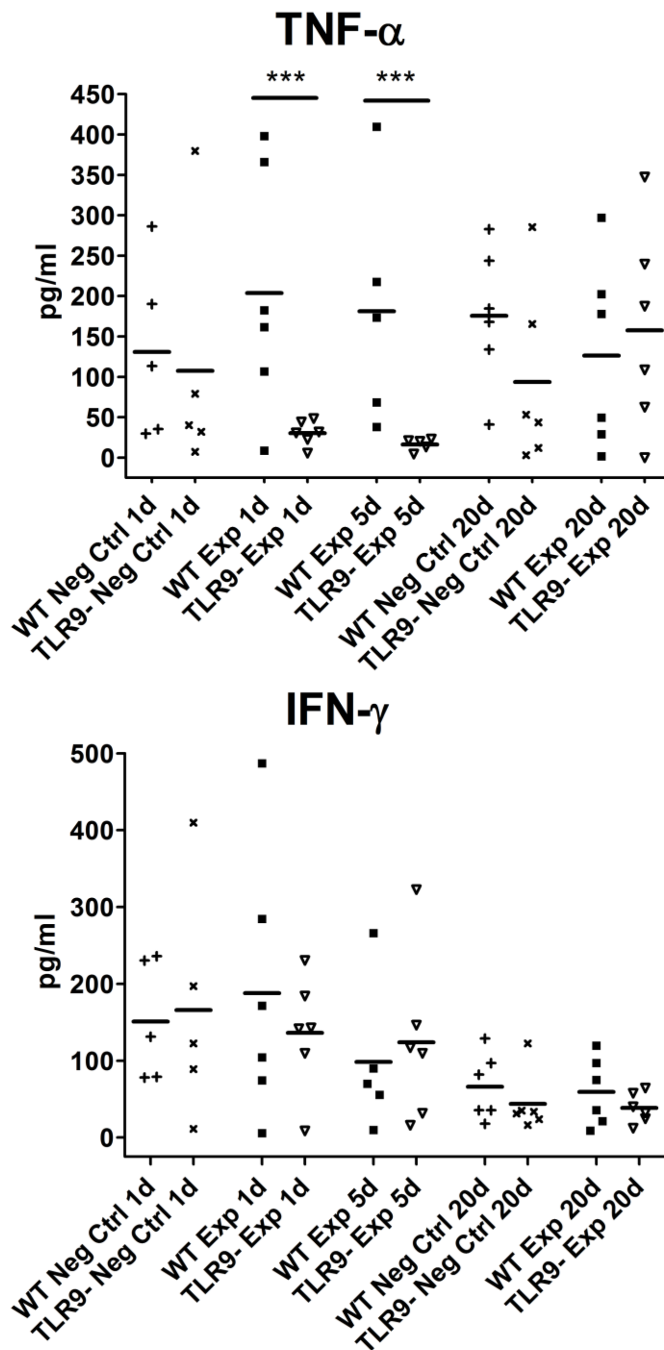

Figure 6.5. Bio-plex ELISA of mouse serum.

Blood was collected by cardiac puncture from exposed and unexposed WT and TLR9-deficient (TLR9-) mice. Samples were centrifuged briefly to remove cells and serum decanted. ELISA was done with antibodies to IL-1β, IL-6, IL-10, IL-12, IFN-γ, and TNF-α. (*** = p≤ 0.01).

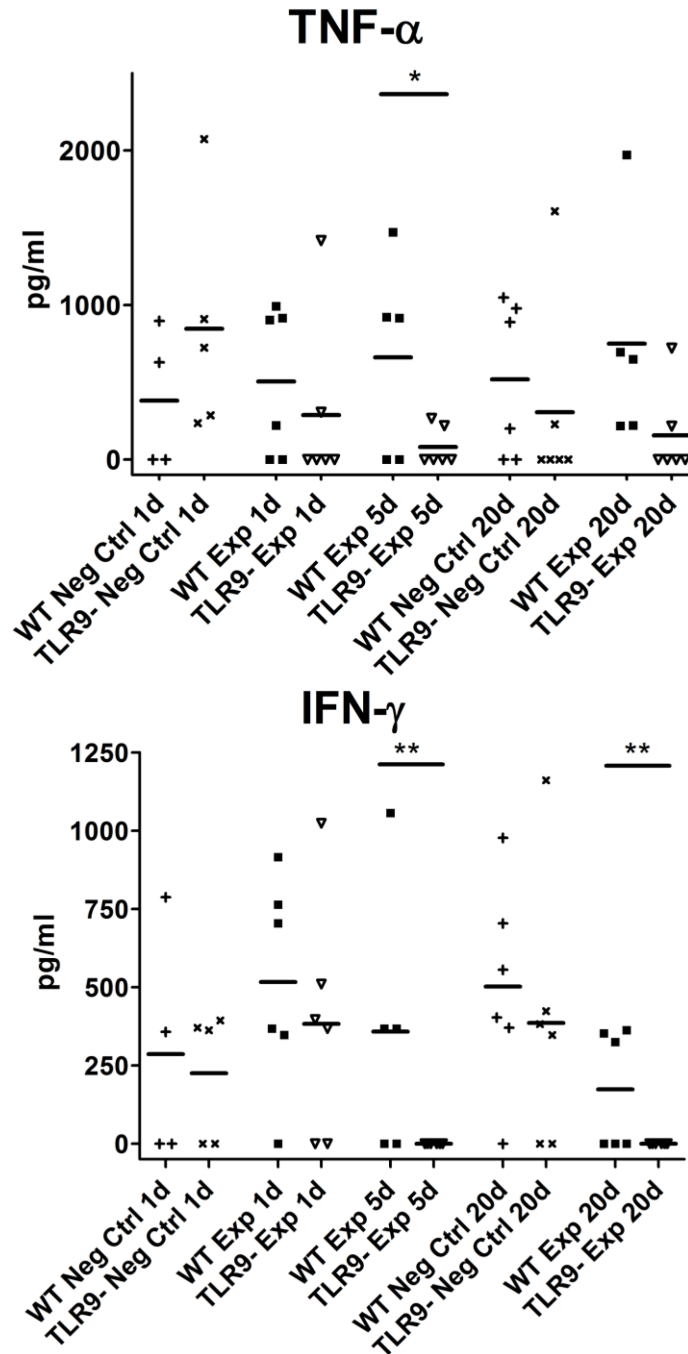

Figure 6.6. Bio-plex ELISA of mouse BAL.

BAL was collected by washing lungs 3 times with cold HEPES buffer from exposed and unexposed WT and TLR9-deficient (TLR9-) mice. Samples were centrifuged briefly to remove cells and fluid decanted. ELISA was done with antibodies to IL-1β, IL-6, IL-10, IL-12, IFN-γ, and TNF-α. (*= $p \leq 0.06$, ** = $p \leq 0.05$).

6.5. Discussion

Previous work on barn dust DNA has been valuable in suggesting a role for this nucleic acid in lung immune responses (Roy et al., 2003). However, it was limited by the use of purified DNA and challenging of PBMCs *in vitro*. The lung is a complex organ with a number of cell types that all contribute to the overall response seen to exposure to a host of lung irritants and infectious organisms. Therefore, an animal challenge model is needed to closely mimic whole organ and whole barn air complexity when attempting to address the possible role of TLR-9 in barn air induced lung dysfunction. Therefore, we used a challenge model established in our previous studies (Charavaryamath et al., 2008).

We first examined cellular differences in the lungs of mice. Lung tissue protein extracts were tested for myeloperoxidase to quantify lung neutrophil levels. While no differences were apparent after 1 or 5 exposures, TLR9-deficient mice showed a significant reduction in lung neutrophils after 20 exposures compared to the WT exposure matched group. Neutrophils are critical for lung defense in response to variety of challenges (Abraham, 2003). Although neutrophils migrate during early phase of acute lung inflammation, their migration may continue over a longer period of time in chronic inflammation (Stockley, 2002). Deficiency of TLR9 may be an indirect regulator of neutrophil migration into inflamed lungs. Interestingly, we observed lower levels of TNF-α in TLR9-deficient mice at 20 days, which could be one of the causes of this reduction as TNF-α is critical for neutrophil migration (Smart & Casale, 1994). These data indicate that TLR9 may partially regulate lung inflammation in mice exposed to chicken barn air.

There was a significant reduction in the number of BAL cells obtained after 5 days of exposure compared to single day exposures for both WT and TLR9-/- animals. By 20 days of exposure, the number of cells in BAL had increased for both exposure groups, but the increase

was much greater in the WT mice. At the 20 day exposure time point there was now significantly fewer lavage cells in the knockout mice compared to WT. The primary cell type found in all BAL samples were alveolar macrophages, with over 90% of cells typically belonging to this group.

Finally, lung tissue was stained for macrophage F4/80 to resolve septal macrophages. Results showed that there was an initial significant decrease in septal macrophages after the first day of exposure in both exposed mouse strains, but that these numbers increased after five exposures. Interestingly, by 20 days the WT exposed mice had a reduction in these macrophages, however, the knockout mice remained at levels similar to those seen at 5 days exposure. This was an unexpected result, particularly in light of BAL cell counts that show a near mirror-opposite pattern over these time points.

When compared to a previous barn exposure experiment with TLR4 knockout mice (Charavaryamath et al., 2008) these changes are much weaker. Changes to BAL cell types were present after a single day exposure in that experiment whereas TLR9 knockout showed changes in total cell numbers by 20 days exposure. Similarly, there were more pronounced changes in histology, and airway hyperresponsiveness in the previous experiment. Interestingly, cytokine changes between TLR knockout and wild-type mice were seen in BAL in our experiment whereas the previous study showed changes only in serum. The greater impact of TLR4 knockout on response of mice to barn air was expected given the number of papers that have shown how important the TLR4 ligand LPS is to barn air induced lung inflammation (Dosman et al., 2006; Kirychuk et al., 2006; Senthilselvan et al., 2009). However, use of different mouse strains and type of barn (pig versus chicken) may account for some of the other differences seen.

One possible explanation for both BAL and septal macrophage results is that the septal macrophages exert an anti-inflammatory effect on the lung, leading to fewer BAL cells. There is evidence for this in a recent study that showed that signaling through TLR4 and TLR9 in septal macrophages induced expression of cytokines such as IL-10 and generally induced an anti-inflammatory response (Hoppstadter et al., 2010). Yet another possibility is that migration of macrophages into the alveolar space requires a transition through the alveolar septa (Landsman & Jung, 2007). If this is the case then a reduction in macrophages at one time point in the septa being mirrored by an increase in the BAL could be a reflection of the increased movement of these cells into the alveoli. This however still raises the question of the reason for such an increased migration.

Finally, we examined a panel of cytokines known to be produced by DNA binding TLR9 (Klinman, 2006) with the expectation of a reduction in one or more of these in the knockout animals. Indeed after a single day exposure there was a significant drop in serum TNF-α in knockout mice compared to WT. After five exposures, we saw a significant reduction in TNF-α and IFN-γ in lung BAL and TNF-α in serum. After 20 exposures only IFN-γ remained significantly reduced in knockout mice, with none of the animals producing detectable levels of the cytokine in their lungs. It should be noted that the reduction in IFN-γ at 20 exposures was below levels of unexposed animals. This would suggest that exposure of TLR9-deficient mice to barn air result in specific inhibition of this cytokines that requires further work to address this issue.

Further considerations exist for the phenomenon of induced tolerance to repeated exposure to immune-stimulatory molecules such as LPS and CpG DNA (De Nardo et al., 2009; West & Heagy, 2002). As a wide variety of exposure systems have been employed, it can be hard to draw

firm comparisons, particularly as many do not extend beyond one to two days exposure. Similarly, while many point to a decrease in cellularity and TNF-α (West & Heagy, 2002), others note that these differences are less apparent in systems employing organic particulates (Schwartz et al., 1994). One possibility is that unmethylated DNA in these organic dusts may be acting to increase TNF-α levels that would otherwise drop due to LPS tolerization.

Barn air is a complex mix of dust and numerous microbial components that can induce lung inflammation. This study was conducted to determine the role of TLR9 and indirectly that of unmethylated DNA in lung inflammation induced following exposure chicken barn air. Because TLR9 signaling can induce expression of TLR4 and TL2 (Luyer et al., 2007), both of which may be critical in responding to bacterial components in barn air, part of the responses seen in TLR9-deficient mice may be due to effects on TLR2 and TLR4 signaling. The same also applies for TLR4, which can increase expression of TLR9 (An et al., 2002). Therefore only a true environmental exposure can really begin to fully reflect the total lung response.

Lung responses to bacterial DNA will depend on the amount of inhaled DNA. Attempts to purify DNA from barn dust from a previous experiment typically produced 1-2μg of DNA from a filter kept in a similar barn for 8 hours (unpublished observations). Of this, a portion of recovered DNA will be methylated eukaryotic DNA, further reducing the amount of stimulatory DNA. Although some differences between WT and TLR9-deficient mice were noticed after single exposure, the divergence in immune responses between mouse populations at 5 days may indicate a requirement for exposure to a sufficient dose of stimulatory DNA that the mice do not see in a single day. Indeed, as Roy and colleagues found, 10μg of barn dust were where responses were detectable (Roy et al., 2003), which corresponds to approximately the dose encountered by the mice after 5 exposures. This accumulated effect though would have to result

from continued exposure, not an accumulation of DNA within the tissue, as other studies have shown that internalized DNA is rapidly degraded (Kawabata et al., 1995).

Recent work has shown that HMGB1 can bind bacterial DNA and increase responses to this DNA (Ivanov et al., 2007; Tian et al., 2007). Still other work has shown that the RAGE can bind HMGB1 coated DNA and greatly increases responses to this DNA (Tian et al., 2007). Because of robust expression of RAGE in the lung including type-I and type-II cells, this receptor could be of particular importance in bacterial DNA induced lung inflammation (Brett et al., 1993; Fehrenbach et al., 1998; Katsuoka et al., 1997). The possibility also exists for expression in vascular endothelium, monocytes/macrophages, and alveolar macrophages (Brett et al., 1993; Schmidt et al., 1993). It is possible that RAGE and HMGB1 may participate in TLR9-mediated lung responses to low doses of bacterial DNA (Abraham et al., 2000).

Thus, we conclude that immune responses seen on single and multiple exposures to poultry barn air are probably complex involving signaling through a number of receptors and to a variety of microbial components. While others have shown endotoxin and TLR4 to be a major part of this immune response, we provide first evidence of TLR9's role in the regulation of lung inflammation following exposure to poultry barn air.

CHAPTER 7: GENERAL DISCUSSION AND FUTURE DIRECTIONS

The main focus of my research was to investigate the role of TLR9, and by extension unmethylated DNA, in the lung in response to barn air. Because the role of an immune receptor in inflammation is largely a function of its cellular expression, I first documented the expression of TLR9 in intact lungs from various species including humans. Having established expression of the receptor, I proceeded to examine cytokine and cellular changes in normal as well as TLR9$^{-/-}$ mice exposed to chicken barn air.

In the first experiment, I characterized TLR9 expression in dog, pig, cattle and horse with the use of multiple methods. Very little information exists on the expression of TLR9 in any of these species, and that little information largely comes from mRNA expression studies on tissue homogenates. First, I adapted and characterized a TLR9 antibody for use in these species because no antibodies are available that were specifically raised for use in these species. The data showed a similarity in TLR9 expression in the lung of these four species. TLR9 was detected in bronchial epithelium, vascular endothelium, alveolar septal cells, and alveolar macrophages. I deliberately used immuno-electron microscopy to resolve sub-cellular localization of TLR9 and to precisely distinguish the alveolar septal cells. Electron microscopy confirmed the identity of many of these cells, and further showed subcellular localization not just in the cytoplasm as has been more widely reported (Ahmad-Nejad et al., 2002; Latz et al., 2004), but also in the nucleus as well as the plasma membrane. Examination of the septal cells showed that type-II cells as well as macrophages expressed TLR9. TLR9 expression was also noticed in pulmonary intravascular macrophages which are a unique highly phagocytic population of macrophages in cattle, pig and horses. It is interesting to note that depletion of PIMs in horses led to a significant decline in the total lung TLR9 mRNA to underscore the extent of TLR9 expression in these cells. This decline

in total TLR9 is similar to that observed for TLR4 in horses following depletion of PIMs (Singh Suri et al., 2006). Interestingly, horses treated with *E. coli* LPS showed an increased expression of TLR9 (Schneberger et al., 2009). This suggested that the PIMs are major contributors of TLR9 expression in the lung and may also play a crucial role in the lungs of horses and cattle, which are housed in barns. The data on TLR9 expression in PIMs are even more relevant considering that PIMs may be recruited into lungs of humans under physiological stress (Thenappan et al., 2011).

Second, mouse and human lungs were assessed for TLR9 protein and mRNA expression. Similar to all domestic species studied, TLR9 was seen in the bronchial epithelium, vascular endothelium, alveolar septa, alveolar macrophages, and type-II cells. Similar subcellular localization was also seen. Protein expression data were further confirmed in both species by *in situ* hybridization for TLR9 mRNA. This further confirmed TLR9 expression in alveolar macrophages, a fact which has been disputed (Fernandez et al., 2004; Kiemer et al., 2008; Suzuki et al., 2005). An examination of asthmatic lungs showed an increase in TLR9 expressing cells in the septa, probably due to influx of known TLR9-positive cell types such as neutrophils, macrophages, eosinophils, and expansion of mast cell population (Hoppstadter et al., 2010; Ikeda et al., 2003; Jozsef et al., 2004; Mansson & Cardell, 2009). The influx of many cells expressing TLR9 would increase the lung burden of TLR9 and make it more sensitive to environmental unmethylated DNA.

Nuclear localization of TLR9 shown here in many cell types has not previously been reported. This may be that to our knowledge immuno-electron microscopy has not been tried with TLR9 previously. Most data on TLR9 localization is from confocal microscopy (Ahmad-Nejad et al., 2002; Latz et al., 2004), where low nuclear fluorescence may not be seen, or

discounted due to higher cytoplasmic expression. While more work needs to be done to test these possibilities, it should be noted that MyD88 has been shown in other studies to be present in the nucleus (Jaunin et al., 1998), suggesting that parts of the MyD88 pathway may occur within this space. Alternate signaling pathways may also exist. While the function of TLR9 nuclear surveillance is unknown, it is possible that monitoring for viruses such as herpesvirus may be accomplished in part by a TLR9-mediated mechanism (Paludan et al., 2011).

Having established the expression of TLR9 in whole lung tissues, I then exposed wild-type as well as TLR9-deficient mice to barn air for 1, 5, or 20 days. Several cytokines were chosen that were consistent with a Th1 mediated response, which unmethylated DNA was known to induce, as well as IL-10, a known TLR9 induced inflammation inhibitory cytokine (Ramaprakash and Hogaboam, 2010). While few cytokines showed any notable differences, TLR9 deficient mice showed reduced TNF-α and IFN-γ expression at 5 days and reduced IFN-γ at 20 days compared to their exposed wild type counterpart. Examination of cellular changes revealed that by 20 days there were significant reductions of neutrophils and BAL cells in the TLR9-deficient animals along with an increase in septal macrophages. Septal macrophages, which may include both interstitial and intravascular macrophages, are a cell population shown to have anti-inflammatory effects in response to several innate immune receptor ligands (Hoppstädter et al. 2010). The data shows a reduction in the number of type-II cells in TLR9 deficient mice exposed for 5 or 20 days compared to wild type (Sethi and Schneberger, submitted). This is the first study to examine the role of TLR9, and by extension unmethylated DNA in a real environmental exposure setting compared to studies using cultured cells and purified DNA (Roy et al., 2003). The culture and purified DNA system does not account for

stimulation and possible synergism from other microbial components, nor particular features of the lung that may enhance or inhibit such.

In summary, there is a significant amount of similarity among species in the types of lung cells that express TLR9 and are capable of responding to unmethylated DNA. It is reasonable to suspect, therefore, that the changes in immune response seen in response to TLR9 knockout mice exposed to barn air may not only be an issue for human barn worker health, but also for animals housed in such facilities that may have elevated dust and bacterial levels.

Having just established that TLR9, and by extension bacterial DNA play a role in lung inflammation due to barn air exposure there are many aspects which now need to be explored. First, what are the mechanisms responsible for decreased IFN-γ and TNF-α levels? One theory is that lack of direct TLR9 signaling may be responsible. Alternately, a reduction in TLR9 signaling may result in reduction of levels of expression of other TLRs (An et al., 2002; Luyer et al., 2007). Second, if TLR9-expressing type-II cells are altered by exposure to chicken barn air (Sethi and Schneberger, submitted) leading to changes to the surfactant, is there an effect on immunity or breathing? Third, we have only examined a few cytokines, but are there changes to other cytokines and chemokines such as IFN-α/β, IP-10 and CXCL1? There also is a need to examine the mechanisms through which TLR9 regulates migration of neutrophils and also the response of animals to secondary challenges after recruitment of inflammatory cells expressing TLR9. Lastly, the horse data showing increased expression of TLR9 following administration of LPS raises the question of if there are alterations in expression of multiple TLRs in animals deficient in a single TLR?

Another set of questions revolves around DNA dose. As mentioned in Chapter 6, the suspected daily dose of unmethylated DNA stimulatory motifs is quite small. An experiment

using isolated dusts re-aerosolized in water with increasing concentrations of eukaryotic (non-stimulatory) and non-eukaryotic DNA (stimulatory) could be given over 5 days to approximate increased or decreased DNA input from different sources. This may also suggest means for reducing responses to bacterial DNA if non-stimulatory or inhibitory DNA could be used to reduce such pro-inflammatory DNA responses.

Additionally, work by others (Senthilselvan et al., 2009) has shown that polymorphisms in the TLR4 gene have been linked to increased inflammation in response to barn air. By showing that TLR9 knockout mice show reduced indicators of inflammation over time, it is reasonable to conclude that TLR9 mutations may have similar effects on lung response in barn workers and should be tracked. That these changes to TLR9 knockout were apparent over prolonged exposure further suggests that such studies of worker illness in response to barn air will need to be conducted over longer time periods, and that these responses undergo an evolution of response that may rely on some receptors earlier in exposure, but on others over time.

Finally, recent work with HMGB1 and RAGE have posed new questions about their role in such lung responses. Double knockout mice for TLR9 and RAGE could be examined for their response to barn dusts or aerosolized DNA from different sources.

Our results in mice suggest that humans exposed to environments containing elevated levels of bacterial molecules, such as unmethylated DNA may see an increase in lung inflammation. This has been shown in the past to be the case with LPS and TLR4 (Senthilselvan et al., 2009; Dosman et al., 2006), but we show that knockout of TLR9 can reduce cytokines as well as influx of neutrophils into the lung over longer periods of exposure. Similarly, these findings point to the possibility of TLR9 receptor mutations contributing to or hindering

responses to environmental bacterial products, possibly similar to what is seen with TLR4 (Senthilselvan et al., 2009). Finally, the expression of TLR9 in a number of cell types in the lungs of mice, humans, and veterinary species suggest that a number of cell types may be sensitive to bacterial DNA and thus may contribute to inflammation due to exposure to this ligand. The similar expression across multiple species further suggests that TLR9 may play a role in lung inflammation in a number of veterinary species as well.

Taken together these findings confirm that there is a degree of similarity of TLR9 expression across different species. Further, when TLR9 was knocked out in mice exposed to barn, there were several changes in their immune responses such as altered cellular and cytokine levels, showing that TLR9 signaling does play some role in the lung immune responses seen when mice are exposed to barn air.

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