IMMUNE RESPONSE IN *RHODOCOCCUS EQUI* INFECTED FOALS.

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ABSTRACT

Rhodococcus equi (*R. equi)* is an intracellular, gram-positive coccobacillus that causes pneumonia in foals aged 2 to 4 months. Neonatal foals are susceptible to *R. equi* infection probably due to inefficient Toll-like receptor (TLR)-2 signaling and inability to produce interferon gamma. One of the reasons for inefficient receptor signaling and recognition of *R. equi* by the foal's immune system may be the inefficient sequestration of TLRs in lipid rafts, which act as signaling platforms. However, there are no protocols to isolate lipid rafts from equine cells and, therefore, no data on the association of TLRs with the lipid rafts in the lung cells of normal and infected foals. Because of the clinical importance of the disease, there is considerable interest in developing effective prophylactic methods, which in turn requires a better understanding of fundamental immunology of the foals. In this study, I have examined the effect of *R. equi* vaccination on the lung inflammation induced following challenge with *R. equi*. I also developed a protocol to isolate lipid rafts from broncho-alveolar lavage (BAL) cells and investigated the association of lipid rafts with TLRs.

In the first study, 15 mixed breed draft-type foals up to 7 weeks of age were studied. The foals were divided into control (n=7) and a vaccinated (n=8). The control foals were given 10 mL phosphate buffered saline intramuscularly while the vaccinated group was vaccinated on day 0 of the study followed by a booster on day 14. All the foals were challenged with *R. equi* ($5x10^6$ cells/mL into the dorso-caudal region of the right lung lobe). BAL was performed on day 14, 28 and 35 and all the foals were euthanized on day 49 of the study.

The study design did not leave any non-infected foal at the end of the experiment. Therefore, lung samples were obtained from two untreated control (non-vaccinated non-infected) foals from the Department of Veterinary Pathology, University of Saskatchewan were used. The data showed similar levels of lung inflammation in both the control and vaccinated foal groups based on BAL cytology, gross pathology and histopathology. Gross and histopathological studies indicated that both control and vaccinated foals developed granulomatous lesions. Immunohistology showed increased expression of TLR4, TLR2 and TNFα in alveolar septa and in some cases in the vascular endothelium and airway epithelium in the lungs of both groups compared to the untreated

control foals. Western blots showed increased expression of TLR2 but not TLR4 in the lung extracts from both the vaccinated and the control foals. Vaccinated foals showed higher concentrations of TNFα ($p=0.0219$) in their BAL on day 28 but lower concentrations of IL-10 ($p=0.0172$) in their lung extracts collected on day 49 compared to the controls. There were no differences in IFNγ and protein concentrations between the two groups.

To understand the role of lipid rafts in TLR4 and TLR2 signaling, I developed an efficient and simpler protocol to isolate lipid rafts from BAL cells of foals and confirmed their identity by localizing Flotillin-1 and GM-1 (fractions 6-9), which are lipid raft markers, and transferrin receptor (fractions 1-4) which is present in non-lipid raft fractions. Lung macrophages from naïve foals lacked sequestration of Flotillin-1 and GM-1 in the higher fractions compared to the vaccinated foals. Further, the data showed that while TLR4 and TLR2 were localized in most of the fractions (1-9) in control foal BAL collected on day 14 and 28, the TLR4 and TLR2 association was restricted to fractions 6-9 in the lipid rafts isolated from BAL cells of vaccinated foals. These data suggest that BAL cells of neonatal foals may not have effective signaling machinery because of lack of association of TLR2 and TLR4 with lipid rafts.

Taken together, the data show similar levels of lung inflammation in the control and vaccinated foals upon infection with *R. equi*. The vaccination, however, appeared to have some effect on the immunohistologic expression of TLR2, TLR4 and TNFa in the lung tissues, and increased association of TLR2 and TLR4 with the lipid raft fractions. Based on the higher expression of TNFα and lower expression of IL-10, the vaccinated foals may be more competent to mount an immune response against *R. equi*.

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Dedicated to my husband

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LIST OF ABBREVIATIONS

BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
ELISA	Enzyme linked immunosorbent assay
GM1	Ganglioside asialo
IFN?	Interferon gamma
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL10	Interleukin 10
n	Number
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
R. equi	Rhodococcus equi
TLR2	Toll-like receptor 2
TLR4	Toll-like receptor 4
TNFa	Tumor necrosis factor
VapA	Virulence associated protein A
vWF	Von Willebrand factor

CHAPTER 1: INTRODUCTION

Animals regularly encounter pathogens that are eliminated by a complex set of immune mechanisms. The immune system consists of two compartments called the innate (natural/native) and the adaptive (acquired) immune systems. The innate immune response is short lived and retains no memory of exposure to antigen. The adaptive immunity encompasses the defense mechanisms providing protection against various pathogens encountered during life and recognizes them on subsequent exposure (Giguere *et al.*, 2005).

Granulocytes, also called polymorphonuclear cells (PMN), and macrophages play pivotal roles in protection against infections immediately after birth. Foals are immune-competent at birth although transfer of maternal immunoglobulin to the fetus is restricted by the epitheliochorial placentation (Giguere *et al.*, 2005). However, at birth foals do not have fully functional lymphoid tissue and macrophages lack phagocytic functions (Giguere *et al.*, 2005). Although the pulmonary immune defense system develops much slower than peripheral blood cells (Giguere *et al.*, 2005), it acts as a reservoir for recruitment of a greater number of lymphocytes (T and B lymphocytes) than peripheral blood (Blunden *et al.*, 1999). It is believed that lack of a fully functional immune response makes foals susceptible to infection by various bacteria and viruses.

Rhodococcus equi (R. equi), a gram positive coccobacillus, is a facultative intracellular bacterium that causes pulmonary infections in foals resulting in pyogranulomatous lesions (Darrah *et al.*, 2004; Muscatello *et al.*, 2009). It is believed that foals between 2-4 months of age are most susceptible to disease caused by *R. equi* infection (Prescott, 1991; Giguere *et al.*, 1997; Chaffin *et al.*, 2003). As well, *R. equi* has been identified as an opportunistic pathogen of environmental origin, which can infect immunocompromised humans (Perez *et al.*, 2002; Meijer *et al.*, 2004; Muscatello *et al.*, 2009). The impact of *R. equi* pneumonia on the equine industry is significant (Toyooka *et al.*, 2005). In the United States, respiratory disease ranked as the third most common cause of disease in foals less than 6 months of age (Anonymous, 2006). To date, there is no effective vaccine available against *R. equi* infection (Lopez *et al.*, 2002). *R. equi* has the ability to persist, replicate within macrophages and kill them (Hondalus *et al.*, 1994) due to mycolic acid-enriched lipids in the cell wall (Barry *et al.*, 1998; Meijer *et al.*, 2004). We lack a precise understanding of immune responses in foals infected with *R. equi* with or without vaccine.

The cell membrane is a semi-permeable lipid bilayer. It is comprised of a variety of biological molecules, primarily proteins and lipids, which are involved in a vast array of cellular processes such as ion conductance, cell adhesion and cell signaling. The cell membrane contains cholesterol and glycosphingolipid-rich microdomains called lipid rafts. Lipid rafts are a few nanometers to a few hundred nanometers in diameter (Pralle *et al.*, 2000; Hancock, 2006), and are heterogeneous and dynamic microdomains that compartmentalize cellular processes. They are enriched in cholesterol, sphingolipids and transmembrane proteins and represent up to 50% of the cell membrane (MacDonald, 1980; Hanada *et al.*, 1995; Fridriksson *et al.*, 1999; Brown *et al.*, 2000; Horejsi, 2003). Lipid rafts play a key role in cell signaling, activation of monocytes and macrophages, and normal functioning of the digestive, respiratory and immune system (Drevot *et al.*, 2002). Foals are susceptible to many pulmonary diseases and there is no information on the lipid rafts in their cells such as alveolar macrophages which are central to pulmonary immune responses. There are no data on the isolation of lipid rafts and association of TLR2 and TLR4 with lipid rafts from foal lung lavage. Because of the role of TLR2 in the engagement of *R. equi* by alveolar macrophages (Darrah *et al.*, 2004), it is important to understand the role of alveolar macrophage lipid rafts in the aggregation of TLR2 and subsequent interactions with *R. equi*.

CHAPTER 2: LITERATURE REVIEW

2.1. Prevalence and significance of *R. equi* infection in foals

R. equi is gram positive coccobacillus, facultative intracellular bacterium that has been known as a pulmonary pathogen of horses for more than 80 years (Vyslouzil *et al.*, 1984). *R. equi* causes pyogranulomatous (Darrah *et al.*, 2004; Muscatello *et al.*, 2009) pneumonia in foals between 2 and 4 months of age (Zink *et al.*, 1986; Yager, 1987; Prescott, 1991; Giguere *et al.*, 1997; Chaffin *et al.*, 2003). *R. equi* has been identified as an opportunistic pathogen of environmental origin, which can infect immunocompromised humans, especially AIDS patients (Prescott, 1991; Drancourt *et al.*, 1992; Mosser *et al.*, 1996; Perez *et al.*, 2002; Meijer *et al.*, 2004; Muscatello *et al.*, 2009). The impact of *R. equi* pneumonia on the equine industry is significant (Toyooka *et al.*, 2005).

2.2. Ecology

Different geographic areas possess different *R. equi* serotypes (Makrai *et al.*, 2008). There is high density (17.3 times) of this bacterium in densely populated stables compared to paddocks (Muscatello *et al.*, 2006) and soil contaminated with herbivore manure, as the bacterium has a predilection for volatile fatty acids (Prescott, 1991), and in stables with poor ventilation especially in warm climates (Prescott, 1991; Hondalus,1997; Prescott *et al.*, 1997; Giguere *et al.*, 1999; Muscatello *et al.*, 2006; Cohen *et al.*, 2008; Flaminio *et al.*, 2009). However, bacteria can also be transmitted from the breath of infected foals (Muscatello *et al.*, 2009). Infection rates of 1-10% and a mortality of less than 1% has been reported from Australian farms while nearly 47% of 138 horse breeding farms in the USA were found to be infected (von Bargen *et al.*, 2009). The incidence of infection increases mostly in summer climate when the temperature is favorable for the growth of bacteria, the dusty environment initiates the aerosol transport of contaminated dust particles (Meijer *et al.*, 2004) and the soils are favorable for growth of bacteria (Muscatello *et al.*, 2006). Manure acts as reservoir of infection as the volatile fatty acids present in equine feces enhance the replication of *R. equi* by 10,000 fold (Hughes *et al.*, 1987) and the bacteria are recovered from the intestinal tract of healthy horses (Woolcock *et al.*, 1980).

The surrounding environment, higher foal density and increased numbers of transiently infected mares play an important role in the spread of infection (Grimm *et al.*, 2007). Infected foals shed higher numbers of *R. equi* in their feces compared to the adult horses, and this shedding is the major cause of contamination of the soil (Prescott *et al.*, 1984). After 7 weeks of age, the shedding is reduced because of the development of gut immunity (Takai *et al.*, 1986). *R. equi* exists in virulent and non-virulent strains, and the virulent strain is characterized by its ability to persist, replicate within alveolar macrophages and kill them (Hondalus *et al.*, 1994). Survival of *R. equi* is dependent upon carbon from lipids (Vazquez-Boland *et al.*, 2009) and organic acids such as proprionate or acetate (Hughes *et al.*, 1987) derived from herbivores manure (Barton *et al.*, 1984; Prescott, 1987). *R. equi* also requires low levels of divalent cations such as iron (Fe^{2+}), calcium (Ca^{2+}), magnesium (Mg^{2+}) for its survival and expression of VapA on its surface (Takai *et al.*, 1992; Takai *et al.*, 1996; Benoit *et al.*, 2001; Benoit *et al.*, 2002; Jordan *et al.*, 2003; Ren *et al.*, 2003) The primary route of infection is inhalation of dust particles from surroundings that are contaminated with *R. equi* from feces (Takai *et al.*, 1986; Prescott,1991; Mosser *et al.*, 1996; Muscatello *et al.*, 2009). Prolonged oral exposure to *R. equi* leads to intestinal lesion in infected foals (Johnson *et al.*, 1983).

R. equi is endemic on certain farms and sporadic on many farms. There is considerable variation in the severity and prevalence of this disease. Recent epidemiological data have shown no relationship between preventive measures such as use of hyper-immune serum and prevalence of disease due to *R. equi* (Muscatello *et al.*, 2007).

2.3. Clinical signs

The classical form of the disease, commonly referred as “rattles”, was mostly observed in the past and only in the highly advanced cases of disease. This particular manifestation shows high fever, increased counts of neutrophils in broncho-alveolar lavage (BAL) and mucus and purulent discharge from the nostrils (Muscatello *et al.*, 2007). Infection with *R. equi* is most commonly characterized by a subacute to chronic bronchopneumonia. Foals in the early stages of the disease show fever (rectal temperature 38.8-40^{0}C, up to 41^{0}C), increased respiratory rate, nasal discharge, neutrophil infiltration into the lungs and formation of giant cells. The advanced stages of the disease show necrosis in the pulmonary parenchyma, development of

granulomatous lesions, infection of lymph nodes, and along with these signs, some foals also suffer from severe diarrhea with ulcerative enteritis (Cimprich *et al.*, 1977; Zink *et al.*, 1986; Yager,1987; Prescott,1991; Giguere *et al.*, 1997). On auscultation, abnormal lung sounds such as inspiratory and expiratory wheezes and crackles are audible in foals infected with *R. equi* (Giguere *et al.*, 1997). Furthermore, about 50% of *R. equi* infected foals showed intestinal manifestations, characterized by typhlitis and multifocal ulcerative enterocolitis along with granulomatous or suppurative inflammation of mesenteric and colonic lymph nodes (Zink *et al.*, 1986). In the chronic phases of the disease, peritonitis may result and *R. equi* can be isolated from the peritoneal fluid (Morton *et al.*, 2001). Infected foals may show stiff gait due to development of septic arthritis/osteomyelitis, especially in the tibiotarsal and stifle joints (Sweeney *et al.*, 1987). Other immune mediated conditions such as uveitis may also develop. The total white blood cell count (≥13,000/μL) and serum fibrinogen concentration (≥600mg/dL) are indicative of severe infections and should prompt further diagnostic testing (Heidmann *et al.*, 2006).

Depending upon the clinical manifestation, for diagnosis fecal culture and abdominal ultrasonography are helpful for diagnosis (Chaffin *et al.*, 2003). However, bacterial cultures from tracheobronchial exudates and cytological examinations are more reliable tools (Giguere *et al*., 1997).

2.4. Vaccination against *R. equi*

Antibiotic therapy for the disease caused by *R. equi* is not effective in all the foals and is prolonged and expensive. So, there is a need to develop an effective vaccine against *R. equi* infections. It is clearly understood that neither heat killed nor DNA-based vaccines are effective against *R. equi* infection (Lopez *et al.*, 2003; Taouji *et al.*, 2004; Haghighi *et al.*, 2005) . Vaccination strategies against *R. equi* are still under investigation especially in young foals as successful immunization against *R. equi* is not yet developed (Pei *et al.*, 2007; Lopez *et al.*, 2008). Both innate and acquired immune mechanisms are required to clear *R. equi* infection. Therefore, live attenuated vaccines might be an effective strategy of protection against this infection (Muscatello *et al.*, 2007). Some protection against experimental *Rhodococcus* infection in mice has been demonstrated by the use of a attenuated strain of *Salmonella* expressing *VapA* protein (Oliveira *et al.*, 2007).

2.5. Pathogenesis involved in *R. equi* infection

Foals are exposed to *R. equi* through various routes such as the respiratory (most common route), intestinal and genital route (Yager, 1987). In the early stages of disease development following oral route of infection, the bacteria reside in the intestine of the infected foals (Takai *et al.*, 1986). Following inhalation of the bacterial, alveolar macrophages form the first line of defense along with the epithelial lining of the airways. The phagocytosis of *R. equi* by the alveolar macrophages is increased in the presence of opsonins and complement fragments (von Bargen *et al.*, 2009). The bacteria are transported into lysosomes leading to the development of phagolysosomes. The bacteria are killed through the activation of various hydrolytic enzymes (proteases) at low pH in the phagolysosomes and through the actions of reactive oxygen species (hydrogen peroxidases, nitric oxide, superoxide radicals) (Haas, 2007). Through multiple mechanisms, some still to be fully elucidated, *R. equi* manages to evade these usually potent anti-bacterial mechanisms in the macrophages. The phagosomes containing virulent *R. equi* mature normally but lose early markers such as cathepsin-D followed by appearance of late molecular markers such as lysosomal associated marker protein 1 (LAMP-1). These *R. equi* containing vacuoles do not proceed to late endocytic organelles, nor do they fuse with lysosmes or acquire proton-pumping vacuolar ATPase (Fernandez-Mora *et al.*, 2005; Toyooka *et al.*, 2005). This allows the bacteria to escape toxic enzymes and continue replication inside the cells (Takai *et al.*, 1985; Samies *et al.*, 1986; Hietala *et al.*, 1987). The survival of *R. equi* is due to the presence of a lipid rich cell wall, especially enriched in mycolic acids, which shield it from adverse environmental conditions such as oxidative stress and low pH within macrophages (Barry *et al.*, 1998; Meijer *et al.*, 2004). As the disease progresses, the vacuoles containing the bacteria become multilobed and filled with vesicles, but still remain intact (Fernandez-Mora *et al.*, 2005). After 8 hours of infection, degradation of the host cell occurs, releasing lysosomal material into macrophage cytoplasm and thereby causing macrophage necrosis (Zink *et al.*, 1987; Hondalus *et al.*, 1994; Luhrmann *et al.*, 2004) and granuloma formation in the lungs (Luhrmann *et al.*, 2004; Meijer *et al.*, 2004). The presence of *R. equi* in the lungs in the early stages of disease leads to inflammation characterized by the migration of neutrophils and monocytes followed by development of giant cells (Johnson *et al.*, 1983). With progression of the disease, the lung parenchyma becomes necrotic, followed by affection of bronchial and mesentric lymph nodes (Zink *et al.*, 1986; Yager, 1987). Neutrophils are effective in killing extracellular bacteria and link

the innate and adaptive immunity (Meijer *et al.*, 2004; Nerren *et al.*, 2009). Neutrophil migration into the lungs is regulated through the expression and engagement of adhesion molecules in the lung capillaries; these processes have not been investigated in horses (Lazarus, 1986; Downey *et al.*, 1993; Guo *et al.*, 2002; Soethout *et al.*, 2002). Shortly after the onset of vascular changes, the migrated neutrophils initiate phagocytic activity and thus release enzymes that damage the tissue, induce production of chemokines to attract more inflammatory cells, and produce pro-inflammatory cytokines (TNFα, IL-1α, IL-1β, IL-6) to increase inflammation at the site of injury (Thacker, 2006). On the other hand, macrophages and other cells of the immune system such as fibroblasts and airway epithelial cells also become activated and release proinflammatory cytokines and increase the expression of adhesion molecules for chemotaxis of neutrophils and monocytes, and thereby further increase the inflammatory response (Thacker, 2006).

There are seven different types of 80 to 90-kb plasmid, such as VapA and VapC to VapH (Takai *et al.*, 2000) in *R. equi*. The major virulence factor associated with *R. equi* is the lipid-modified virulence-associated protein A (VapA) (Takai *et al.*, 2000), which is expressed on the bacterial surface as proved by its susceptibility to trypsin digestion (Lazarus,1986; Burns *et al.*, 1996; Murtaugh *et al.*, 1996), especially between 34°C and 41°C. Plasmid cured derivates of *R. equi* strains become unable to replicate and persist inside macrophages (Giguere *et al.*, 1999) and are incapable of inducing pneumonia in foals as they are easily cleared by pulmonary immune defense mechanisms (Wada *et al.*, 1997; Giguere *et al.*, 1999). On the other hand, VapA can induce activation of Toll-like receptor 2 (TLR2) and is essential for the replication of the bacteria inside the macrophage, which is one of the most important mechanisms employed by *R. equi* to evade the host immune response (Jain *et al.*, 2003; Darrah *et al.*, 2004). Interestingly, there is evidence of enhanced susceptibility of neonatal foals to *R. equi* due to inefficient TLR2 signaling and activation of NF-κB and production of inflammatory cytokines (Darrah *et al.*, 2004). The ability of *R. equi* to persist in macrophages is fundamental to its escape from elements of the pulmonary defense (Giguere *et al.*, 1999; Jain *et al.*, 2003; Meijer *et al.*, 2004;Takai *et al.*, 1985; Samies *et al.*, 1986; Hietala *et al.*, 1987). The low number of resident alveolar macrophages (Zink *et al.*, 1984) and their reduced ability to kill bacteria (Zink *et al.*, 1985) may contribute to a higher incidence of *R. equi* infection in young foals (less than 20 days).

2.6. Pulmonary immune response to *R. equi* infection

It is generally accepted that the immune system in the lungs of young foals is not fully developed compared to adult horses. However, precise information on the specifics of immune system development in foals is not available. Foals mount a type 2 immune response and are susceptible to *R. equi* infection (Boyd *et al.*, 2003; Hooper-McGrevy *et al.*, 2003; Breathnach *et al.*, 2006). Foals with a mature immune system can resist and clear *R. equi* infection (Mosser *et al.*, 1996). Expression of IFNγ in foals starts at 1 week of age and reaches its peak level at approximately 3 months of age (Breathnach *et al.*, 2006). Thus, susceptibility to intracellular pathogens such as *R. equi* is likely due to the inability of foals to mount an adequate Th-1 immune response and also due to lack of TLR 2 expression on alveolar macrophages (Darrah *et al.*, 2004). In addition, the role of cell-mediated immunity is important in clearance of *R. equi* (Mosser *et al.*, 1996), and there is a requirement for a balanced Th-1 (cell mediated) and Th-2 (humoral) immune response (Hines *et al.*, 1997). T cell-mediated immunity plays a significant role in the clearance of intracellular bacteria through cytotoxicity against infected cells (macrophages). There is an increase in expression of pro-inflammatory cytokines such as TNFα and anti-inflammatory cytokines (IL-10, and IL-12), while expression of IFNγ remains unaltered in foals infected with a virulent strain of *R. equi* (Giguere *et al.*, 1999). These cytokines play a significant role in cell to cell communication as they act as signaling molecules (Benton *et al.*, 1988).

Pulmonary alveolar and intravascular macrophages in the horse lung produce inflammatory cytokines such as TNFα and IL-1β (Baarsch *et al.*, 1991; Parbhakar *et al.*, 2005). Inflammatory cytokines are produced by activated cells and these cytokines in turn activate phagocytic cells to eliminate bacteria. However, excessive levels of TNFα are detrimental to the health of the individual as increased levels lead to severe damage to the lung parenchyma, cardiopulmonary shock and eventually death of the animal (Cohen, 2002; Parbhakar *et al.*, 2005). These cytokines activate endothelium, induce increased expression of adhesion molecules and lead to recruitment of inflammatory cells such as neutrophils (Benton *et al.*, 1988; Murtaugh *et al.*, 1996; Stylianou *et al.*, 1998). TNFα induces the production of IL-6 from macrophages, fibroblasts, endothelial cells and smooth muscle cells (Cohen, 2002). IL-6 stimulates acute phase response proteins such as increased levels of transferrin and regulates the specific humoral

immune response (Muraguchi *et al.*, 1988; Takatsuki *et al.*, 1988; Van Snick *et al.*, 1988; Murtaugh *et al.*, 1996). Depending upon the inflammatory stimulus by *R. equi* and immune response of the host, granulomas, fibrin deposition, hypertrophy of smooth muscle and bronchoconstriction occurs (Schluger, 2005).

Although considerable progress has been made in our understanding of pathogenesis of *R. equi* infection in the foals, the exact mechanisms underlying the ability of *R. equi* to infect foals and the inability of foals to eradicate the bacteria are not known. One of the possibilities may be the ability of *R. equi* to "silently" enter the macrophages or the immaturity of monocytes and macrophages in the lungs of foals to recognize *R. equi*. Recent evidence has alluded to the role of TLR2 in the engagement of *R. equi* and activation of immune cells (Darrah *et al.*, 2004). The mere presence of TLR2 on plasma membrane is not sufficient to activate the cells. There is a requirement that TLR2 molecues are brought in close proximity through the actions of lipid rafts for effective recognition of microbial threat, cell activation and appropriate immune response.

2.7. Structure of lipid rafts: Biochemical components and size

The plasma membrane contains 26% phosphatidylcholine, 24% sphingomyelin, and 12% glycosphingolipids and consists of various adhesion structures such as the cellular synapses, cell to cell junctions and membrane invaginations such as caveolae and clathrin coated pits (Hietala *et al.*, 1987; Zink *et al.*, 1987). Caveolae are stable flask shaped invaginations of the plasma membrane found in various cell such as endothelial cells and adipocytes. They are rich in proteins and lipids (cholesterol and sphingolipids), contain 100-200 regulatory proteins such as caveolin-1(cav-1) and play a significant role in signal transduction (Acosta-Perez *et al.*, 2008; Lajoie *et al.*, 2009). Cav-1 regulates lipid raft dependent endocytosis (Parton *et al.*, 2007). Lipid rafts are plasma membrane domains, enriched in cholesterol, glycosphingolipids and transmembrane proteins (MacDonald, 1980; Hanada *et al.*, 1995; Fridriksson *et al.*, 1999; Brown *et al.*, 2000; Horejsi, 2003) found in all cells (Fig.2.1.). Lipid rafts are insoluble in cold detergent extraction and therefore are also known as detergent-insoluble glycolipid-enriched complexes (DIGs) or the detergent resistant membrane (DRM) (Schuck *et al.*, 2003). Lipid raft size is usually in the nanometer range (10-200 nm), and lipid rafts have a complex biochemical structure and are transient in nature. One of the functions of lipid rafts is to compartmentalize cellular processes (Hancock, 2006; Lajoie *et al.*, 2007). There are no data on the actual area of

cell surface occupied by lipid rafts (Hancock, 2006). Lipid rafts play a key role in the activation of monocytes and macrophages as many receptors and signaling proteins are present in and are aggregated by the lipid rafts. Also, major histocompatibility complex-I is associated with DRM as it interacts with proteins such as Ganglioside asialo (GM1) (Shaw, 2006). Lipid rafts are tightly packed because of the saturated hydrocarbon chains (Brown *et al.*, 2000; Rodgers *et al.*, 2005; Knorr *et al.*, 2009). Many proteins such as CD14, which are important in endotoxin-induced cell activation, are tethered via the glycosylphosphatidylinositol molecule on the outer layer of the cell membrane (Chatterjee *et al.*, 2001; Triantafilou *et al.*, 2002; Sharom *et al.*, 2004). There are examples of anchoring of signaling proteins such as Src-family kinases (e.g. Lck, Fyn and Lyn) to the inner leaflet via dual acylation modification (Brown *et al.*, 2000) and myristoylation (Bickel *et al.*, 1997; Simons *et al.*, 2000; Rajendran *et al.*, 2003). The outer shell of the membrane consists of sphingolipid and cholesterol, which is connected to the inner shell, composed of phospholipids and cholesterol, through a lipid bilayer (Rajendran *et al.*, 2003). This arrangement of proteins in the lipid rafts leads to formation of physical platforms to link the outside of the cell with the signaling pathways inside of the cell (Brown *et al.*, 1998; Simons *et al.*, 1999; Simons *et al.*, 2000). This association is brought about through clustering of lipid rafts and quickly brings proteins into proximity of each other and speeds up cell signaling and generation of an inflammatory response (Simons *et al.*, 2000). In addition to the role of lipid rafts in inflammatory responses, the rafts are also implicated in the normal physiology of digestive, respiratory and immune systems (Simons *et al.*, 2002).

There are no data on lipid rafts in foal tissues. Because of the susceptibility of foals to many respiratory diseases such as endotoxin-associated lung inflammation and pneumonia caused by infections such as *R. equi*, and considering the importance of lipid rafts in TLR-induced inflammatory cell signaling, it is important to develop protocols to isolate lipid rafts from alveolar macrophages of foals and to study their association with TLRs in normal and inflamed lungs.

HYPOTHESIS

- Vaccination against *R. equi* will reduce lung inflammation in foals challenged with *R. equi.*
- Lipid rafts associate with TLR2 and TLR4 in lung tissues and broncho-alveolar lavage cells of foals.

OBJECTIVES

The study was undertaken to investigate the following objectives.

1. To examine lung inflammation in control (non-vaccinated + *R. equi* challenge), vaccinated (vaccinated + *R. equi* challenge), untreated control foals.
2. To examine the expression of TNFα, IL-10 and IFNγ in BAL supernatants and lung tissues (vaccinated foals and control foals)
3. To examine the expression of TLR2 and TLR4 in lung tissues (untreated controls foals, vaccinated foals and control foals).
4. To develop protocol for the isolation of lipid rafts from broncho-alveolar lavage (BAL) cells of foals.
5. To study the association of TLR2 and TLR4 with lipid rafts isolated from BAL cells of foals.

CHAPTER 3: MATERIALS AND METHODS

3.1. Animals

All procedures were approved by University of Saskatchewan Animal Research Ethics Board and conducted in compliance with the guidelines of the Canadian Council on Animal Care (CCAC). A total of 15 mixed breed draft-type foals up to 7-weeks of age were used in this study. Foals were born healthy and normal between May 09, 2008 (foal 1) and June 06, 2008 (foal 15). They were kept together in one pen along with their mares, with free access to grass hay and water throughout the study (Figure 3.1). Routine physical examination that included body weight, heart rate and rectal temperature were performed on days 0, 14, 28, 35 and 49. Respiration rate was monitored twice daily. All animals were subjected to euthanasia by an overdose of Euthanyl (Pentobarbital Sodium) at the end of the study.

3.2. Vaccine

Each vaccine dose contained 100 μg of rVapA, 2.5 g of CpG ODN (class C, ODN 2395), 4 mg carbopol and 10% (v/v) Emulsigen in a total of 10 ml PBS. Control foals received 10 ml PBS. All vaccines and control injections were given intramuscularly.

3. 3. *R. equi* culture

R. equi 103+ (virulent strain) was kindly provided by Dr. J. Prescott (Department of Pathobiology, University of Guelph, ON, Canada). It possesses an 80.6 kb virulence-associated plasmid encoding for virulence-associated protein A (VapA) and other virulence-associated protein genes. The bacterial culture was grown in Phytone media supplemented with riboflavin (BBLTM Phytone TM Peptone, BD, Sparks, MD, USA) for 16 h at 37°C in an orbital shaker. The final concentration of $5x10^6$ bacteria/mL was achieved after re-suspending the bacteria in PBS and stored at −80°C. Before each treatment, serial dilutions of bacterial culture were plated on BHI plates with 1.5 % agar and colony forming units (CFU)/mL were calculated.

3.4. Study design

The study plan is summarized in Figure 3.2. Fifteen foals aged up to 7-weeks were divided into two groups. All of the even numbered foals (n=7; foal numbers 2, 4, 6, 8, 10, 12 and

14) represented the control (non- vaccinated) group and were given 10 mL PBS intramuscularly. All the odd numbered foals (n=8; foal numbers 1, 3, 5, 7, 9, 11, 13 and 15) represented the vaccinated group. Foals were between 0-6 days of age on day 1 of the study and were euthanized on day 49. The foals were examined for weight, rectal temperature, heart rate, respiratory rate, presence of cough, quality of lung sounds on inspiration and expiration and colour of oral mucous membranes on day 0, 14, 28, 35 and 49. Foals that developed unexpected clinical signs such as colic were separated from the group with their dams, examined thoroughly daily and treated as necessary or euthanized. With the exception of one foal (foal number 4), all foals completed the study. Foal 4 showed depression, colic and abdominal distension on day 42 and was euthanized on day 45.

The first vaccination against *R. equi* was given on day 0 (Vaccination 1), when foals were between 1 and 6 days of age. The booster was given 14 day after the first vaccination at the time of the first bronchoalveolar lavage (BAL). On day 28, the second BAL was performed and animals were challenged intra-bronchially with *R. equi* ($5x10^6$ cells/mL into the dorso-caudal region of the right lung lobe). The third BAL was collected on day 35. All the foals were subjected to euthanasia on day 49 to examine gross, histopathological and immunohistological changes in the lungs.

3.5. Lung tissue sampling

The different experiment groups were summarized in table 3.1 and figure 3.2. Both left and right lung lobes were analyzed grossly, weighed and photographed (dorsal and ventral views). The lung lobes were sectioned at three regions: section 1 mid-way along the length of the cranial lobe; section 2 at the level of the hilus; and section 3 half-way between the hilus and the caudal extent of the lobe. Although the challenges were done in only right lung, the histopathology showed inflammation in both the lungs. Therefore, the untreated control foal lung samples (n=2) were kindly provided by Dr. Andrew Allen, Department of Veterinary Pathology, University of Saskatchewan for lung histopathology, immunohistology and confocal studies. One of the foals (N95-1884) was a normally developed female foal, born on April 26, 1995, at about 336 days of gestation and euthanized between 4 and 10 hours after birth. The pregnant dam has undergone general anesthesia 132 days prior to parturition. The other foal (N95-2813) was a

normally developed female foal, born on July 8, 1995, at about 331 days of gestation and euthanized about 5 hours after birth.

The pregnant dam would have undergone general anesthesia 115 days prior to parturition and both the dam and fetal foal in both cases of foals has undergone abdominal surgery and received a variety of drugs, including antibiotics.

Lung sections were photographed and scored according to the following criteria: Normal (score 0) = Absence of lesions and no inflammation; Mild (score 1) = A few, widely scattered foci of inflammation; Moderate (score 2) = many clustered, small foci of inflammation or accompanied by congestion and abscess; Severe (score3) = large (50mm or greater) foci of inflammation accompanied with severe lung inflammation.

3.6. Bronchoalveolar Sampling

Table 3.1 shows the BAL sampling plan. Foals were sedated with 0.2-1.0 mg/kg xylazine (Novopharma, Markham, ON, Canada) intravenously, followed by induction with 2 mg/Kg ketamine (Wyeth-Ayerst, Saint-Laurent, QC, Canada) and 0.1 mg/Kg Diazepam (Sandoz, Boucherville, QC, Canada). After induction, an 18 gauge 1.5 inch sterile catheter was placed in a jugular vein. In order to prolong the duration of the anesthesia and to prevent coughing, 0.02 mg/Kg Butorphanol (Wyeth-Ayerst,) was given intravenously. Anesthesia was maintained with ketamine and diazepam (1/4 to 1/2 of the induction dose) or triple drip. Supplemental oxygen (2-5 L/min) was given to foals via a nasal tube during the collection of BAL to maintain oxygen saturation level above 95%. Various physical parameters such as heart rate, respiratory rate, colour of mucus membrane, capillary refill time, pulse quality and depth of anesthesia were monitored.

A 3m video-endoscope (Olympus SIF-100, Olympus Canada Inc., Markham, ON, Canada) was passed intranasally and directed into the left lung to lavage it first by instilling 500mL of warm sterile PBS solution (0.9% sodium chloride, 0.06% sodium bicarbonate, pH 6.5 Abbott Laboratories, Saint-Laurent, QC, Canada) at 37°C followed by the right lung. Immediately after BAL2 at day 28 (on the day of challenge),

Figure 3.1. Photo showing housing of foals along with their mares

we lavaged the left side first followed by the right side, then challenged the right caudal lobar bronchus intrabronchially with 10 mL of PBS containing $5x10^6$ *R. equi*.The endoscope was flushed with 50 mL of air just before removal. The aspiration of BAL fluid was done using a vaccum pump (Millipore GE, Mississauga, ON, Canada) that directed the fluid into sterile flasks on ice for further analysis (Figure 3.3). The total volume of BAL fluid administered and recovered was recorded. Foals were kept under observation. Once able to stand and walk, they were returned to their dams.

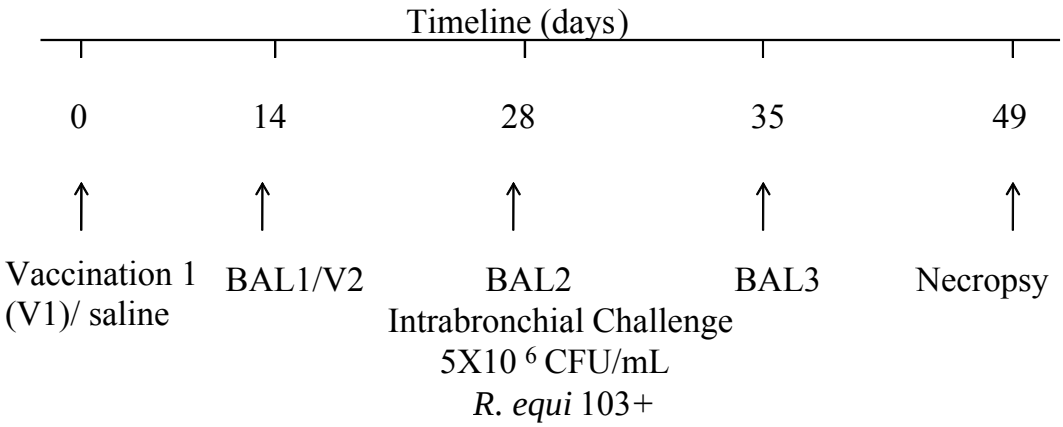

Figure 3.2. Summary of study plan

Figure 3.3. An endoscope with a suction pump

BAL Sampling	BAL Experiment Groups
BAL 1 on day 14	Vaccinated group (Vaccine + *R. equi)* n=8 Control group (Saline + *R. equi*) n=7
BAL 2 on day 28	
BAL 3 on day 35	

Lung Sampling	Experiment Groups
Histopathology	Untreated control (non-vaccinated, non-infected) group (n=2)
Immunohistochemistry	Vaccinated group (Vaccine + *R. equi)* n=8 Control group (Saline + *R. equi*) n=7
Confocal microscopy	Untreated control foal group (n=2)

Table 3.1. Summary of BAL and lung experiment groups

3.7. Differential cell count

Total nucleated cell counts (TNCC) were determined by using a hemocytometer (Fischer scientific, Ottawa, ON, Canada). The differential cell counts were determined by examination of cytospin preparations, stained with Trypan blue, and counting 100 leukocytes in a microscopic field (40X).

The BAL cells (right lung) were washed with RPMI-1640 medium (Invitrogen, Burlington, ON, Canada), supplemented with 2 mM L-glutamine, 10% fetal bovine serum (Invitrogen) and 5 µg/mL gentamicin (Invitrogen) and were centrifuged at 400*g* for 10 min at 10°C. The pellet was re-suspended in 10 mL of supplemented RPMI-1640. Cells were grown in a sterile plastic culture flask (VWR, international, LLC, Edmonton, AB, Canada) and incubated overnight in a humidified CO_2 incubator (5% CO_2) at 37°C to facilitate adherence of macrophages (Drevot *et al.*, 2002).

3.8. Lipid raft isolation

I pooled BAL samples from different foals to have enough number of cells to isolate lipid rafts. The pooling information is included in Table 3.2. The supernatant of the control (n=7) and vaccinated (n=8) foals on BAL1 (day 14), BAL2 (day 28) and BAL3 (day 35) from the right lung was discarded and the adhered macrophages were treated with 4mL trypsin 1X (Sigma-Aldrich, Oakville, ON, Canada) at room temperature and shaken well to remove adhered macrophages. These macrophages were re-suspended in 10mL RPMI-1640 and centrifuged at 400*g* at 4°C for 10 mins. The supernatant was discarded and the pellet was suspended in 10 mL RPM-1640, centrifuged at 4000 rpm at 4°C for 10 mins, the pellet was re-suspended in 600 µL of lysis buffer and further subjected to extraction by needle (30 times) and centrifugation at 400*g* for 10 mins. The supernatant (500µL) was collected in ultra centrifuge tubes (Kendro laboratory products, Sorvall, Newtown, USA) and 500 µL of 50 % Opti-prep density gradient medium was added. This was followed by (40 to 5%) step gradient of sucrose and ultra-centrifugion at 50,000*g* for 2 h at 4°C. Then, different layers (100uL) were collected and stored at -80°C until further analysis. Lipid raft isolated from each pooled sample was analysed separately. Figure 3.4 summarizes steps involved in lipid raft isolation.

BAL1/day14	BAL2/day28	BAL3/day35
F (1)	F (1+3)	F (1+3)
F (2+4)	F (2+4)	F (2+4)
F (3+5)	F (5)	F (5)
F (6+8)	F (6+8+10)	F (6+8)
F (7+9+11)	F (7+9+11)	F (7+9+11)
F (10+12)	F (12)	F (10+12)
F (13)	F (13)	F (13)
F (14)	F (14)	F (14)
F (15)	F (15)	F (15)

Table 3.2. Pooling of cells for lipid raft fractions from foal BAL

3.9. Protein extraction

The tissue samples from normal and abnormal area from caudal dorsal region (Explained in Section 3.5) of the right lung of control (n=7) and vaccinated (n=8) foals were ground in liquid nitrogen using a sterile pestle and mortar and lysed in 250 µL of lysis buffer and allowed to sit on ice for 20 mins. Cell lysate was centrifuged at 10,000 rpm at 4°C for 5 mins. The supernatant was collected and stored in -80°C until further analysis.

3.10. Western blotting

Protein for Western blots was isolated from right lung tissues collected from the foals or the lipid raft fractions. Protein from lung tissue or lipid raft fractions was isolated from different treatment groups such as control (n=7) and vaccinated (n=8). The sample (45 µL) from each treatment was mixed with 5 µL of loading buffer (990 µL bromophenol blue, 300 µL of 10% SDS, 50 µL b-mercaptoethanol). Samples were denatured by boiling for 6 mins. The proteins were separated on 12% SDS-polyacrylamide gel using a 1X running buffer (3.3 g/L Tris base, 14.4 g/L Glycine, 0.5 g/L SDS) at 170 V. Proteins were electroblotted onto a PVDF membrane (GE Healthcare life Sciences, Canada) at 100 V for 65 mins using a protein transfer buffer (3.03 g/L Tris base, 14.4 g/L Glycine in 20% methanol). Membranes were blocked with 5% non-fat skimmed milk in 0.1% Tween-20 in PBS buffer (pH 7.4) for 1 h at room temperature and incubated overnight with flotillin-1 (goat anti-mouse Flotillin-1 antibody; 4 µg/mL; BD Transduction Laboratories, New Jersey, USA), ganglioside asialo-GM1 (goat anti-rabbit; 5 µg/mL; Abcam Cambridge, USA), transferrin receptor (goat anti-rabbit; 5 µg/mL; Abcam), TLR2 (goat anti-rabbit; 1 µg/mL; Abcam Cambridge, USA) or TLR4 (goat anti-rabbit; 2 µg/mL; Santa Cruz Biotechnology Inc.) antibodies as recommended by the manufacturer. While we have characterized the TLR4 antibody for use with horse tissues (Suri *et al.,* 2004), the other antibodies are not specific for the equine antigens. Unbound antibody was removed by washing the membrane with PBS + 0.2% Tween-20 (PBST) followed by washings with PBS (pH 7.4). Membranes were incubated with an appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (polyclonal goat anti-mouse immunoglobulin (0.2 µg/mL; Dako, Mississauga, ON, Canada) or polyclonal goat anti-rabbit immunoglobulin (0.5 µg/mL; Dako).

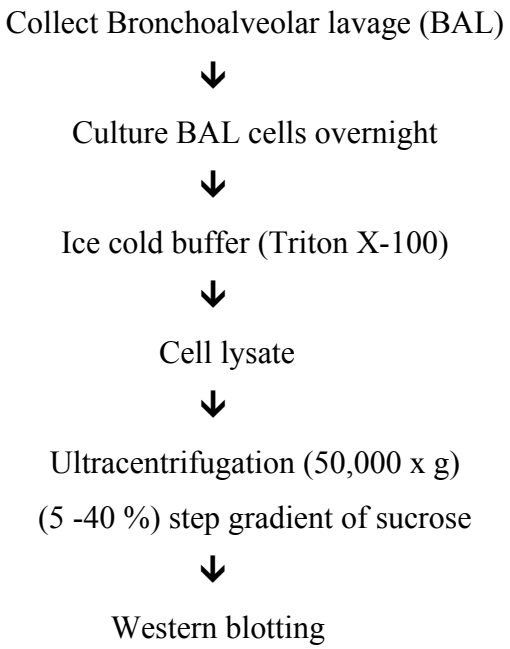

Figure 3.4. Protocol for lipid raft isolation

The unbound antibody was removed by washing with PBST and PBS (pH 7.4). The antigen-antibody complex was detected using a chemiluminescence kit (GE Healthcare Life Sciences) and membranes were exposed on X-ray films (GE Healthcare Life Sciences).

3.11. Enzyme linked–immunosorbant assay (ELISA) for tumor necrosis factor-α (TNF-α), Interferon gamma (IFN-γ) and Interleukin 10 (IL10)

The expression of TNF-α protein concentration in right lung BAL supernatants at different time points (Table3.1) such as BALI (day 14), BAL2 (day 28), BAL3 (day 35) and lung tissue extracts from control (n=7) and vaccinated (n=8), at day 49 were analyzed using sandwich ELISA kit (Endogen, Pierce Biotechnology, inc., IL, USA) as per manufacturer's instructions. These antibodies are not specific for the equine antigens. Briefly, wells of a microtitre plate (VWR, 96 well plates) were coated with 110 µL of rabbit anti-goat TNFα antibody (diluted in Carbonate/Bicarbonate buffer (50 mM, pH 9.6)) and incubated overnight at 4°C. Wells were washed with wash buffer (0.05% Tween-20 in PBS pH 7.4), standards and samples diluted in reagent diluent (4% BSA in PBS pH 7.4) were added to the wells and incubated at 37°C for 1 h. The wells were washed with wash buffer (0.05% Tween 20 in PBS, pH 7.4) and 100 µL of detection antibody was added to each well after dilution with reagent diluents. The plate was incubated at room temperature for 30 mins. After washing wells with wash buffer, 100 µL streptavidin-HRP (1:400 in reagent diluent) was added and the plate was incubated at room temperature for 30 min. Substrate solution (100 µL) was added and plates were incubated in the dark at room temperature for 20 min. On colour development, reaction was stopped by adding 100 µL of stop solution (0.18 M sulfuric acid) to each well. Absorbance was measured at 450 nm using a microplate reader (BMG-Lab tech NOVO star- Microplate-Fluorometer, Biocompare, South San Francisco, CA, USA).

IFN-γ protein concentration in right lung BAL supernatants at different time points (Table 3.1) such as BALI (day 14), BAL2 (day 28), BAL3 (day 35) and lung tissue extracts from control (n=7) and vaccinated (n=8), at day 49 was determined by using a sandwich ELISA kit (Mabtech, Cincinnati, USA) according to the manufacturer's instructions. Wells were coated with 40 µL of equine IFNγ coating antibody (diluted with 10 mL PBS) and incubated overnight at 4^0C. Following washings with PBS pH 7.4, wells were blocked by adding 0.1 % BSA in PBS

pH 7.4 and incubated at 37°C for 1 h. Wells were washed five times with PBS-Tween. Standards were diluted in incubation buffer and added to the wells and incubated at 37°C for 2 h. After removing standards and samples, wells were washed with PBS-Tween and 100 µL of detection antibody (mAb PAN-biotin) was added to each well after dilution with incubation buffer. The plate was incubated at 37°C for 1 h at room temperature. Wells were washed with PBS-Tween, 100 µL of streptavidin-HRP (1:000 in incubation buffer) was added to each well and incubated at room temperature for 1 h. The wells were washed five times with PBS-Tween and 100 µL of substrate solution (TMB) was added to each well and incubated in the dark at room temperature for 20 min. On color development, the reaction was stopped by adding 100 µL of stop solution (0.18 M sulfuric acid) to each well and absorbance was measured at 450 nm wavelength.

IL-10 protein concentration in right lung BAL supernatants at different time points (Table 3.1) such as BALI (day 14), BAL2 (day 28), BAL3 (day 35) and lung tissue extracts from control (n=7) and vaccinated (n=8), at day 49 was determined by using a direct ELISA according to the R&D manufacturer's instructions. The antigen was diluted to final concentration of 20 µg/mL and the wells were coated with 100 µL of sample (diluted with 10 mL PBS).Then, the ELISA plate was incubated overnight at 4^0C. Following two washings with PBS pH 7.4, wells were blocked by adding 1 % BSA in PBS pH 7.4 and incubated at 37°C for 2 h. Wells were washed two times with PBS. 100 µL of anti-equine IL-10 antibody was added to each well after dilution with blocking buffer (10 µl/mL). The plate was incubated at 37°C for 2 h, on a shaker at room temperature. Wells were washed four times with 200 µL of PBS. Then, wells were coated with 100 µL of goat anti-equine HRP conjugated secondary antibody (2µl/10mL PBS) and incubated at room temperature for 2 h. The wells were washed five times with PBS and 100 µL of substrate solution (TMB) was added to each well. On color development, the reaction was stopped by adding 100 µL of stop solution (0.18 M sulfuric acid) to each well and absorbance was measured at 450 nm wavelength.

3.12. Histopathology

The study design was such that it did not leave any non-infected control foals at the end of the experiment. Therefore, the untreated control foal lung samples (n=2) provided by Dr. Andrew Allen, Department of Veterinary Pathology, University of Saskatchewan were used.

Lung tissue samples of approximately one cubic centimeter from normal and abnormal area were collected from caudal dorsal region (section 3) of both control (n=7) and vaccinated (n=8) groups. The lung tissues were fixed in 4% par formaldehyde (in PBS pH 7.4) for 24 h at 4°C. The tissues were transferred to PBS pH 7.4, dehydrated in ascending concentrations of ethanol and cleared in xylene followed by embedding in paraffin**.** Sections (5 µm) were prepared from each tissue block and were stained with hematoxylin-eosin for histological examination.

3.13. Immunohistochemistry

Immunohistochemistry was done on the sections obtained from the right lung of control (n=7) and vaccinated (n=8) foals and from the paraffin blocks of the untreated control foals (n=2). Tissue sections were de-waxed with xylene and rehydrated in an ethanol series. The sections were incubated in hydrogen peroxide (0.5% V/V in methanol) for 20 min to quench endogenous peroxidase followed by pepsin treatment (2 mg/mL in 0.01N HCl) for 45 min for antigen retrieval. Non-specific sites were blocked by incubating sections with BSA (1% in PBS pH 7.4; Sigma-Aldrich) for 30 min. The sections were incubated with primary antibody such as TLR4 (goat anti-rabbit; 6.5 µg/mL; Santa Cruz Biotechnology Inc.), TLR2 (goat anti-rabbit; 4.5 µg/mL; Abcam,) or TNF-α (rabbit anti-goat; 0.25 µg/mL; Santa Cruz Biotechnology Inc.) overnight 4°C. Following washings with PBS pH 7.4, sections were incubated with an appropriate secondary antibody as polyclonal rabbit anti-goat immunoglobulin-HRP (10 µg/mL BSA; Dako, Mississauga, ON, Canada), polyclonal goat anti-rabbit (10 µg/mLBSA; Dako) and polyclonal rabbit anti-goat immunoglobulin-HRP (10 µg/mL BSA; Dako) respectively for 30 min. The colour development kit (Vector Laboratories, Burlington, ON, Canada) was used to determine the antibody reaction. Slides were counterstained with methyl green (Vector Laboratories Burlington, ON, Canada). Lung sections stained with only the secondary antibody, and those treated only with color development reagent were used as negative controls. Untreated control lungs were stained with rabbit anti-human von Willebrand Factor (vWF) antibody (6.66µg/mL), which recognizes vascular endothelium (DAKO, Mississauaga, ON, Canada). Then, the slides were counter-stained with methyl green (Vector Laboratories).

3.14. Laser confocal microscopy for lipid raft proteins

Lung tissue sections (5 μm) from the untreated control foals (n=2) were prepared from each tissue block and placed on poly L-lysine (20 KDa) 0.1 % w/v in H_2O (Sigma, Aldrich) coated glass slides. The lung tissues were cleared in xylene and rehydrated in descending concentrations of ethanol followed by two washings with sterile water and three washings in PBS pH 7.4**.** Blocking was performed in BSA (1% w/v in PBS pH 7.4) for 30 min. After one rinse with PBS (pH 7.4), lung tissues were probed with primary antibody mouse anti-Flotillin-1 antibody (10 μg/mL; Abcam), goat anti-rabbit GM1 antibody (10 μg/mL; Abcam Cambridge, USA), goat anti-rabbit TLR4 (5 μg/mL; Santa Cruz Biotechnology Inc.), goat anti-rabbit TLR2 (5 μg/mL; Abcam,) antibodies. After three washings with PBS (pH 7.4), slides were incubated with an appropriate secondary antibody diluted in 1 % BSA , anti-mouse IgG conjugated to fluorescence isothiocyanate (FITC; 40 μg/mL) or anti-rabbit IgG conjugated to Cy5 (2.5 μg/mL ; Millipore, Bedford, USA) in the dark for 1 hour at room temperature. This was followed by three washings with PBS (pH 7.4) for 10 min each. The slides were mounted with Prolong Gold antifade mounting reagent (Invitrogen) and examined under a confocal microscope (Leica model TCS SP5, Wetzler, Germany). Images were processed using Leica IM1000.

3.15. Statistical analysis

All statistical analyses were performed using Prism™ software (version 2.0; GraphPad Software Inc., San Diego, CA, USA). The groups were compared at specific time points using the Wilcoxon Rank Sum test. Results are expressed as the median values ± SD. The P value $\leq$ 0.02 was considered significant.

CHAPTER 4: RESULTS

4.1. Influx of inflammatory cells

There was no significant difference in total number of cells (p=0.3687) in BALF from the right side of lung in control and the vaccinated groups at different sampling days (Figure 4.1). Cytospin from BAL taken from right lung of control (n=7) and vaccinated (n=8) foals at day 14, day 28 and day 35 were prepared (Figure 4.2) and differential cell count was done (Table 4.1). Also, the protein concentration (p=0.2482) estimated by Bio-Rad Protein Assay, based on the method of Bradford, in BAL supernatant did not differ significantly (Figure 4.3) between the groups.

The gross appearance (surface and cut) of lungs from the control and the vaccinated groups was not consistent. On post-mortem evaluation, in the control group, lungs were classified as unaffected in 2/7 foals (28.6%; foals 2 and 6), moderately affected in 3/7 foals (42.8%; foals 4, 8, 12), and severely affected in 2/7 foals (28.6 %; foals 10, 14). In the vaccinated foal group, lungs were classified as unaffected in 4/8 foals (50 %; foals 3, 5, 9,13) and moderately affected in 4/8 foals (50 %; foals 1, 7, 11, 15) (Table 4.2). Lungs from some of the foals clearly showed granulomatous lesions lung (Figure 4.4A) compared to some of the vaccinated foals (Figure 4.4B). Similar variation was observed on the cut surface of lungs collected from different control foals (Figure 4.4C, E) and the vaccinated foals (Figure 4.4D, F). There were no differences in the gross lesions between the groups (Figure 4.4G).

Lung sections from untreated control (non-vaccinated, non-infected) foals obtained from the department of Veterinary Pathology and stained with hematoxylin-eosin (H&E) revealed normal morphology of alveolar septa (Figure 4.5 A) and bronchus (Figure 4.5 D). The control foal group showed marked septal congestion and influx of inflammatory cells in alveoli (Figure 4.5 B) accompained by mucus plug inside the bronchus (Figure 4.5 E). However, similar results were examined in vaccinated foal group in alveoli (Figure 4.5 C) and bronchus (Figure 4.5 F). The bronchial epithelium showed prominent cilia along with stratified appearance of epithelium in untreated control foal (Figure 4.5 G) compared to altered epithelium accompained by fuzzy and matted cilia in control (Figure 4.5 H) and vaccinated (Figure 4.5 I) foal groups. Marked congestion of blood vessels along with thickening of the tunica externa was appreciated in vaccinated (Figure 4.6C) and control foal (Figure 4.6B) groups compared to untreated foal group

(Figure 4.6A). Also, histopathology studies indicated advanced granulomatous lesion accompanied by deposition of edematous fluid, fibrin and marked vasculature changes and the loss of normal architecture of lung in control (Figure 4.7A) and vaccinated (Figure 4.7B) foal groups. Further examination of granulomas from control foal (Figure 4.7C) and vaccinated (Figure 4.7D) foal groups revealed accumulation of inflammatory cells such as macrophages, lymphocytes and giant cells. Considering variation in the foals within the same group, there appeared to be no differences in the inflammation between the control and the vaccinated groups.

4.2. Western blotting and immunohistologic expression of Toll-like receptors (TLR2 and TLR4) in foal lung tissue

Western blotting revealed expression of TLR2 (89kDa) and TLR4 (89kDa) in lung tissue extracts from vaccinated group and the control (Figure 4.8A) foal group. The 48 KDa band of ß-actin, expressed in both the treatment groups showed equal loading of protein in each lane. Densitometric comparison of Western blots showed significant increase in the expression of the TLR2 (Figure 4.8B) but not of TLR4 (Figure 4.8C) in the lung extracts from the vaccinated foals compared to the control foals.

Untreated control foal lung sections stained with only secondary antibody lacked any color reaction while those incubated with the von Willebrand Factor antibody showed staining of vascular endothelium (Figure 4.9A). Immunohistology revealed increased expression of TLR2 in the alveolar septa of the control and the vaccinated foals compared to the untreated control foals (Figure 4.9B). The expression of TLR2 in the airways and the large blood vessels remained nearly similar although there was an appearance of reduced expression in the vaccinated group (Figure 4.9C) compared to other treatment groups. TLR4 expression was faint in the alveolar septa in the lungs of untreated control foals but was increased in the septa of control and especially the vaccinated foals (Figure 4.10A). While the vascular expression of TLR4 appeared similar in all the groups, the airway epithelial expression increased in the vaccinated foals (Figure 4.10B). Alveolar septa of the untreated control foals showed weak staining for TNFα compared to the control and the vaccinated foals (Figure 4.11A). Vascular and airway epithelial expression of TNFα was also increased in the lungs of vaccinated foals compared to the untreated control and the control foals (Figure 4.11B). Overall, there was similar expression of TLR4, TLR2 and TNFα in the lungs of the control and the vaccinated foals.

4.3. Expression of inflammatory cytokines

4.3.1 Tumor necrosis factor-alpha (TNF α) expression

TNFα protein concentration was assessed with sandwich ELISA both on BAL and lung extracts from control and vaccinated groups. The foals in the vaccinated group showed higher concentrations of TNFα in BAL (p=0.02) compared to the control foals on day 28 (Figure 4.12A). However, there were no differences in TNFα concentrations in lung extracts from the two groups (p=0.9451) (Figure 4.12B).

4.3.2 Interferon gamma (IFNγ) protein expression

IFNγ protein concentration was analyzed in BAL (Figure 4.13A) and lung extract Figure 4.13B) by sandwich ELISA. There was no significant difference in IFNγ concentration in BAL (p=0.8211) and lung extract (p=0.1672) between both the groups.

4.3.3 Interleukin 10 (IL-10) protein expression

IL-10 protein concentration was examined in BAL and lung extract by direct ELISA in control and vaccinated groups at different sampling days. In BAL, there were no significant differences (p=0.8937) in the expression of IL-10 between the groups (Figure 4.14A). However, significantly lower concentrations of IL-10 were observed in lung extracts from the vaccinated group compared to the control group (p=0.0172) (Figure 4.14B).

4.4. Lipid raft isolation protocol from bronchao-alveolar lavage macrophages

A method of lipid raft isolation from equine alveolar macrophages was developed by pooling foal BAL and preparing cell lysates from a minimum of $1X10^7$cells in Triton X-100, a non-ionic detergent and further separation by sucrose (5-40%) step gradient. Lipid rafts were obtained at the interphase of 20 and 25 % sucrose.

4.5. Identification of lipid raft and non- lipid raft proteins

The pooled samples from the foals BAL cells were used for lipid raft extraction. Each of the pooled samples (Table 3.2) at different sampling days was Western blotted and screened for the presence or absence of lipid raft resident proteins (Flotillin-1 and GM1) and non-lipid raft

protein – transferrin receptor (Tfr). Western blot results showed the expression of lipid raft and non-lipid raft proteins in foal BAL cells collected on day 14 from control and vaccinated foals (Figure 4.15 A) . In control foals (Figure 4.15A), the faint expression of a 48 kDa band for Flotillin-1 was appreciated in fractions 2, 3, 4 while stronger bands for Flotillin-1 were seen in fractions 7, 8 and 9. However, in vaccinated foals (Figure 4.15A), the expression of Flotillin-1 was appreciated from fraction 4 to 9 and the bands were more intense in fractions 6 to 9 of the lipid raft. Western blot results also showed GM1 as a 13 kDa band from fractions 4 to 9 in control group (Figure 4.15A) and from fractions 6 to 9 in vaccinated group (Figure 4.15A). Transferrin receptor (TfR) is a characteristic non-raft protein and it was detected in fractions 1, 2 and 3 but not in fractions 7, 8 and 9 in control foals (Figure 4.15A) and in fractions 1 and 2 in vaccinated foal group (Figure 4.15A).

Similarly, foal BAL supernatants collected on day 28 were pooled from control foals and vaccinated foals were screened for the presence or absence of lipid raft resident proteins (Flotillin-1 and GM1) and non-lipid raft protein, transferrin receptor (Tfr). In control foals (Figure 4.15 B), the expression of a 48 kDa band for Flotillin-1 was appreciated from fraction 2 to 9 as it was getting more intense towards the lipid raft fractions. However, in vaccinated foals (Figure 4.15 B), the expression for Flotillin-1 was also seen in almost all the fractions but strong expression was in fractions 5 to 8. Furthermore, GM1 as a 13 kDa band was expressed strongly in the 7^{th} fraction as compared to fractions 8 and 9 in control foal group (Figure 4.15B) and from fractions 6 to 9 in vaccinated group (Figure 4.15B). Transferrin receptor (TfR) protein was detected in fractions 1, 2, 3 and 4 in both control foals (Figure 4.15B) and in vaccinated foal group (Figure 4.15B).

Again on day 35, foal BAL supernatants were pooled from control foals and vaccinated foals, respectively, and were screened for the presence or absence of lipid raft and non-lipid raft proteins. In control foals and vaccinated foal groups (Figure 4.15 C), the expression of a 48 kDa band for Flotillin-1 and GM1 was appreciated from fraction 6 to 9. Transferrin receptor (TfR) was detected in fractions 1, 2, 3 in non lipid raft fractions in both control foals (Figure 4.15C) and in the vaccinated foal group (Figure 4.15C).The expression of both lipid raft resident proteins and non-lipid raft protein were well appreciated in vaccinated group compared to control group on day 35.

I probed untreated foal lung tissues for the expression of Flotillin-1 and GM1 with confocal microscopy. The data showed co-localization for Flotillin-1 and GM1 in lung sections (Figure 4.16).

4.6. Association of lipid rafts with TLR2 and TLR4 in BAL and foal lung tissue

In the present investigation, I examined whether lipid rafts co-localize with the TLRs. Western blot results indicate TLR2 and TLR4 as 89 kDa bands by probing with appropriate antibodies at different sampling days. Pooled foal BAL supernatants collected on day 14 (Figure 4.17A upper panel) showed TLR2 as 89 kDa band from fractions 1 to 9. The expression of TLR2 was appreciated in lipid raft fractions 7 to 9 fractions in the vaccinated group. On day 28 (Figure 4.17A middle panel), in case of the control foal group, TLR2 expression was seen from fractions 5 to 9 but again strongly towards lipid raft fractions. In the vaccinated foal group, TLR2 expression was appreciated in lipid raft fractions 8 to 9. Similarly, on day 35 (Figure 4.17A lower panel), TLR2 in the controls was associated with fractions 6 to 9, but intense expression was seen in 7^{th} fraction. In vaccinated foal group, TLR2 expression remained the same as on day 28, as it was limited to fraction 8 and 9.

Pooled foal BAL cells collected on day 14 (Figure 4.17B upper panel) were western blotted and the results showed faint expression of TLR4 as 89 kDa band from fractions 1 to 9. Similarly, the expression of TLR4 was appreciated as faint bands in lipid raft fractions 7 to 9 in the vaccinated group. On day 28 (Figure 4.17B middle panel), TLR4 expression was seen in fraction 5 to 9 in the control foals and fractions 7 to 9 in the vaccinated group. On day 35 (Figure 4.17B lower panel), in the control foal group, TLR4 was associated with fractions 5 to 9. However, in the vaccinated foal group, TLR4 expression was limited to fractions 7 to 9. The pattern of TLR4 on day 28 and day 35 remained almost the same, but stronger expression was appreciated on day 35. The overall picture indicated that both TLR2 and TLR4 represented similar results on different sampling days in both treatment groups.

The relationship between TLRs and lipid rafts was further studied by confocal microscopy in untreated foals (n=4) by using various combinations of antibodies. Confocal microscopy data depicts that both Flotillin-1 and TLR2 (Figure 4.18) colocalise in the alveolar

septum (Figure 4.18, upper panel) and in airway epithelium (Figure 4.18, lower panel). Similar results were obtained TLR4 and Flotillin-1 (Figure 4.19).

Day/group	Macrophages (%)	Segmented neutrophils (%)	Lymphocytes (%)	Monocytes (%)	Eosinophils (%)
BAL 14					
Control	85± 2	5± 3	5± 2	1±1	0±0
Vaccinated	84± 7	5±3	5± 4	2± 1	0± 0
BAL 28					
Control	90±2	2± 2	5± 2	2± 1	0± 0
Vaccinated	90±3	2±0	5± 3	2± 1	0± 0
BAL 35					
control	81± 7	14±8	3± 1	0± 0	0± 0
vaccinated	84±9	11±10	4±2	0± 0	0±0

Table 4.1: Differential cell counts of Control (n=7) and Vaccinated (n=8) foal BAL supernatant. Data is presented as mean ± SD

Control group	Lung lesion	Score		Vaccine group	Lung lesion	Score
F2	Normal	0		F3	Normal	0
F6	Normal	0		F5	Normal	0
F4	Moderate	2		F9	Normal	0
F8	Moderate	2		F13	Normal	0
F12	Moderate	2		F1	Moderate	2
F10	Severe	3		F7	Moderate	2
				F11	Moderate	2
F14	Severe	3		F15	Moderate	2

Table 4.2: Post-mortem lung lesions (right lung) in control (n=7) and vaccinated (n=8) foals. There were no significant differences between the groups. Foals lung lesions scored as described in Materials and Methods.

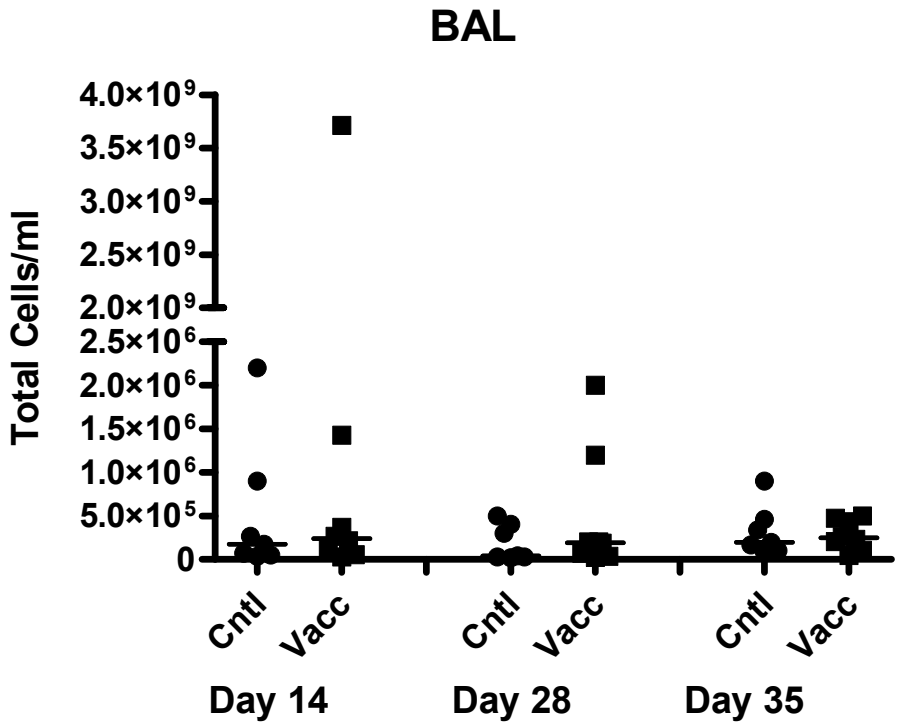

Figure 4.1. Total number of cells in the right side foal BAL collected from control (n=7) and vaccinated (n=8) groups at different sampling days.There was no significant difference (p=0.3687) between groups. Horizontal line indicates median.

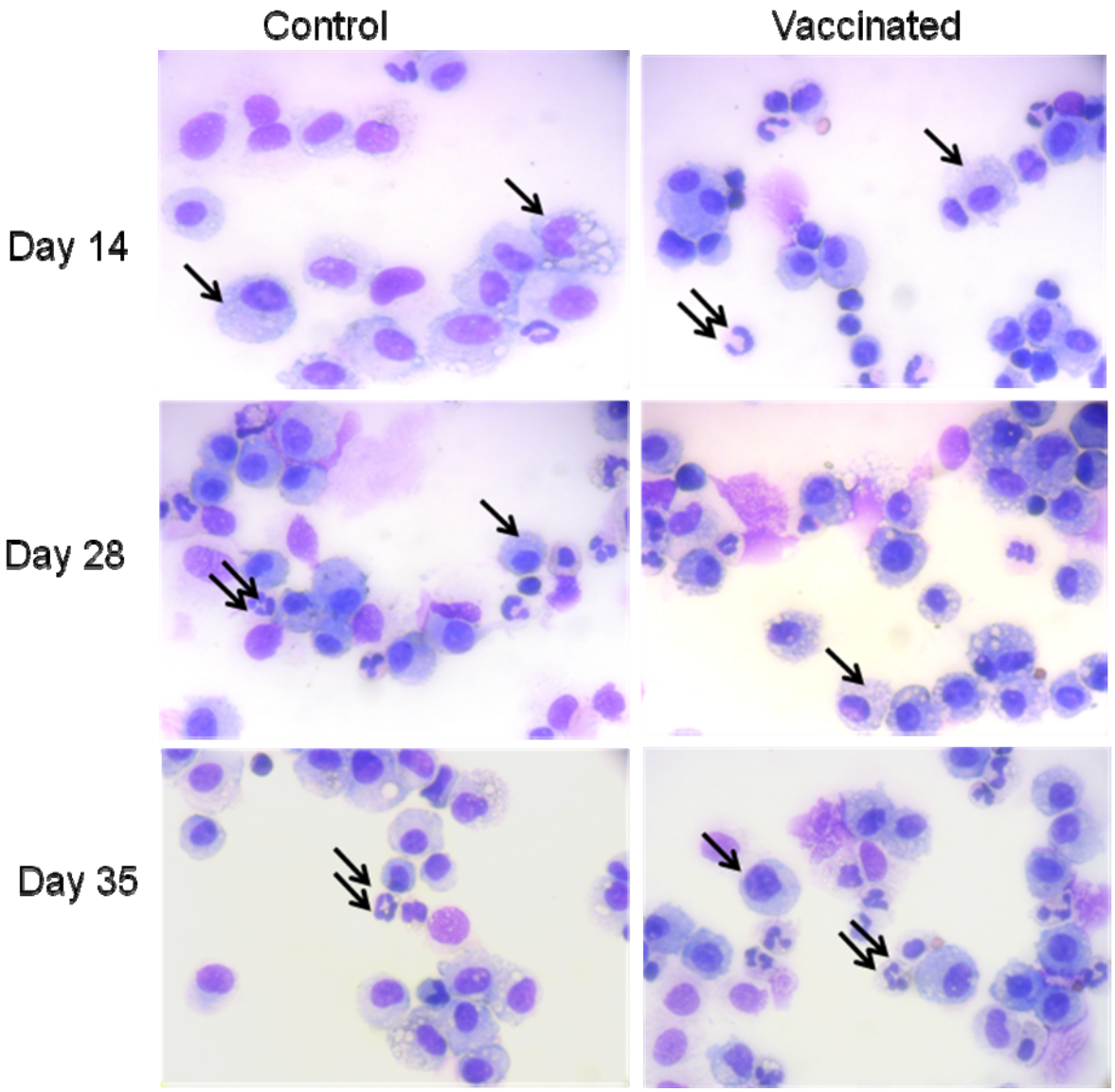

Figure 4.2. Cytospins of the foal BAL supernatants from control and vaccinated foals at different sampling days showed generally similar distribution of alveolar macrophages (single arrows) and neutrophils (double arrows). X40

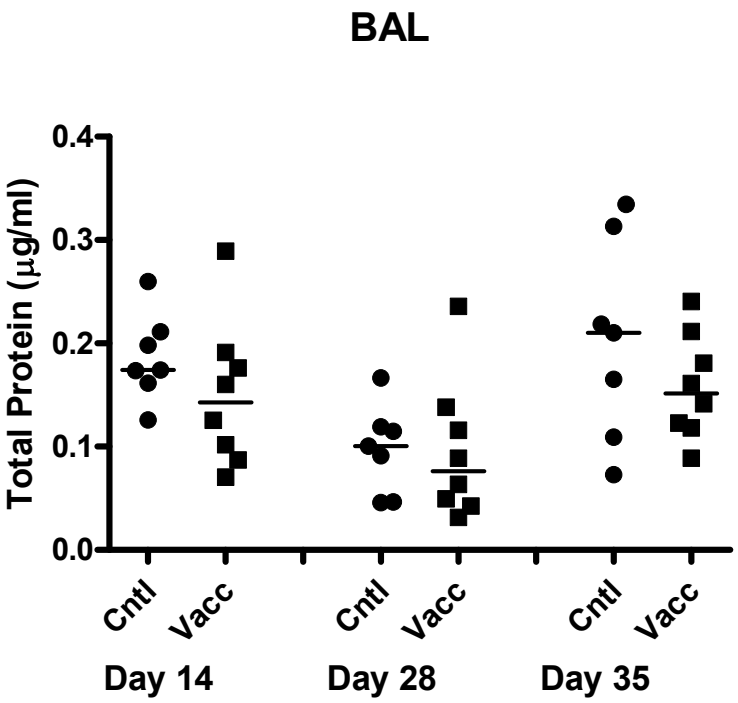

Figure 4.3. Protein concentration in foal BAL supernatant recovered from control (n=7) and vaccinated (n=8) groups at different sampling days.There was no significant difference (p=0.2482) between groups. Horizontal line indicates median.

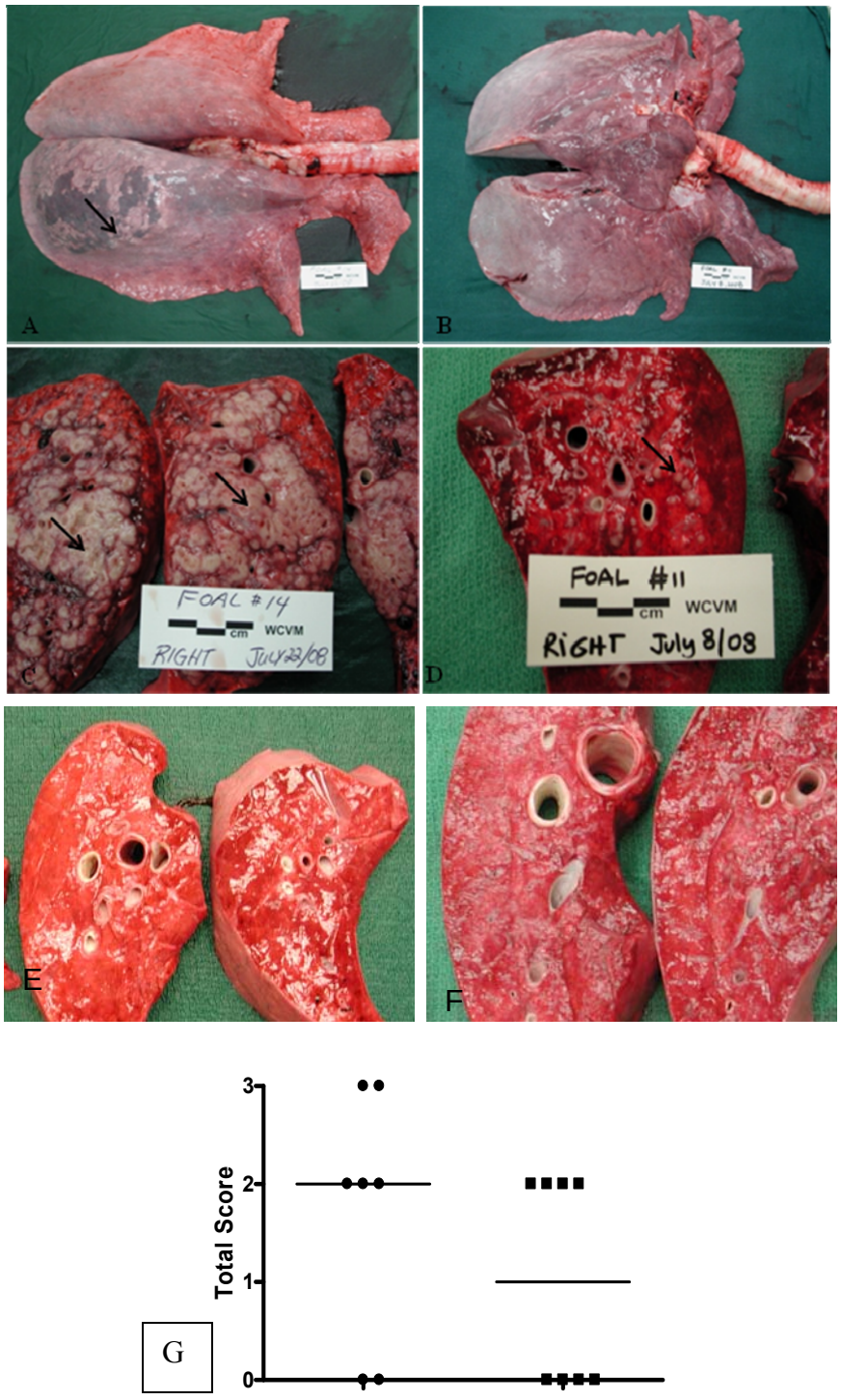

Figure 4.4. Gross evaluation (upper panel) and cut sections (lower panel) of the lungs at day 49 showing lesions in lungs from control (A, C, E) and the vaccinated (B, D, F). While control lungs (C) from one foal show more pathology than those from a vaccinated foal (D), the lungs from another control foal (E) show much reduced lesions. G shows no differences in the lung pathology between the groups.

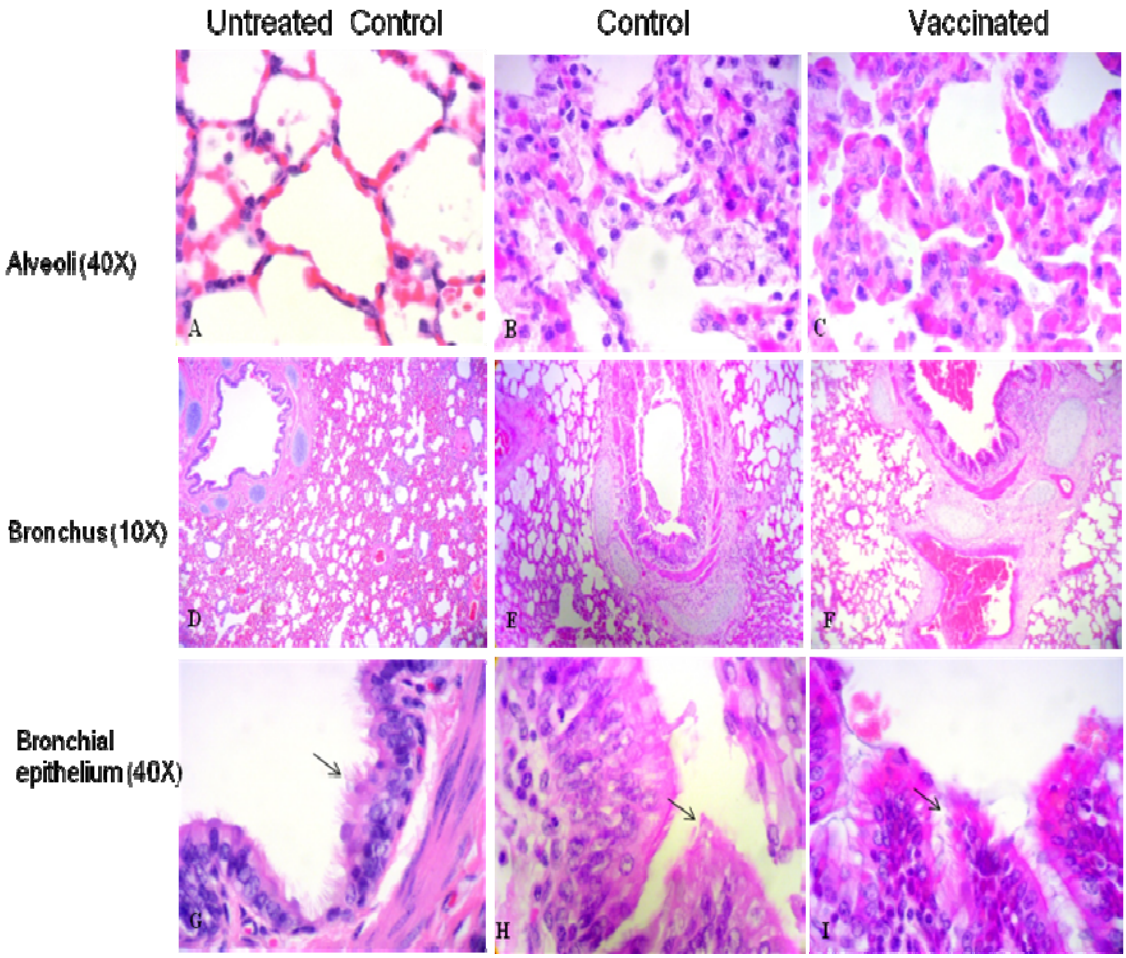

Figure 4.5. Histopathology: Foal lung sections stained with hematoxylin-eosin (H&E) revealed no inflammation in alveoli (A) and bronchus (D) in untreated control foals. Whereas, marked septal congestion and influx of inflammatory cells in alveoli (B,C) accompained by mucus plug inside the bronchus (E,F) was appreciated in control and vaccinated foals respectively.The bronchial epithelium showed prominent erect cilia in untreated control foal (G) as compared to fuzzy and matted clia in control (H) and vaccinated (I) groups. Overall there were no differences between the control and the vaccinated foals lungs. A-C and G-I:X40 . D-F:X10.

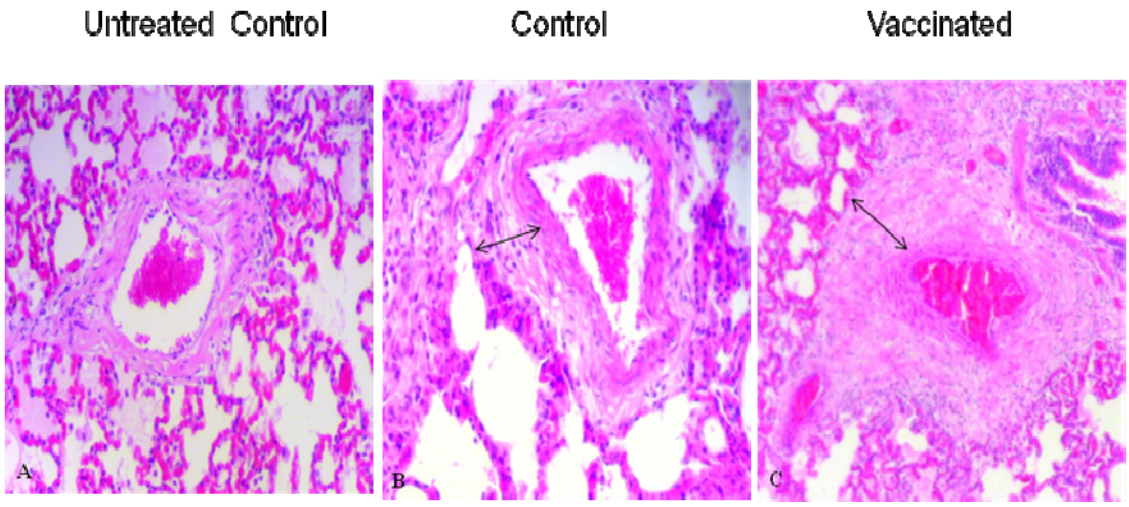

Figure 4.6. Histopathological studies: Foal lung section stained with hematoxylin-eosin (H&E) from untreated control foal (A) showed minimal vasculature changes as compared to marked congestion of blood vessels and thickening of tunica externa (marked by arrow) layer of blood vessel in control (B) foal and vaccinated (C) foal group. A-C:X40

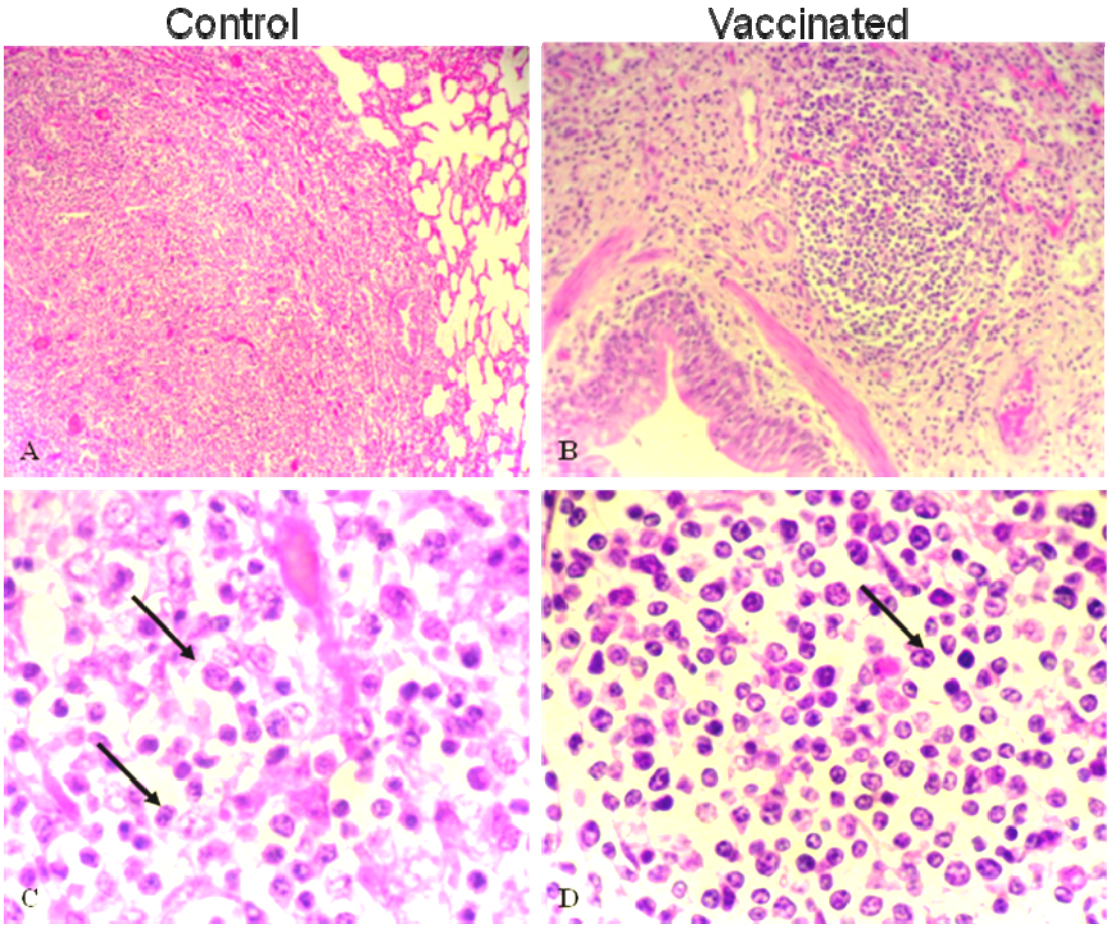

Figure 4.7. Histopathological studies: Foal lung section stained with hematoxylin-eosin (H&E) from control foal (A) and vaccinated (B) foal groups revealed large aggregates of inflammatory cells accompanied with deposition of edematous fluid, vasculature changes and the loss of normal architecture of lung. Further examination of granulomas from control foal (C) and vaccinated (D) foal groups revealed influx of inflammatory cells such as macrophages (marked by arrows), lymphocytes and giant cells .A-B: X10.C-D:X40.

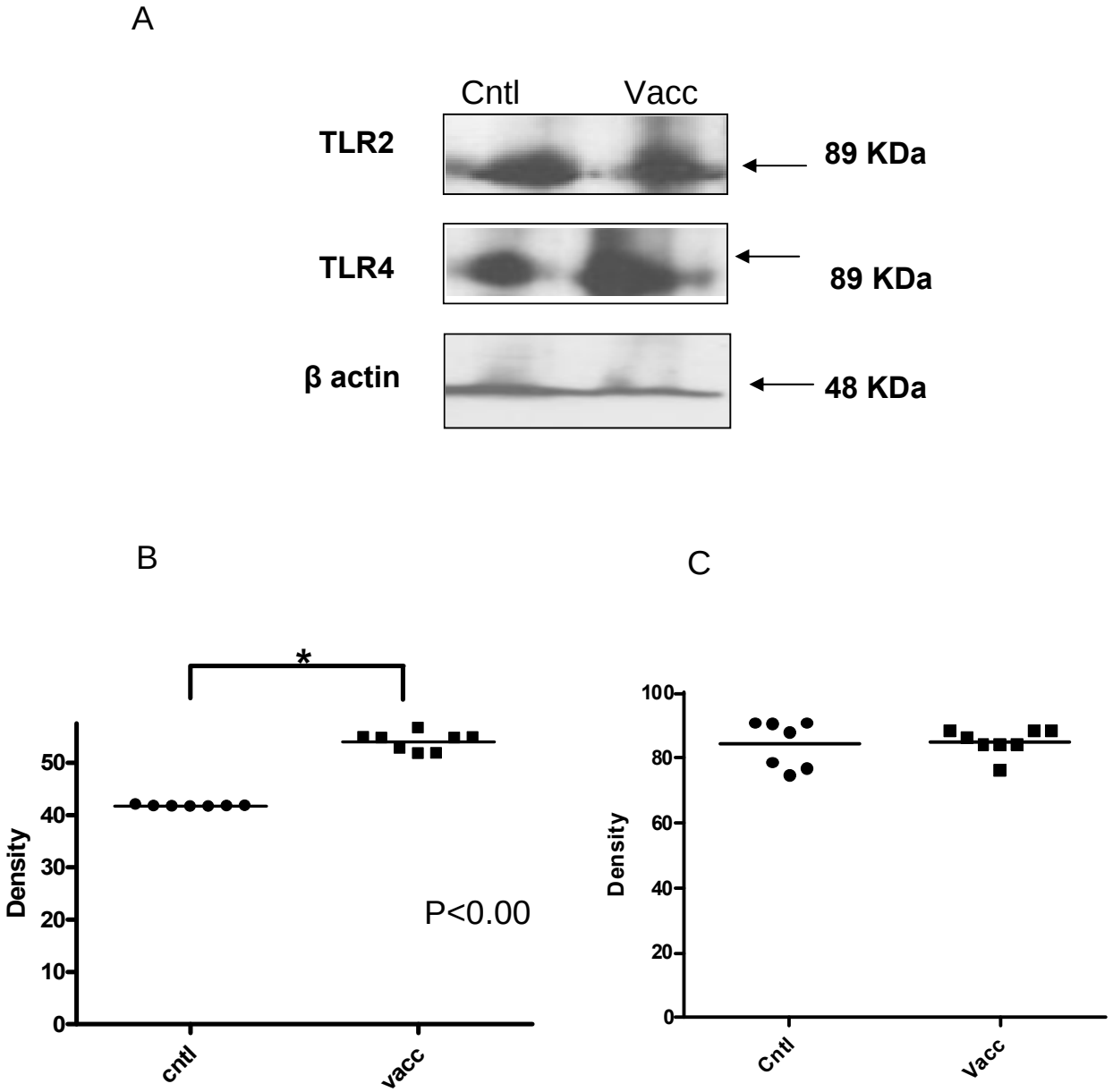

Figure 4.8. Western blot showing the expression of TLR2 (A, B) and TLR4 (A, C) in foal (N=7/group) in lung extracts recovered control and vaccinated foal groups on day 49. TLR2 but not TLR4 expression was significantly higher (*) in the lung extracts of vaccinated foals.

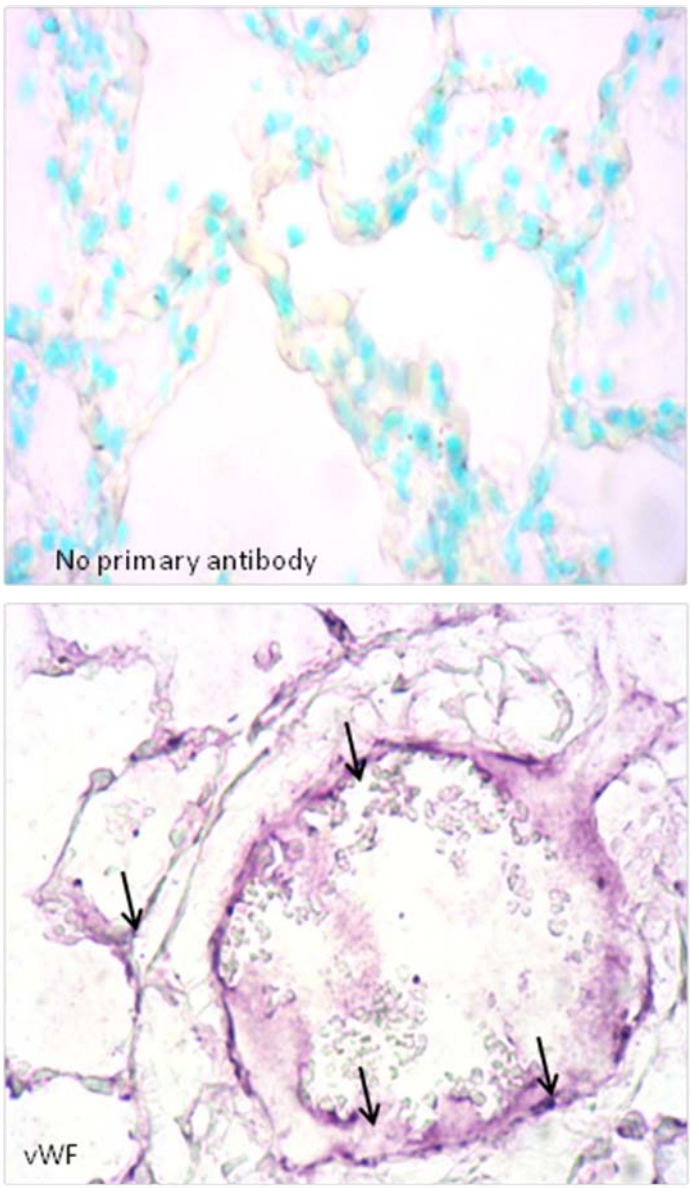

Figure 4.9A. Lung section stained without primary antibody did not show any staining while those incubated with vWF antibody revealed staining of vascular endothelium (arrows). X40

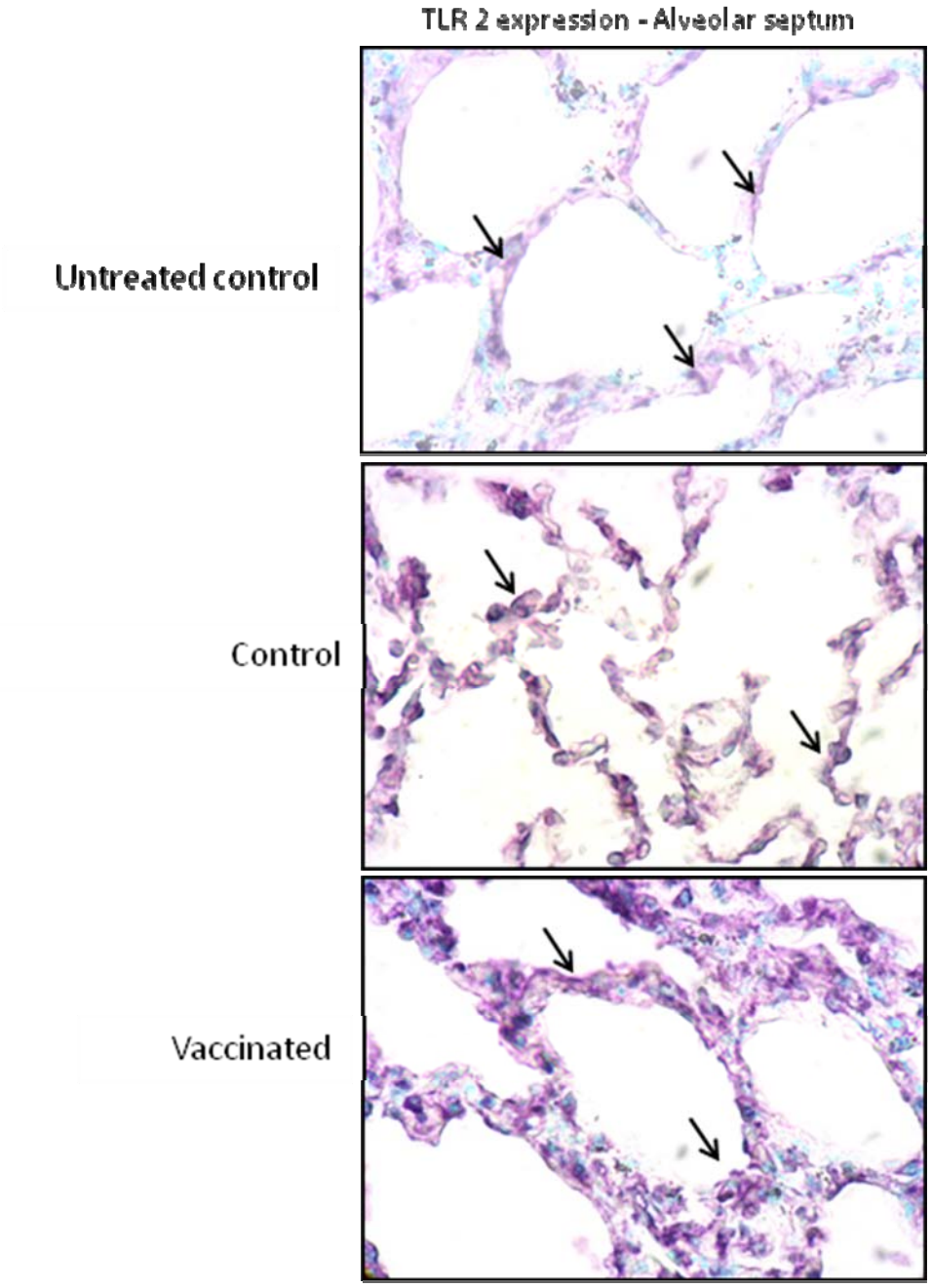

Figure 4.9B. Lung section stained with TLR2 antibody showed weak staining (arrows) in alveolar septum of untreated control foal lung compared to the lungs from the control and the vaccinated foals which showed overall similar staining. All X40.

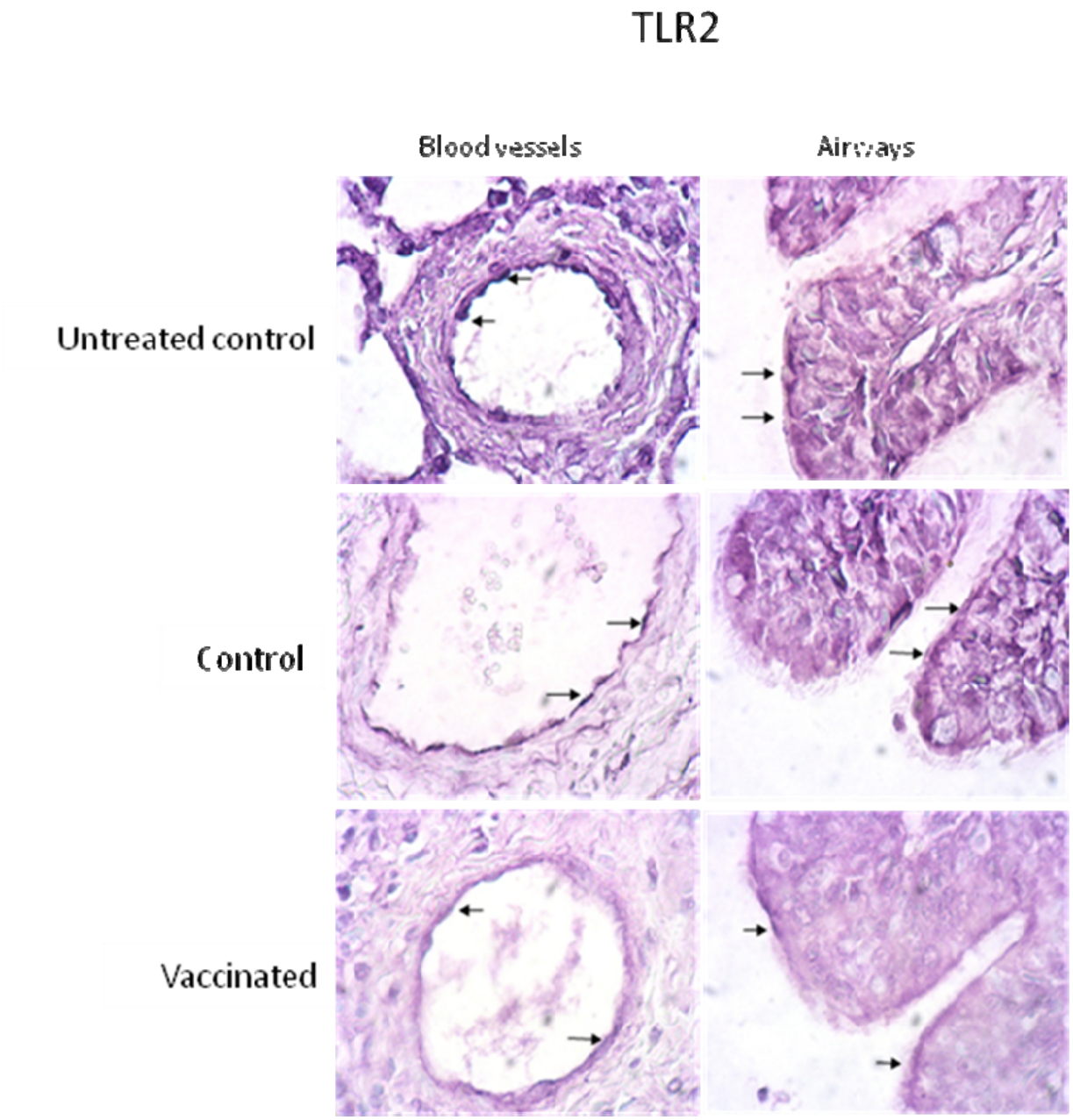

Figure 4.9C. Lung section stained with TLR2 antibody showed nearly similar reaction (arrows) in the vascular endothelium of blood vessels and the epithelium of airways of the untreated control and the control foals but the expression appeared less intense in the vaccinated foals. All X40.

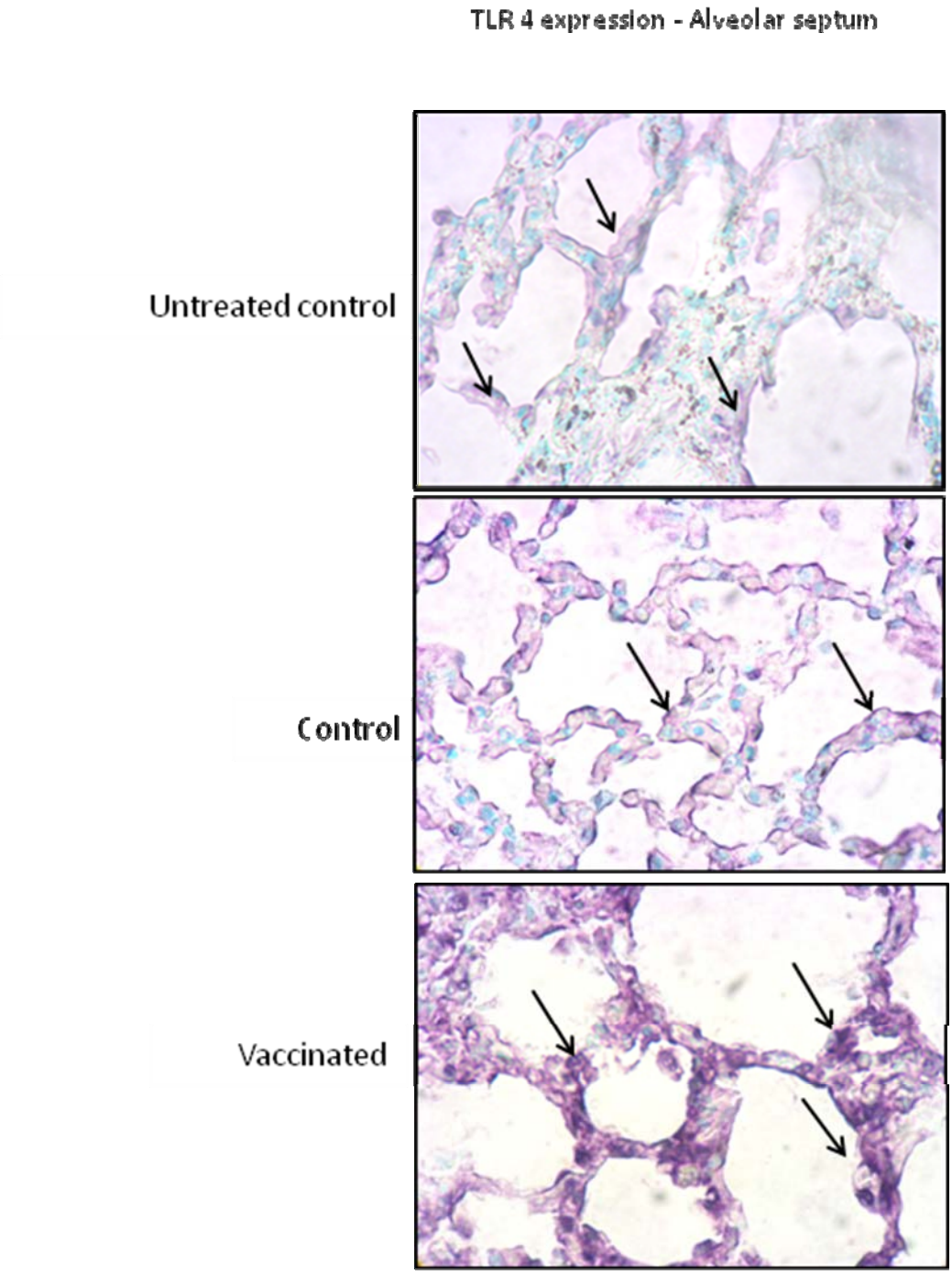

Figure 4.10A. Lung section stained with TLR4 antibody showed weak staining (arrows) in alveolar septum of untreated control foal lung compared to the lungs from the control and the vaccinated foals. There appeared some increase in staining of TLR4 in the lungs from vaccinated foals. All X40.

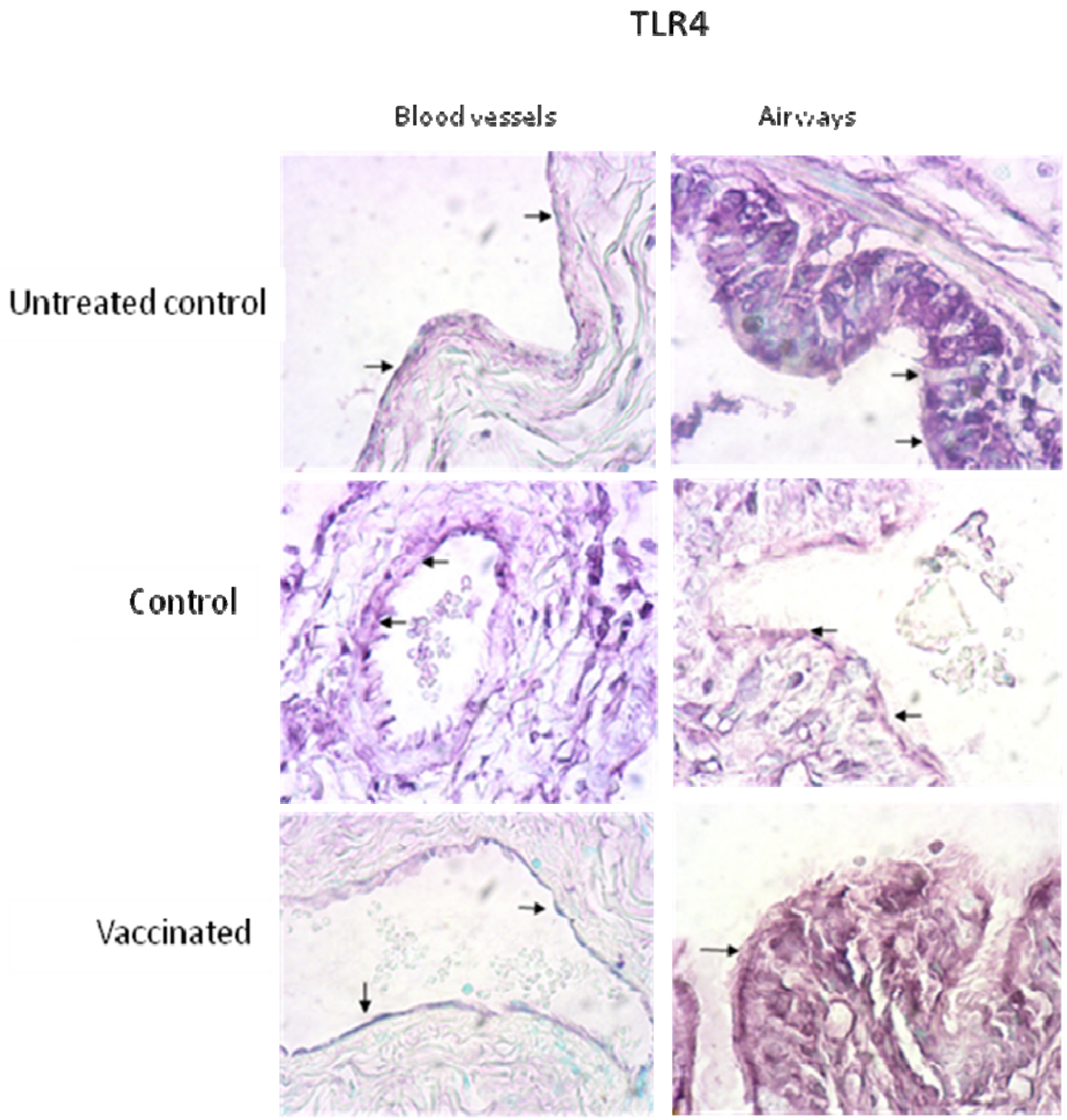

Figure 4.10B. Lung section stained with TLR4 antibody showed nearly similar reaction (arrows) in the vascular endothelium of blood vessels. However, the TLR4 reaction appeared more intense in the epithelium of the airways of the vaccinated foals compared to other groups. All X40.

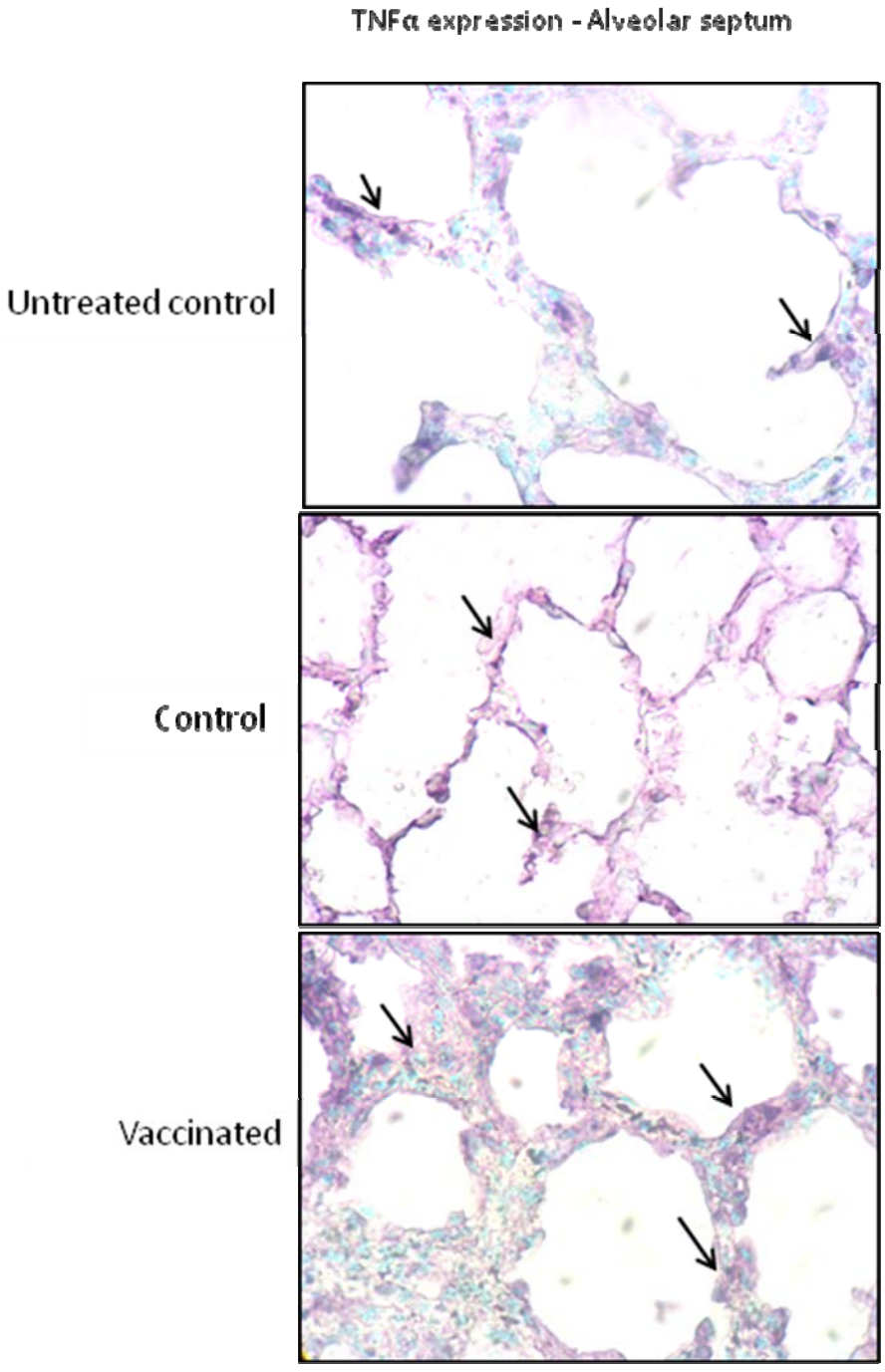

Figure 4.11A. Lung section stained with TNFα antibody showed weak staining (arrows) in alveolar septum of untreated control foals compared to the lungs from the control and the vaccinated foals. All X40.

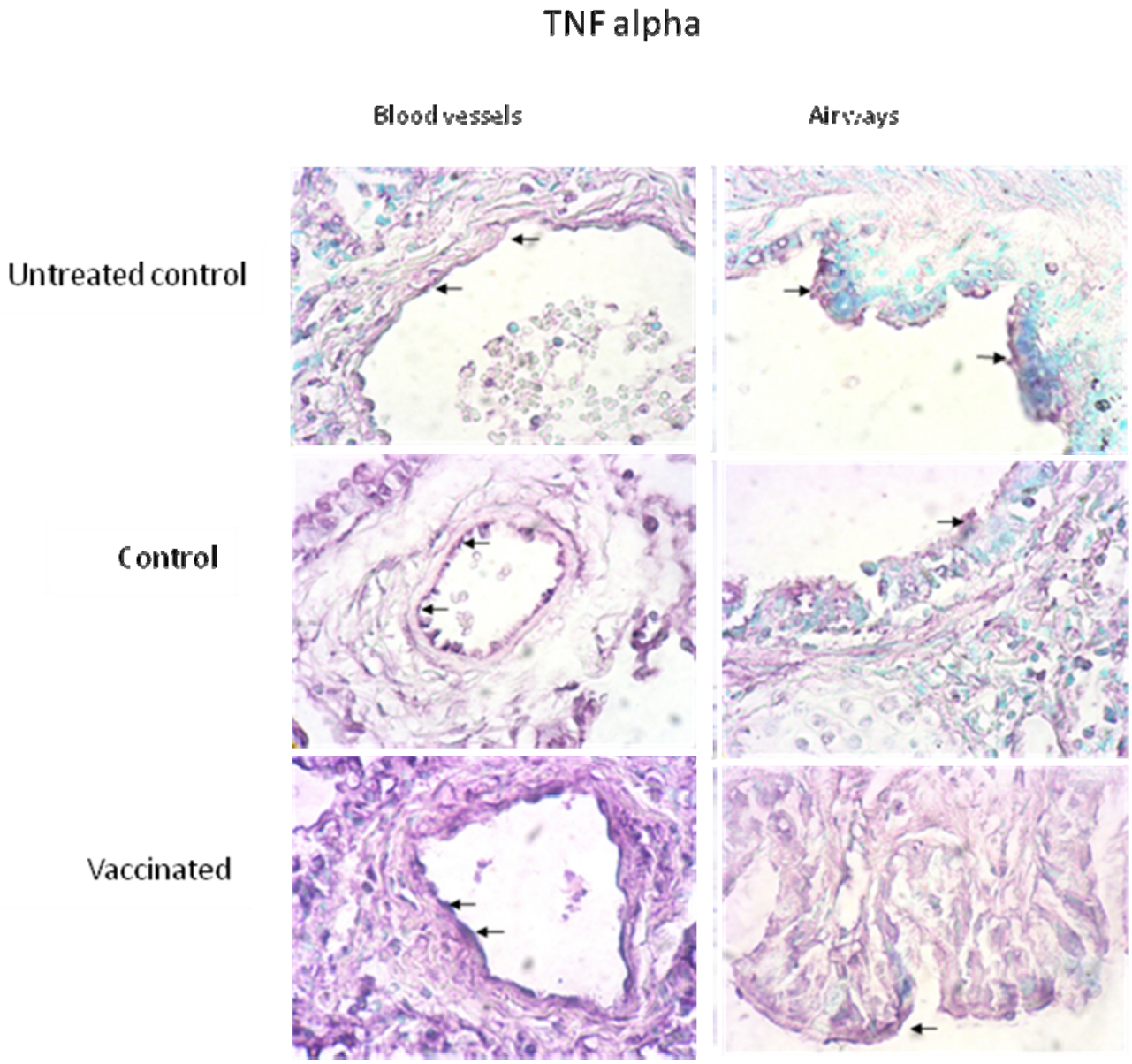

Figure 4.11B. Lung section stained with TNFα antibody showed more intense staining (arrows) in the vascular endothelium of blood vessels and the epithelium of the airways of the vaccinated foals compared to other two groups. All X40.

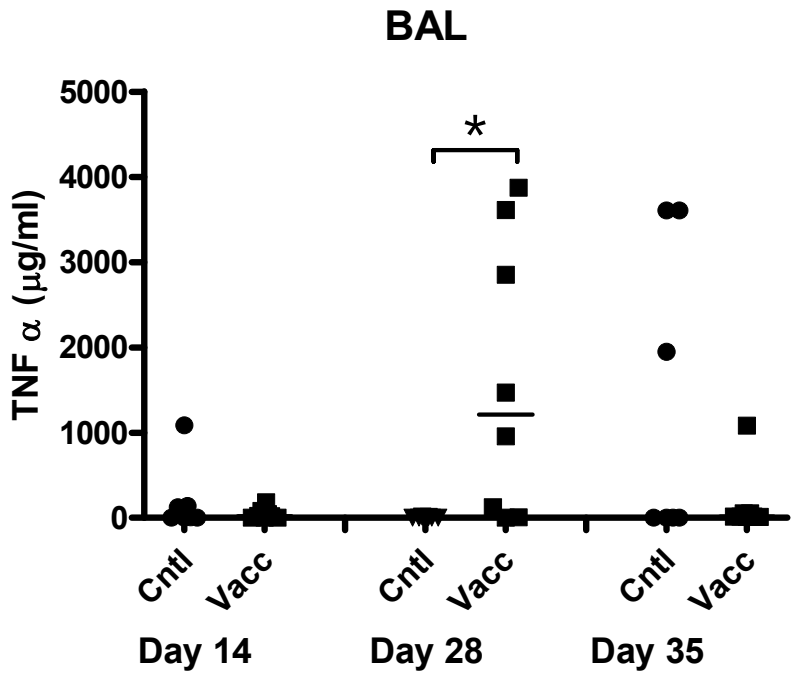

Figure 4.12A. TNFα concentration as determined with ELISA on foal BAL supernatant recovered from control (n=7) and vaccinated (n=8) groups at different sampling days.There was significant difference (p=0.02) (indicated by *) between control and vaccinated groups at day 28. Horizontal line indicates median.

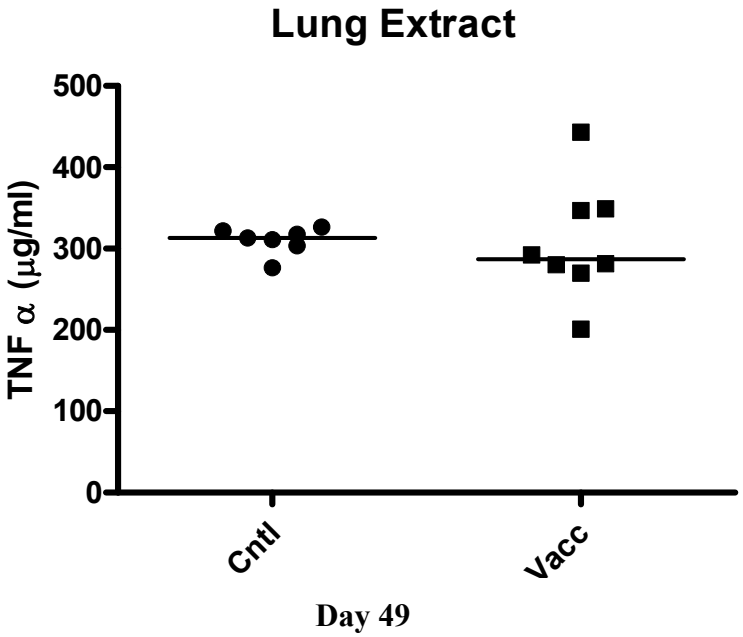

Figure 4.12B. TNFα concentration as determined with ELISA on foal lung extract recovered from control (n=7) and vaccinated (n=8) groups at day49.There was no significant difference (p=0.9457) between groups. Horizontal line indicates median.

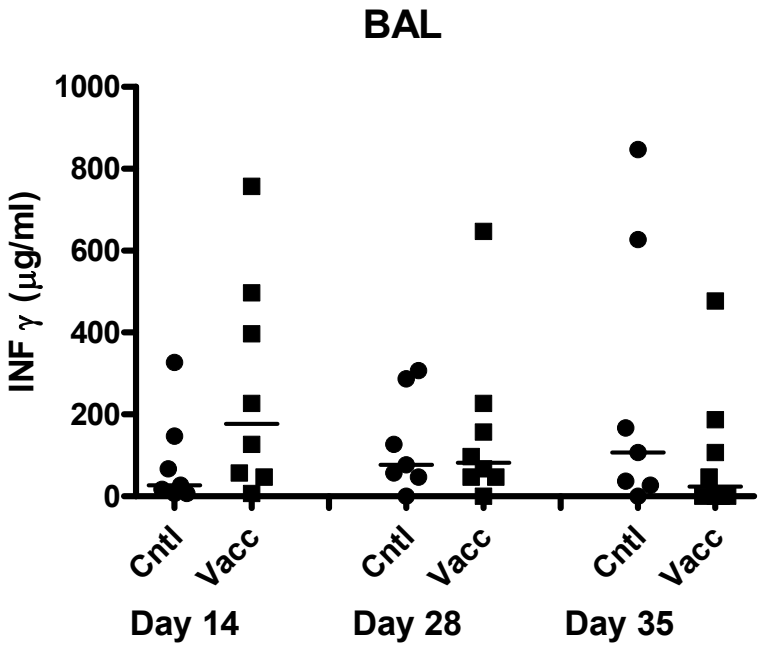

Figure 4.13A. IFNγ concentration as determined with ELISA on foal BAL supernatant recovered from control (n=7) and vaccinated (n=8) groups at different sampling days.There was no significant difference (p=0.8211) between groups. Horizontal line indicates median.

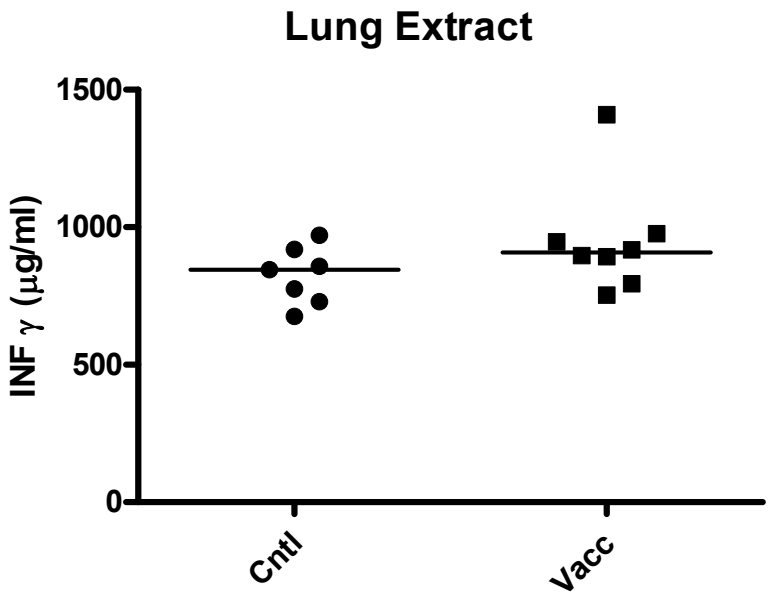

Figure 4.13B. IFNγ concentration as determined with ELISA on foal lung extract recovered from control (n=7) and vaccinated (n=8) groups at day 49. There was no significant difference (p=0.1672) between groups. Horizontal line indicates median.

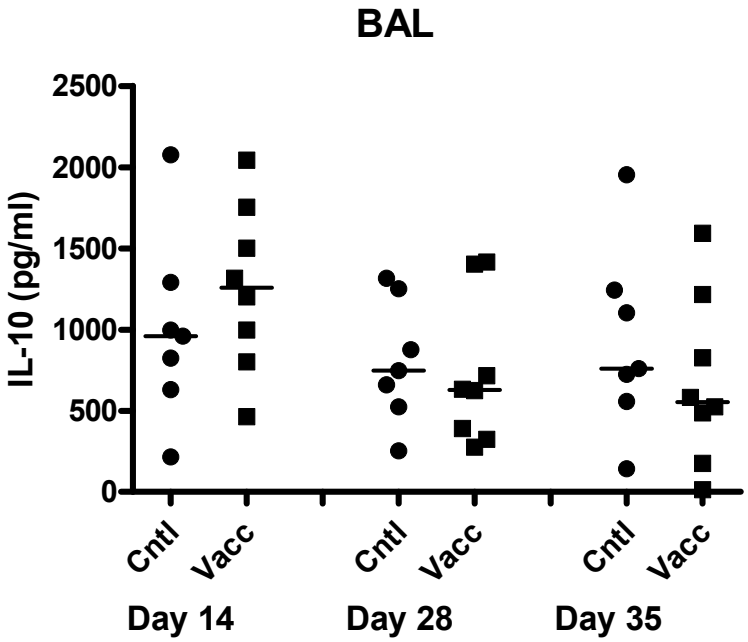

Figure 4.14A. IL-10 concentration as determined with ELISA on foal BAL supernatant recovered from foals in control (n=7) and vaccinated (n=8) groups at different sampling days.There was no significant difference (p=0.8937) between groups. Horizontal line indicates median.

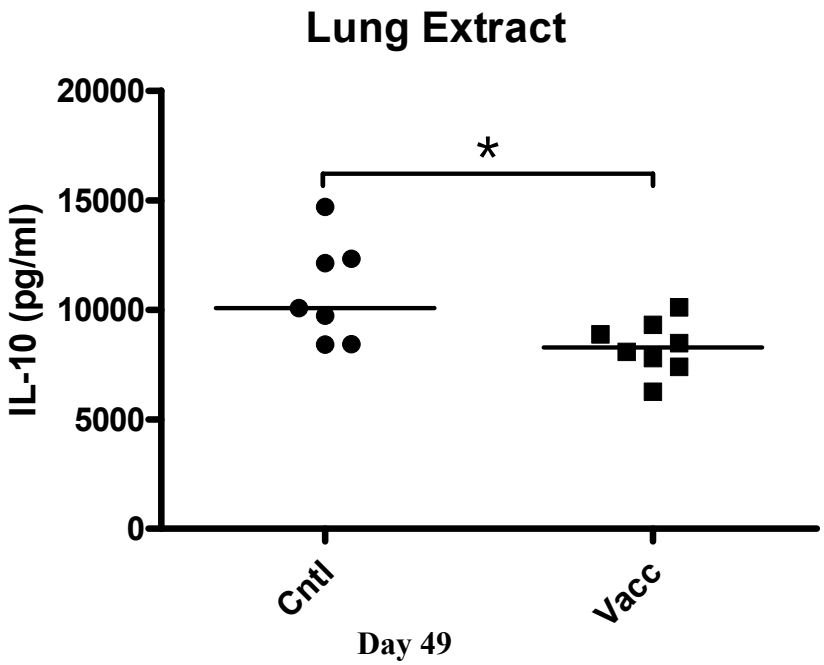

Figure 4.14B. IL-10 concentration as determined with ELISA on foal lung extract recovered from foals in control (n=7) and vaccinated (n=8) groups at day 49. There was significant difference (p=0.0172) (indicated by *) between control and vaccinated groups. Horizontal line indicates median.

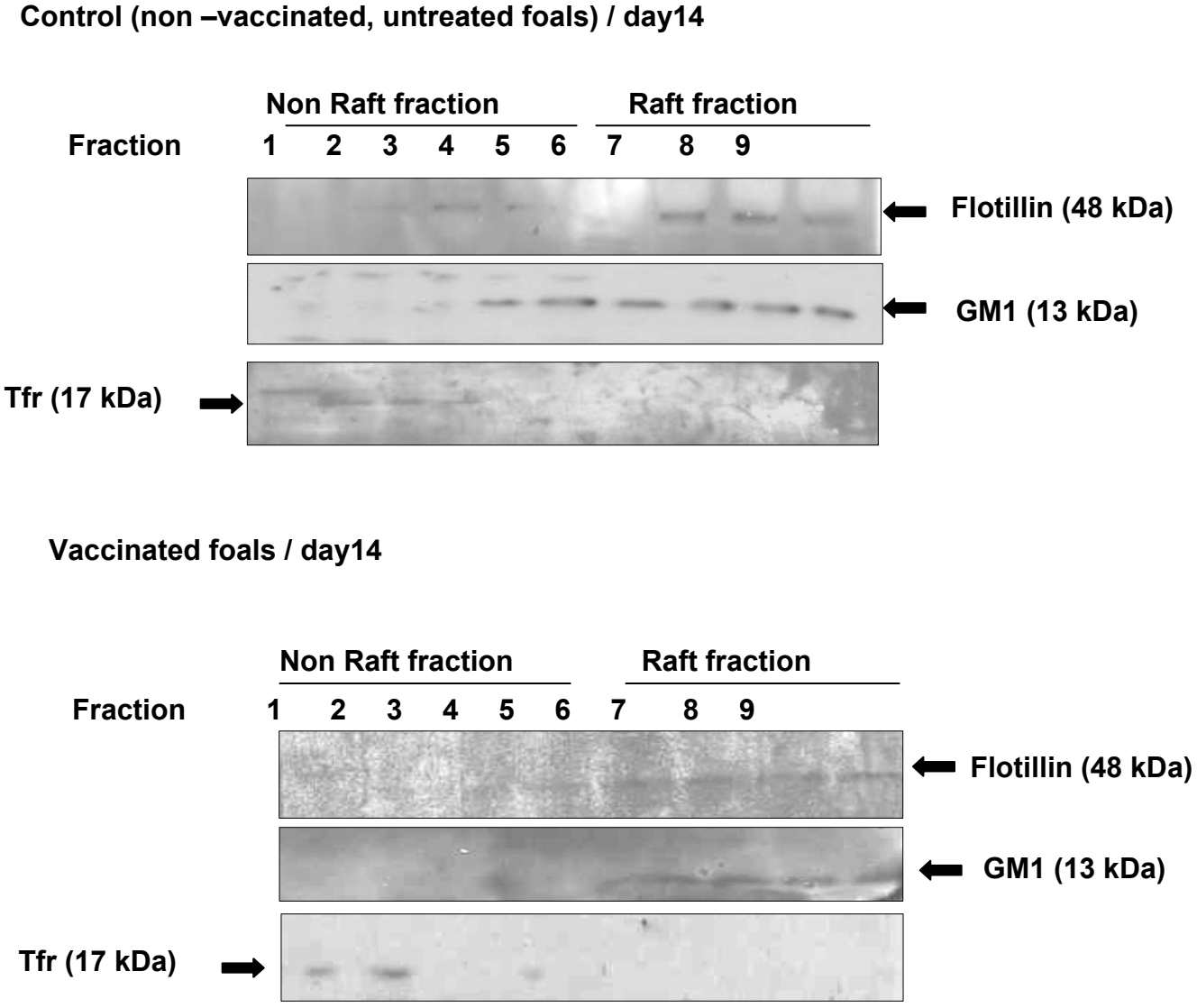

Figure 4.15A. Western blot showing the expression of lipid raft proteins (Flotillin-1 and GM1) and non-lipid raft protein (Transferrin receptor-Tfr) in various membrane fractions isolated from foal BAL cells on day 14.

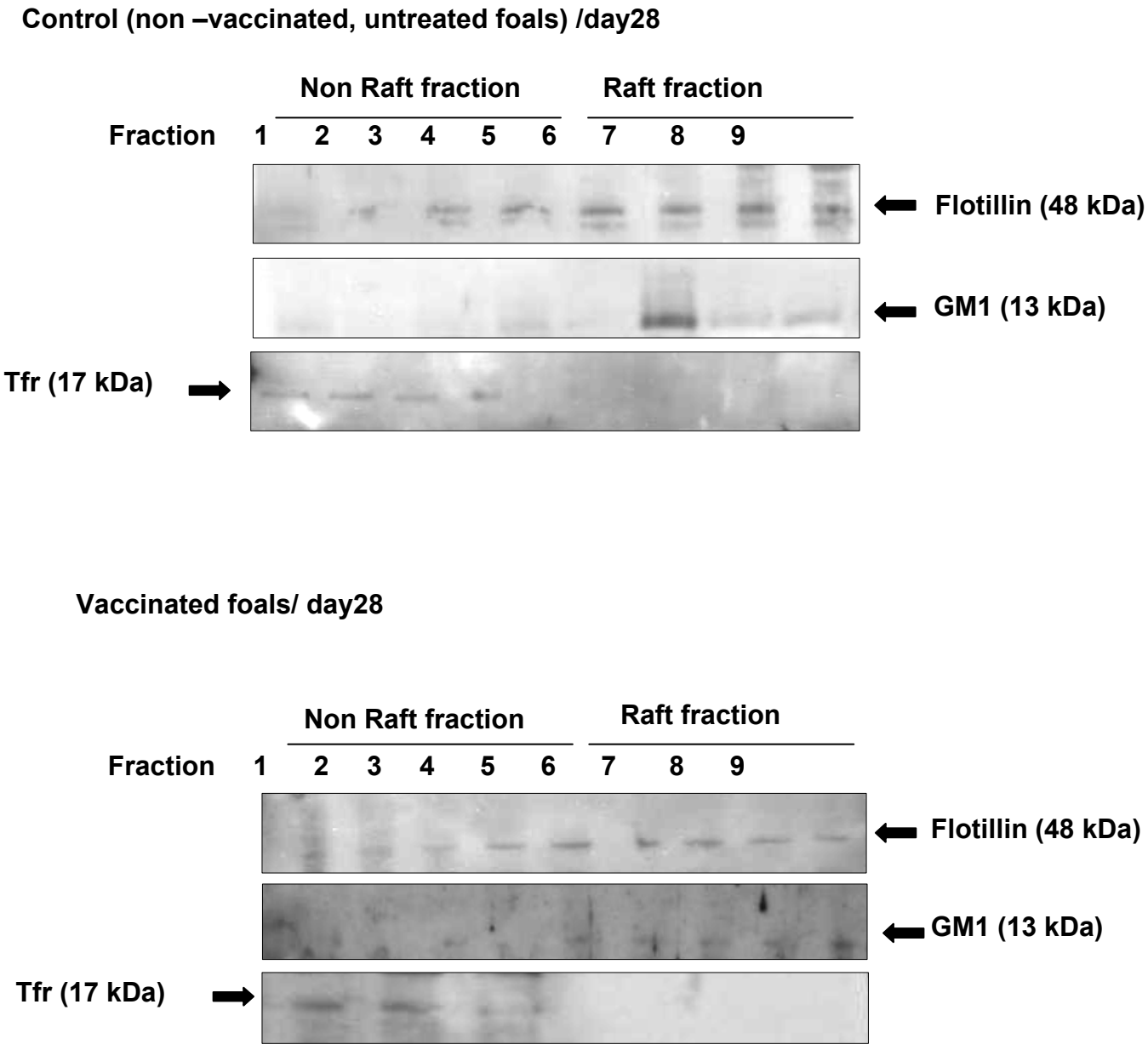

Figure 4.15B. Western blot showing the expression of lipid raft proteins (Flotillin-1and GM1) and non-lipid raft protein (Transferrin receptor-Tfr) in various fractions of plasma membranes isolated from foal BAL supernatant on day 28.

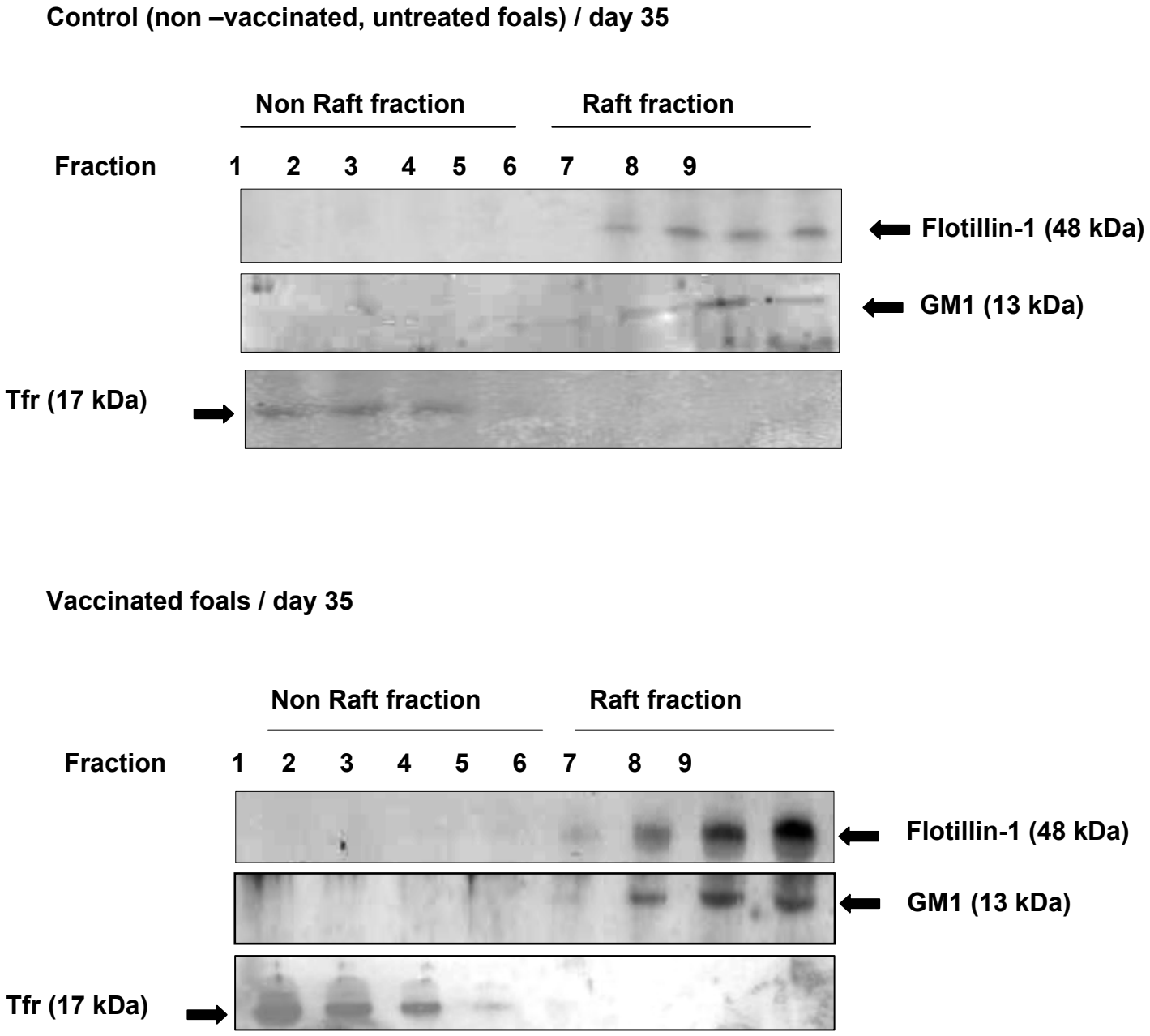

Figure 4.15C. Western blot showing the expression of lipid raft proteins (Flotillin-1and GM1) and non-lipid raft protein (Transferrin receptor-Tfr) in various fractions of plasma membranes isolated from foal BAL supernatantat day 35 .

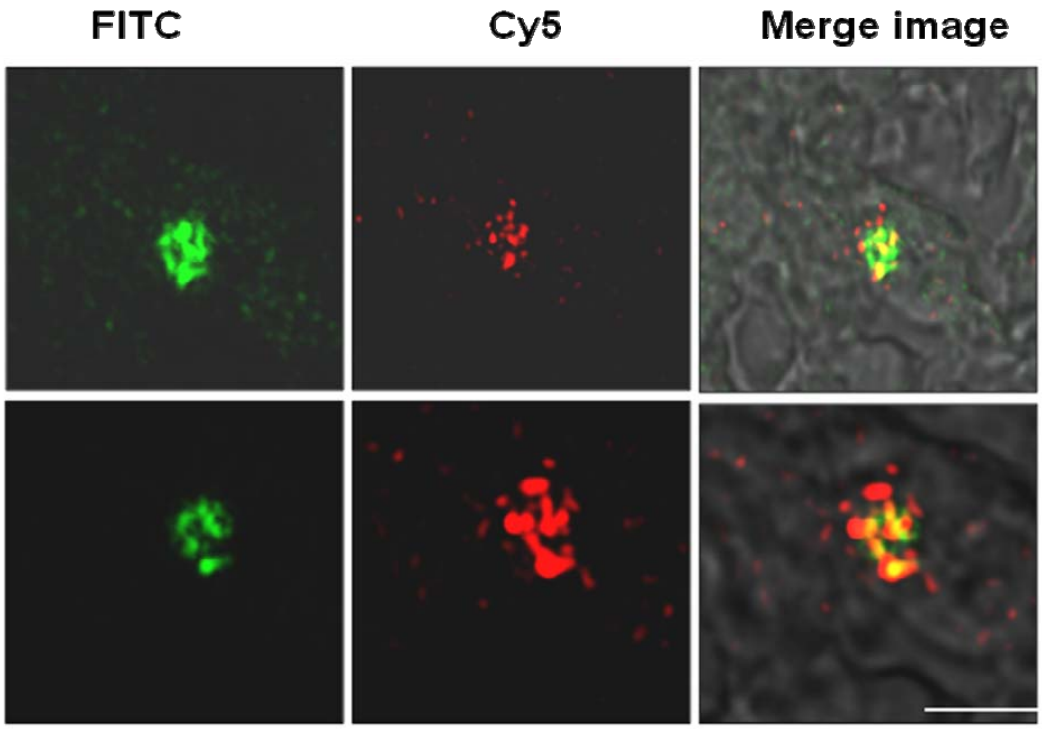

Figure 4.16. Co-localisation of lipid raft proteins (Flotillin-1 and GM1) in untreated control foal lung tissue by confocal microscopy (Bar=100 μm)

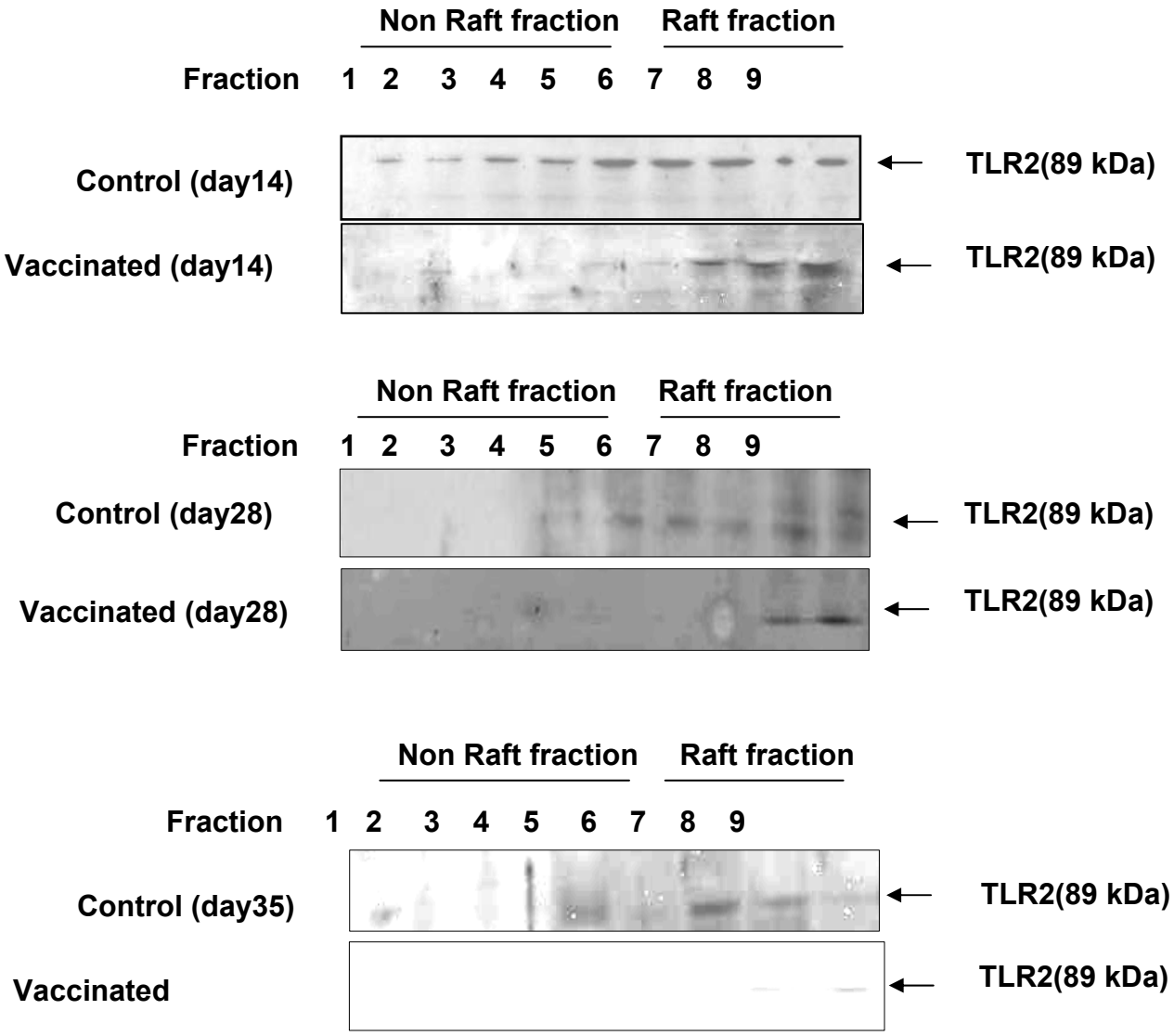

Figure 4.17A. Western blot showing the expression of TLR2 in various fractions of plasma membrane isolated from foal BAL at indicated sampling days. TLR2 was generally restricted to the lipid raft fractions (7-9) in the vaccinated foals.

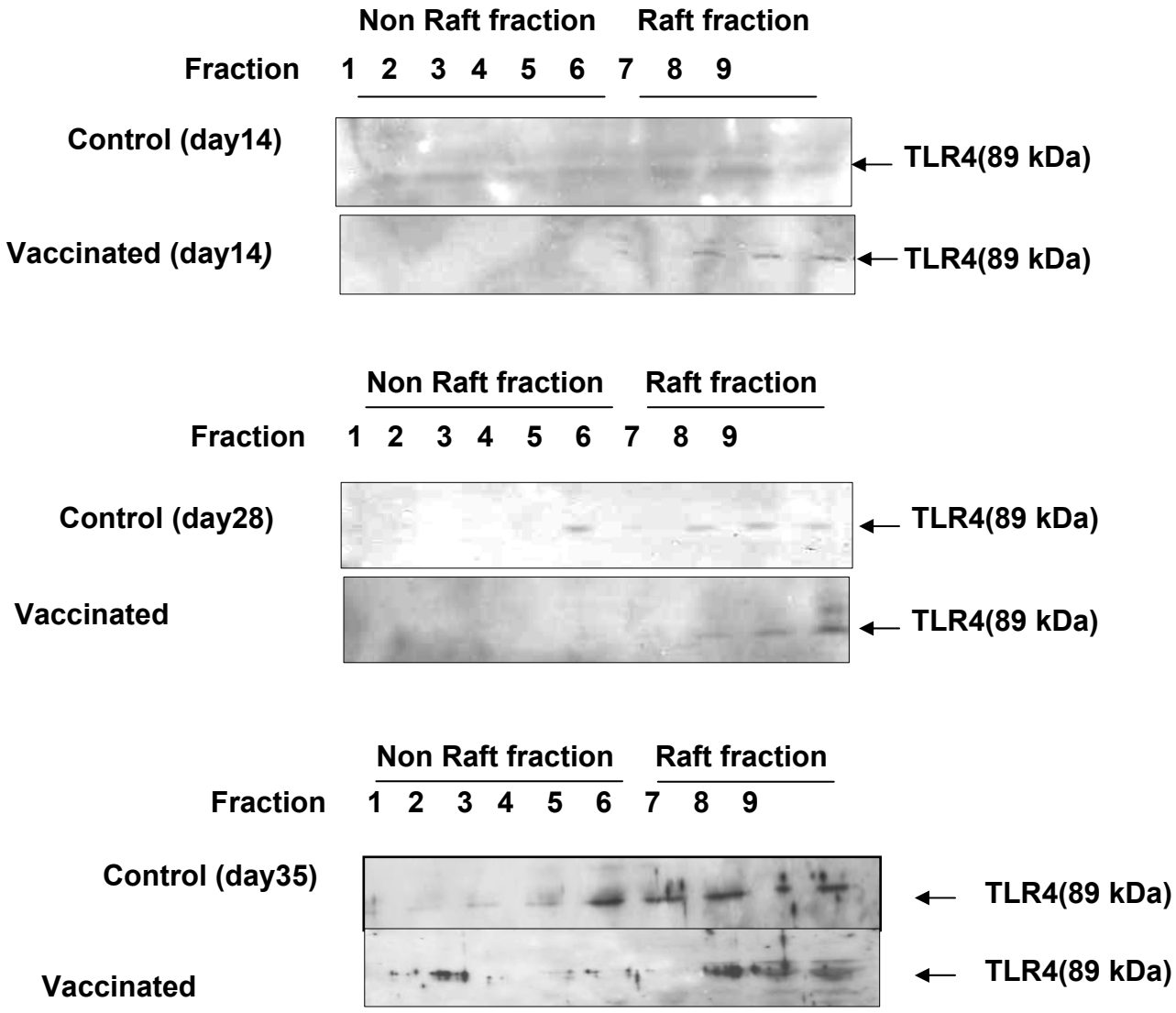

Figure 4.17B. Western blot showing the expression of TLR4 in fractions of plasma membrane obtained from foal BAL cells from control and vaccinated foal groups at different sampling days. TLR4 was generally restricted to lipid raft fractions (7-9).

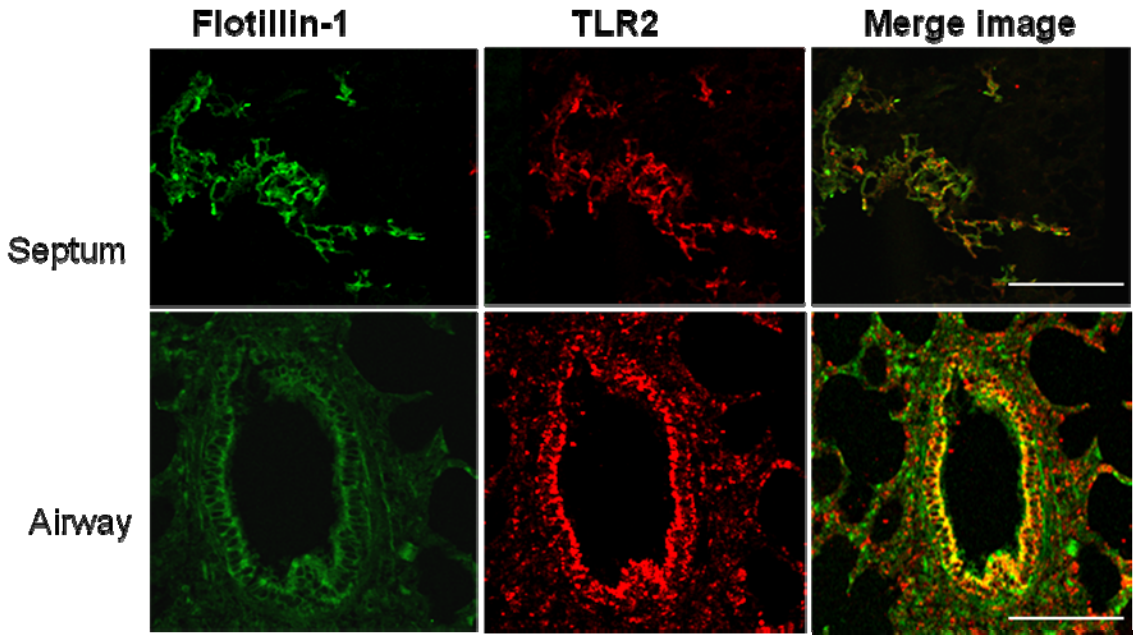

Figure 4.18. Expression of Flotillin-1 and TLR2 in untreated control foal lung tissue (day 49) by confocal microscopy. Flotillin-1 and TLR2 colocalises in the alveolar septa (upper panel) and the airway epithelium (lower panel). (Bar=100μm)

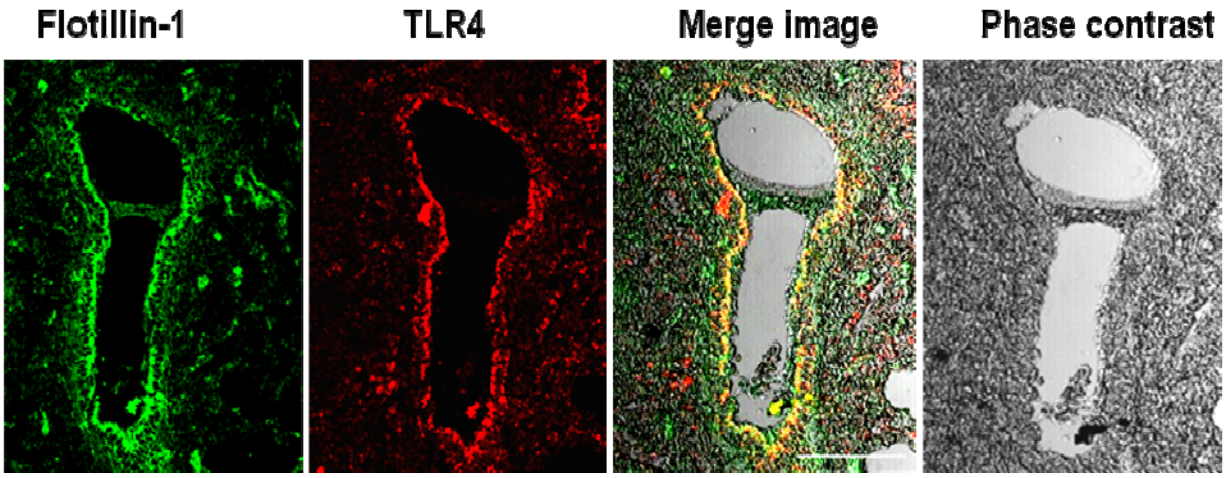

Figure 4.19. Expression of Flotillin-1 and TLR4 in untreated control foal lung tissue (day 49) by confocal microscopy. Flotillin-1 and TLR4 colocalises in the plasma membrane in cells (Bar=100μm)

CHAPTER 5: DISCUSSION

In this study, I have compared the lung inflammation in the control (non-vaccinated and challenged) and vaccinated (vaccinated and challenged) foals. I also provide the first protocol to isolate lipid rafts from equine cells. Gross and histological data showed lung inflammation in both control and vaccinated foals. Immunohistology showed altered expression of TLR2, TLR4 and TNFα on alveolar septa, airway epithelium and endothelium in both groups compared to the untreated control lungs. Vaccinated foals showed increased expression of TNFα in the BAL fluid, decreased expression of IL-10 in lung tissues and increased association of TLR4 and TLR2 with the lipid rafts isolated from alveolar macrophages.

Young foals are susceptible to *R. equi* infection between 1-6 months of age (Prescott, 1991; Giguere *et al.*, 1997; Chaffin *et al.*, 2003). The establishment of *R. equi* infection is a process that is probably 'ignored' by the immune system and has clinical consequences for the host. The mechanisms of establishment of *R. equi* infections in young foals are poorly understood and probably are intertwined with the development of the immune system in the lung. Because of the clinical consequences of *R. equi* infection there has been increasing interest in understanding the basic immune responses in young foals with an aim to elucidate the mechanisms of establishment of infections. Furthermore, a better understanding of basic immune responses will lead to development of effective vaccines against *R. equi* infection.

In the present study, I studied inflammatory responses in the lungs of *R. equi* infected foals and vaccinated foals to determine the efficacy of the vaccine. The inflammation in the lung was studied through the use of BAL, histopathology, ELISA and immunohistology. Although the central objective of the study was to evaluate the efficacy of the vaccine against *R. equi* infection, one of the weaknesses of the study is the lack of BAL and lung tissue homogenate data from non-infected control foals. We partially overcame this problem by using paraffin-embedded lung tissues from normal foals which were obtained from the department of Veterinary Pathology as controls for histopathological and immunohistological studies.

Broncho-alveolar lavage is a powerful tool to study lung pathophysiology in horses and other species and provides a sample for quantification of inflammatory cells and inflammatory mediators in the supernatant of BAL. In many instances the isolated cells can be further used to analyze their inflammatory capacities in vitro (Kawasaki *et al.*, 2006; Feng *et al.*, 2008;

Hoffman, 2008;Couetil *et al.*, 1999; Couetil *et al.*, 2001; Sanchez *et al.*, 2005; Allen *et al.*, 2006; Riihimaki *et al.*, 2008).

Lung inflammation is characterized by the migration of inflammatory cells into the alveolar spaces (Parbhakar *et al*., 2005). The cell migration is a complex process that includes activation and expression of cytokines on endothelial and epithelial cells (Janardhan *et al*., 2004). These cellular and molecular processes result in migration of inflammatory cells such as neutrophils and macrophages into alveolar spaces in response to lung infections with bacteria. Analyses of BAL are useful in evaluating the migration of inflammatory cells into the alveolar spaces. The data from this study showed no differences in total cell numbers in the BAL between the control and vaccinated group at different sampling days to suggest that the vaccination did not alter the inflammatory response induced by *R. equi* infection.

The accumulation of inflammatory cells in the alveolar spaces is a terminal step in their migration from the lung microvessels. It is possible that more inflammatory cells are trapped in the interstitum and the blood vessels in the lung which may not be evident from the analyses of BAL. Therefore, we performed gross and histological examination of the lungs from both groups. There appeared to be some differences in the extent and nature of gross lesions in the lungs. But it is difficult to quantify the lesions at the gross level of examination. Semi quantitative scoring of gross sections cut from the lungs did not show any differences in lung inflammation between the two groups. However, compared to the lung sections from the untreated control foals, both the groups showed gross and histological signs of lung inflammation. Based on the BAL, gross and histological analyses, it appears that *R. equi* induced lung inflammation was not modulated by the vaccination.

The manifestation of lung inflammation through changes in the BAL and tissue architecture is an advanced step, which is initiated following sophisticated changes in the expression of various inflammatory molecules and activation of receptors of the immune system. Toll-like receptors (TLRs) are transmembrane proteins and germline coded receptors present on the cells of the immune system (Lu *et al.*, 2008). TLRs are a part of the conserved IL-1 superfamily and act as sensors against microbial conserved pathogen associated molecular patterns such as LPS, lipotechoic acid, CpG, peptidoglycans and unmethylated DNA on the surface of pathogens (Takeda *et al.*, 2003; Takeuchi *et al.*, 2001; Abreu *et al.*, 2005; Suri *et al*.,

2006). TLR signaling results in cell activation, expression of adhesion molecules and secretion of inflammatory cytokines (Suri *et al*., 2006). To date, 11 TLRs have been found in humans and 13 TLRs have been found in mice, that were isolated based on their ligand specificity (Sheedy *et al.*, 2007; Uno *et al.*, 2007). CD14, a 55KDa protein mediates the recognisation of LPS (Pugin *et al.*, 1993; Gioannini *et al.*, 2007; Miyake, 2007). CD14 is present both in and outside of the lipid raft in normal conditions. Previously, expression of TLR4 has been described in the endothelium, pulmonary intravascular macrophages and the airway epithelium of the horse (Suri *et a*l., 2006). In the study presented here, immunohistology showed increased expression of TLR2 and TLR4 in the alveolar septum of the lungs of both control and vaccinated groups compared to the untreated control foal lungs. Interestingly, Western blots also showed increased expression of TLR2 but not TLR4 in the lung extracts from the vaccinated foals compared to the control foals. Previously, the engagement of a particular TLR by its ligand has been shown to alter the expression of other TLR in the horse lungs (Suri et al., 2006). It is also expected that failure to signal the presence of *R. equi* or any other infectious agent in the lungs of newly born foals may result in the establishment of infection. The weak expression of TLR2 and TLR4 in the alveolar septum, which is critical for the sensing of bacteria and signaling across the blood-air barrier, may underlie the failure to mount an effective immune response against *R. equi.* The increased expression of TLR2 and TLR4 following bacterial challenge with or without vaccination indicates some effect on the maturation of the immune system in the lungs of the foals. It is possible that even though *R. equi* infection up-regulated the expression of TLRs, especially TLR2, the immune response may be one step behind the infection and may not be able to fully clear the infection. In future studies, it may be useful to examine the expression of TLR4 and TLR2 on the BAL cells with flow cytometry and examine the role of these receptors in their responses to *R. equi* infection in vitro.

The activation of cells through receptors such as TLR4 and TLR2 finally leads to the expression of inflammatory mediators such as TNFα, IFNγ and IL-10. Cytokines play critical roles in inflammation and their fine balance is important in clearing infection without causing too much tissue pathology. I examined the expression of TNFα with immunohistology in the lung tissues and that of TNFα, IFNγ, IL-10 with ELISA in foal BAL and lung extracts. Because the protein is the functional end product of gene expression and sole reliance on the mRNA data can lead to faulty conclusions, I decided to examine the protein expression of selected cytokines.

Due to the limitations of the experiment design, it was not possible to compare the BAL changes in the control or vaccinated groups with the age-matched untreated control foals. Therefore, the BAL collected on day 14, 28 and 35 from the control and the vaccinated foals were compared with each other and the lung tissues collected after euthanasia on day 49 were analyzed. Although there are many cytokines that play roles in inflammation, I selected IFNγ for its established role as a immune-modulator and development of foal immunity (Merant *et al*., 2008), TNFα for its central pro-inflammatory role in bacterial infections and lung inflammation and IL- 10 for its role as an anti-inflammatory cytokine (Charavaryamath *et al*., 2006). There were no differences in the expression of IFNγ in foal BAL collected on different sampling days or the lung tissue extracts obtained after euthanasia on day 49. Immunohistology appeared to show increased expression of TNFα in lung tissues especially in the alveolar septum and the airway epithelium, from both the control and the vaccinated groups compared to untreated control foals. There was also significantly higher concentration of this cytokine in BAL from the vaccinated foals compared to the control foals at day 28. Interestingly, there were reduced concentrations of IL-10 in lung extracts of the vaccinated foals compared to the control foals. Because of lack of BAL data from untreated control foals in the present study, it is difficult to compare with the data from previous studies that showed an increase in expression of pro-inflammatory cytokines such as TNFα and IL-1 and anti-inflammatory cytokine such as IL-10, and 1L-12, while expression of IFNγ and IL-12 remained unaltered in foals infected with a virulence plasmid strain of *R. equi* (Giguere *et al*., 1999). Nevertheless, increased expression of the pro-inflammatory cytokine TNFα in BAL on day 28 and reduced expression of the anti-inflammatory cytokine IL-10 in lung extracts on day 49 in the vaccinated foals would suggest an increased immune response to *R. equi* challenge. Indirectly this would suggest a priming effect of the vaccine on the lung cells such as macrophages leading to a more robust inflammation in the lungs in response to the challenge. It however, needs to be stated that balanced rather than exuberant inflammation is a desired outcome in an infection. These differences in the cytokine expression between the two groups may not be profound enough to alter the degree of pathology in the lungs of the foals. The data require further studies to clarify the impact of vaccination on the expression of inflammatory molecules. Despite some differences in the cytokine expression,

the present study shows that the vaccine does not prevent lung pathology induced by *R. equi* infection.

Lipid rafts are detergent resistant membranes, enriched in cholesterol, sphingolipids and transmembrane proteins and make up about 50% of the plasma membrane (Helms *et al.*, 2004; Hao *et al.*, 2001; Pike, 2003). Recently, the role of lipid rafts in cell signaling has been elucidated through various studies. It has been shown that various microbes such as bacteria, viruses and parasites used lipid rafts as a platform or port of entry into the cell. The microbes alter lipid raft dynamics thereby "hijacking" them and thus, preventing immune response against the invading pathogens (Manes *et al.*, 2003). Innate immune receptors such as TLRs are localized in the lipid rafts to facilitate signaling through them (Triantafilou *et al.,* 2002; Dolganiuc *et al.,* 2006). It is possible that *R. equi* infection is established in lung cells of foals because of the failure of sequestration of TLRs in the lipid rafts. Because there are no data on the isolation of lipid rafts from the equine cells, I developed a protocol to prepare lipid rafts from BAL cells collected from the foals. The newly developed method for the isolation of lipid rafts from BAL cells is faster and more efficient compared to the previous methods which are more complex and time consuming (Martens *et al.*, 2001; Gaus *et al.*, 2003; de Mello Coelho *et al.*, 2004; Sleight *et al.*, 2005). The data show isolation of plasma membrane factions from 1 X 10^7 BAL cells. Because there were not enough cells in the BAL taken from a single foal, I had to pool the BAL from 2-3 foals to have enough cells for successful isolation of the rafts. I took care to pool BAL samples from the foals within the same treatment group. The identity of the lipid rafts was established through localization of Flotillin-1 (48kDa) and GM1 (13kDa), which are resident proteins of lipid rafts, with Western blots. The fractions were also probed for the presence of non-lipid raft proteins such as transferrin receptor (17kDa). The comparison of lipid rafts from BAL cells from the control foals with the vaccinated showed no differences in the distribution of Flotillin-1, GM1 and tranferrin receptor proteins. I also show the first localization of lipid raft proteins in the lung tissues of untreated control foals with confocal microscopy. The lipid raft proteins were localized in the alveolar septum, airway epithelium and the vascular endothelium in the lungs.

Lipid rafts from BAL collected on day 14 from the control foals showed Flotillin-1 and GM-1 in fractions 3-9. However, the BAL taken from the vaccinated foals on day 14 showed

these proteins generally in fractions 6-9. The expression of these proteins in lipid rafts taken from BAL collected on day 35 was more intense in fractions 6-9 of the vaccinated foals compared to the control foals. These data suggest the possibility that the first vaccination may have led to activation of cells to segregate lipid rafts while the macrophages from the naïve foals may not be able to form lipid rafts. This interpretation may suggest a subtle effect of vaccination on the cell signaling platforms i.e. lipid rafts of macrophages.

After successfully establishing the raft isolating and identification protocol, I investigated whether TLR4 and TLR2 colocalize with lipid raft fractions because for effective cell signaling TLRs must be sequestered into lipid rafts. While TLR2 was localized in both non-lipid raft and lipid raft fractions of BAL macrophages of control foals on day 14, it was detected in lipid raft fractions on day 28 and day 35. In contrast, TLR2 was detected in fractions 7-9 on day 14, 28 and 35 of vaccinated foals. It appears that the first vaccination affected the localization of TLR2 in lipid raft fractions and is in line with the segregation of lipid raft and non-lipid raft fractions. Nearly similar observations were made for TLR4. TLR4 presence in lipid raft fraction was not in agreement with previous studies, where TLR4 was found in non-lipid raft region of the plasma membrane (Triantafilou *et al.*, 2002; Soong *et al.*, 2004). It seems that the vaccination may have have had a general effect on the ability of BAL cells to sequester receptors in lipid rafts of the cell membrane. The confocal microscopy data showed co-localization of TLR2 and TLR4 with Flotillin-1 in the airway epithelium, vascular endothelium and alveolar septum. Taken together, these data show association of TLR2 and TLR4 with lipid raft fractions. Also, the data indicate that BAL macrophages from newly born foals lack the capacity to develop lipid rafts and also to segregate TLR2 and TLR4 into lipid rafts. Interestingly, the first vaccination did induce association of TLR2 with lipid rafts in macrophages. Considering the role of TLR2 in *R. equi* cell signaling (Darrah *et al.*, 2004), these data show that exposure to vaccination and the age may play important roles in the ability of the foals to launch an effective immune response, and point out the subtle effects of vaccination. The increased association of TLR4 and TLR2 with lipid rafts of the foals may also offer an explanation for more pronounced inflammation in the lungs of vaccinated foals as indicated by increased expression of TNFα and reduced expression of IL-10. These being the first set of data on the localization of TLRs with lipid rafts, there is a need for further studies.

CHAPTER 6: SUMMARY

The data from the experiments reported in this thesis show similar degree of inflammation in the lungs of *R. equi* infected foals with or without vaccination. The immunohistologic data appeared to show increased expression of TLR4, TLR2 and TNFα in the lungs of both the infected groups (with or without vaccination) compared to the untreated control foal group. There were increased concentrations of TNFα in BAL and reduced concentrations of IL- 10 in lung extracts of vaccinated foals compared to control foals. Western blots showed expression of increased expression of TLR2 but not TLR4 in lung extracts of the vaccinated foals compared to the control foals. Taken together, these data suggest similar levels of pathology in the lungs but indicate higher degree of immune response in lungs of foals that were vaccinated before challenge with *R. equi.* Lipid rafts sequester receptors such as TLRs and act as signaling platforms. Therefore, I developed a protocol to isolate lipid rafts from foal BAL cells to investigate distribution of TLR4 and TLR2. The data indicate that lung macrophages from naïve newly born foals may not have efficient lipid rafts and also may be unable to sequester TLR2 and TLR4 to generate effective cell activation and immune response. Combination of increased expression of TLR2 and TLR4 and increased association of these molecules with the lipid raft fractions in the vaccinated foals does suggest subtle effects of the vaccination. These effects do require further investigation.

CHAPTER 7: CONCLUSION

The data from the experiments do not support the hypothesis that vaccination reduces lung inflammation in response to *R. equi* challenge. The data do support the hypothesis that lipid rafts associate with TLR2 and TLR4 in BAL cells of the vaccinated foals but not the control foals.

CHAPTER 8: FUTURE STUDIES

To further understand the role of lipid rafts in *R. equi* infection, it is important to pursue the cellular and molecular effects upon depletion of lipid rafts. It may be important to explore proteins other than TLRs that are involved in pathogen entry inside the cells. Lastly, to explore if lipid raft proteins are restricted to lipid raft region only or they shift to non-lipid raft region upon infection. It would also be interesting to examine the expression of TLR4 and TLR2 on the BAL cells with flow cytometry and examine the role of these receptors in their responses to *R. equi* infection in vitro.

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