REGULATION OF IMMUNE FUNCTION IN NEONATAL FOALS: RESPONSES TO VACCINATION AND ADMINISTRATION OF IMMUNOSTIMULANTS

By

CLARE ANN RYAN

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To Jillian Adele Stirn

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By

Clare Ann Ryan

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Foals are thought to be susceptible to certain infectious diseases because their immune system is naïve or not as competent as that of adult horses. However, the differences in host defense mechanisms between newborn foals, older foals, and adult horses are poorly understood. The objectives of the first part of this study was to compare the frequency of IFN-γ and IL-4 secreting cells of newborn foals to that of older foals and adult horses, and to determine the effect of the type of mitogen used for in vitro stimulation on the relative frequency of cells secreting these cytokines. The frequency of IFN-γ and IL-4 secreting cells was significantly lower in both groups of foals compared to adult horses. Regardless of age, the type of mitogen used for in vitro stimulation had a significant effect on the IFN-γ/IL-4 ratio. The objective of the second part of this study was to determine the effect of comercially available immunostimulants on neutrophil, macrophage, and lymphocyte function following *ex vivo* exposure to *Rhodococcus equi*. Inactivated *Propionibacterium acnes* (PA), inactivated parapoxvirus ovis (PPVO), or saline (control) was administered to foals on days 0 (7 days of age), 2, and 8. Treatment with PPVO significantly increased phagocytosis of *R. equi* and

oxidative burst activity of neutrophils whereas treatment with PA decreased intracellular proliferation of *R. equi* within monocyte-derived macrophages, The objective of the third part of this study was to compare serum immunoglobulin concentrations, antigen-specific lymphoproliferative responses, and cytokine profile of proliferating lymphocytes of 3-day old foals, 3-month old foals, and adult horses following vaccination with a killed adjuvanted vaccine. Both humoral and cell-mediated immune responses to the vaccine were modest in 3-day old foals. Although immune responses improved with age, 3-month old foals did not respond with the same magnitude as adult horses. Newborn foals did not have a bias toward a Th2 response following vaccination with the killed vaccine used.

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CHAPTER 1
INTRODUCTION

Cell-mediated immune responses of murine and human neonates are generally thought to be biased toward a thymocyte helper type 2 (Th2) response (Adkins, 2000). Several studies have documented that newborn foals are deficient in their ability to induce interferon- gamma (IFN-γ) in response to stimulation with mitogens (Boyd *et al.*, 2003;Breathnach *et al.*, 2006). These findings, along with the peculiar susceptibility of foals to infection with *Rhodococcus equi*, a facultative intracellular pathogen known to only cause disease in immunocompetent mice when a Th2 response is experimentally induced (Kanaly *et al.*, 1995), have led to the hypothesis that T cell responses from newborn foals may be biased toward a Th2 cytokine profile. However, experimental infection of neonatal foals with virulent *R. equi* triggers induction of IFN-γ mRNA transcription in a manner that is similar to that of adult horses, indicating that foals can mount adequate IFN-γ responses providing the proper stimulus (Jacks *et al*., 2007a; Jacks *et al*., 2007b).

There are several gaps in our current understanding of the regulation of immune responses in foals. The work presented in this dissertation aims at addressing a few of these gaps. First, a thorough assessment of the Th1/Th2 polarization of the foals' immune responses also necessitates measurement of Th2 cytokines such as IL-4. Unfortunately, interleukin (IL)-4 production has not been investigated in newborn foals. In addition, the relative Th1/Th2 polarization of equine neonatal immune responses would be better assessed by measuring antigen-specific responses after vaccination rather than after artificial stimulation with mitogens. There are no studies evaluating cell-mediated and cytokine responses of newborn foals in response to vaccination.

Finally, data in human, laboratory animals, and adult horses suggest that commercially available immunostimulants are potent inducers of IFN-γ and contribute to neutrophil and macrophage activation. A product capable of increasing IFN-γ induction and activating neutrophils and macrophages in neonatal foals prior to natural infection may be effective in preventing, or at least curtailing, infection with *R. equi* during the narrow window of susceptibility to this pathogen.

The objectives and hypotheses of the first study (Chapter 3) are as follows:

1. To compare the frequency of IFN-γ and IL-4 secreting cells of newborn foals to that of older foals and adult horses

   The hypothesis was that newborn foals have fewer IFN-γ and IL-4 secreting cells than older foals or adults.

2. To determine the effect of the type of mitogen used for *in vitro* stimulation on the relative frequency of cells secreting these cytokines

   The hypothesis was that the type of mitogen used to stimulate PBMCs affects the magnitude of the response.

The objectives and hypotheses of the second study (Chapter 4) are as follows:

1. To determine the effect of immunostimulants on intracellular survival and replication of *R. equi* in foal macrophages

   The working hypothesis for this objective is that macrophages obtained from foals pre-treated with immunostimulants can kill *R. equi* more efficiently *in vitro*.

2. To determine the effect of immunostimulants on cytokine induction by *R. equi*-infected macrophages

   The hypothesis is that mRNA expression of inflammatory (IL-1β, IL-6, TNF-α) and Th1-inducing cytokines (IL-12, IL-18) will be significantly higher in macrophages obtained from foals pre-treated with immunostimulants than in controls.

3. To determine the effect of immunostimulants on phagocytic activity and oxidative burst of blood neutrophils of foals

   The hypothesis is that immunostimulants will significantly enhance phagocytic activity and oxidative burst of peripheral blood neutrophils.

4. To determine the effect of immunostimulants on lymphocyte immunophenotyping, lymphoproliferative responses, and cytokine induction in foals

   The hypothesis was that immunostimulants will significantly enhance lymphoproliferative responses and IFN-γ induction by proliferating lymphocytes in foals.

The objectives and hypotheses of the third study (Chapter 5) are as follows:

1. To determine serum IgM and IgG subclass concentrations of newborn foals, older foals, and adult horses following vaccination with a killed vaccine

   The working hypothesis for this objective is that neonatal foals will produce lower IgGa and IgGb concentrations in response to vaccination compared to older foals or adult horses.

2. To determine antigen-specific lymphoproliferative responses of newborn foals, older foals, and adult horses following vaccination with a killed vaccine

   The hypothesis is that newborn foals have decreased antigen-specific lymphoproliferative responses compared to older foals or adult horses.

3. To determine the cytokine profile of proliferating lymphocytes from newborn foals, older foals, and adult horses following vaccination with a killed vaccine

   The hypothesis for this objective is that lymphocytes from newborn foals will produce less IFN-γ and less IL-4 in response to stimulation with the vaccine antigen compared to lymphocytes obtained from older foals or adult horses.

CHAPTER 2
REVIEW OF THE LITERATURE

Rhodococcus equi is a facultative intracellular bacterium that causes severe pyogranulomatous pneumonia in foals. It has also emerged as an important opportunistic pathogen in immunosuppressed people and patients infected with the human immunodeficiency virus (Arlotti *et al.*, 1996;Emmons *et al.*, 1991;Prescott, 1991). Although *R. equi* is found in soils worldwide, it does not uniformly cause disease in all foals exposed to it. The disease mainly affects foals 1-5 months of age. Some farms may have little to no apparent disease, whereas on other farms the disease is endemic. There is major economic impact on the equine industry due to the cost of treatment, loss of function, delays in training, and death of some animals.

Epidemiology

Rhodococcus equi is a saprophytic organism that survives well in surface soil (Barton & Hughes, 1984). The bacteria are shed in high numbers in the feces of infected foals, and persist in the environment. Adult horses, although they do not display clinical signs of the disease, can also be an important source of the bacteria through fecal shedding. However, because it does not replicate within the adult equine intestinal tract, the amount of bacteria shed in the feces of dams is small (10^1 to10^3 bacteria/gram of feces) relative to the heavy fecal shedding (about 10^5 bacteria/gram of feces) of foals, whose intestinal tract allows for replication until about 3 months of age (Takai *et al.*, 1987). The organism is very hardy in the environment, and is able to multiply in feces on the ground (Hughes & Sulaiman, 1987). *R. equi* is particularly adept at surviving in dry, arid conditions (Barton & Hughes, 1984). The main route of infection is inhalation of the bacteria. Inoculation of foals with the bacteria intra-

bronchially is a consistent experimental method of inducing *R. equi* pneumonia in foals (Martens *et al.*, 1982). However, ingestion of the bacteria certainly does also occur and may be an alternative route of infection (Johnson *et al.*, 1983). *R. equi* has a worldwide distribution. Isolates have been identified on most continents, and in several species of animals including pigs, ruminants, dogs, cats, and horses, Foals are likely exposed very early in life to *Rhodococcus equi* based on the ubiquity of the organism in their environment.

Clinical Disease

The hallmark of *Rhodococcus equi* infection is severe, chronic, progressive pyogranulomatous pneumonia. Initial signs of the disease may manifest clinically as fever and mild exercise intolerance or elevated respiratory rate. The insidious onset of disease makes detection of early disease a challenge. More intermediate signs of lethargy, inappetance, increased respiratory rate and effort, and occasionally cough and nasal discharge may occur. Signs may progress rapidly if untreated to severe respiratory distress, cyanosis, and death. Histopathologic examination of foals that die from, or are euthanized because of the disease, is characterized by large granulomatous, cavitary lesions with intracellular organisms present diffusely throughout the lungs. Infected foals may also frequently develop extra- pulmonary lesions including polysynovitis, osteomyelitis, and septic arthritis, which are characterized by varying degrees of lameness and/ or joint effusion. Immune- mediated uveitis is also a recognized sequela of the disease. Gastrointestinal lesions can be found in up to 50% of *R. equi* patients necropsied (Zink *et al.*, 1986), and can occasionally be associated with the development of diarrhea. Granulomas can often be found in the mesenteric

lymph nodes and Peyer's patches, and there may be associated ulcerative lesions on the colon (Johnson *et al.*, 1983).

A variety of methods have been used for the diagnosis of *Rhodococcus equi* pneumonia in foals. The gold standard remains culture of the bacteria from transtracheal wash samples along with cytological evidence of septic inflammation. Additional diagnotics tools include physical examination, bloodwork, and imaging studies. Thoracic auscultation may reveal a range of abnormalities including diffuse crackles and wheezes, or simply increased bronchovesicular sounds. Ancillary bloodwork can be helpful in monitoring foals on farms with endemic disease. Specifically, an increase in white blood cell counts has been shown to occur prior to development of clinical disease (Giguère *et al.*, 2003b). However, these findings are non- specific and must be interpreted in view of the foal's clinical signs and prevalence of *R. equi* pneumonia on the farm. Imaging of the thoracic cavity is a commonly employed diagnostic. Thoracic radiographs are largely reserved for use at referral institutions. Most commonly, a prominent alveolar pattern is present. Classical radiographic findings indicating the presence of abscesses are discrete cavitary and nodular lesions. Thoracic ultrasonography is commonly used in the field because of widespread availability and ease of use. Initially, practitioners may detect pleural irregularities as the only abnormality. Progression of the pneumonia may allow for detection of consolidated lung and/or abscesses peripherally. Serological assays have been largely unhelpful in the diagnosis of *R. equi* pneumonia, largely due to the number of foals that are exposed but remain healthy (Giguère *et al.*, 2003a).

Treatment

Currently the mainstay of treatment is antimicrobial therapy with macrolides and rifampin. Although many isolates are sensitive to a wide array of antimicrobials *in vitro*, macrolides and rifampin have much better efficacy in vivo because of their ability to penetrate into abscesses and cell membranes of white blood cells. In the 1980's, therapy with rifampin and erythromycin was used as the treatment of choice. The treatment regime consisted of a prolonged course of therapy usually 6-8 weeks, and was successful in decreasing deaths caused by infection. More recently, however, other combinations utilizing newer generation macrolides such as azithromycin and clarithromycin in conjunction with rifampin, have gained popularity because of better efficacy and the shorter course of therapy required (Giguère *et al.*, 2004). Simultaneous supportive care may be required in the form of non- steroidal anti-inflammatories for analgesic and anti- pyretic properties, oxygen supplementation for foals unable to oxygenate properly, fluid therapy for dehydrated animals, and nutritional support in the form of parenteral nutrition for severely debilitated foals. With these drugs and therapies, survival rates have improved. Foals that recover typically do so with no negative impact on performance (Ainsworth *et al.*, 1993;Bernard *et al.*, 1991). Foals recovering from pneumonia enjoyed similar success at the racetrack compared to cohorts without pneumonia. However, *R. equi* pneumonia is still very common and remains a tremendous drain on owner and manager resources.

Microbiology

Rhodococcus equi is characterized as a Gram positive, facultative intracellular bacterium. It is a pleomorphic coccobacillus belonging to the mycolata taxon which also contains the human pathogen *Mycobacterium tuberculosis*. These bacteria

characteristically have a cell envelope rich in high molecular weight branched chain mycolic acids. *R. equi* has a lipoarabiminomannan (LAM) wall, which is similar to that of *M. tuberculosis*. This wall has been shown to contribute to its immunogenicity, based its ability to increase proinflammatory cytokine expression by equine peripheral blood mononuclear cells (PBMCs) exposed to the LAM (Garton *et al.*, 2002). It has also been shown to suppress T cell proliferation and inhibit TNF-α induced functions including microbicidal activity, induction of cytokines associated with macrophage activation (Chatterjee & Khoo, 1998).

Virulent *R. equi* contains an 85-90 kilobase plasmid shown to have 69 open reading frames(Takai *et al.*, 2000). The plasmid contains a 27.5 kb pathogenicity island which encodes genes for virulence associated proteins (VapA, and VapC-Vap I) (Takai *et al.*, 2000). Regulation of *vap* gene expression is dependent on multiple factors including pH, temperature, magnesium, and iron (Benoit *et al.*, 2001;Ren & Prescott, 2003;Takai *et al.*, 1996). *Vap A* and *Vap G* expression are highly up-regulated by exposure to hydrogen peroxide as part of the oxidative burst of activated macrophages (Benoit *et al.*, 2002), and may play a role in mediating survival within macrophages.

In vitro infection of murine macrophages with plasmid- containing *R.equi* results in effective replication of the bacterium, whereas macrophages infected with plasmid-cured strains successfully clear the bacteria (Giguère *et al.*, 1999b). Vap A, expressed on the cell surface when *R. equi* is grown between 34 and 41ºC (Takai *et al.*, 1996), is associated experimentally and clinically with increased incidence and severity of disease (Takai *et al.*, 1994;Takai *et al.*, 1991). Foals infected with *R. equi* strains containing the virulence plasmid and expressing VapA develop clinical signs of the

bronchopneumonia, while foals infected with plasmid cured strains do not develop disease (Giguère *et al.*, 1999a;Wada *et al.*, 1997). In one study by Jain *et al*., a mutant strain of *R. equi* lacking *vapA* and *vapC-vapI* genes was shown to avirulent; complementation with *vapA* restored virulence whereas complementation with *vapC, vapD, or vapE* did not (Jain *et al.*, 2003). Conversely, a recombinant plasmid-cured derivative expressing wild-type levels of VapA failed to survive and replicate in macrophages and remained avirulent for foals showing that expression of VapA alone is not sufficient to restore the virulence phenotype (Giguère *et al.*, 1999a). These findings show that although VapA is essential for virulence, other plasmid-encoded products also contribute to the ability of *R. equi* to cause disease

Mechanisms of Cellular Infection

R equi is a facultative intracellular pathogen. Once inside macrophages, live bacteria persist and multiply within membrane-bound phagocytic vesicles (Zink *et al.*, 1987). Approximately 60-75% of *R. equi* survive after ingestion by macrophages (Zink *et al.*, 1985). Once in the cell, the normal host defenses to bacterial pathogens include fusion of the phagosome and lysosome, and exposure of the pathogen to toxic reactive oxygen intermediates including oxygen (O_2), superoxide anions (O_2-), hydrogen peroxide (H_2O_2), hydroxyl radical (-OH), and reactive nitrogen intermediates including nitic oxide (NO), and peroxynitrate (ONOO-). Production of these toxic metabolites is increased in macrophages that have been activated by exposure to the pro-inflammatory cytokine IFN-γ. *R. equi* has proven to be resistant to several of these metabolites when exposed *in vitro*, including H_2O_2 (Benoit *et al.*, 2002), and superoxide (O_2^-) (Brumbaugh *et al.*, 1990). Killing of intracellular *R. equi* in a murine macrophage

model is largely dependent on production of peroxynitrate (ONOO-) by activated macrophages (Darrah *et al.*, 2000).

R. equi enters the cell via complement mediated mechanisms utilizing the complement type 3 receptor (CR3), also called Mac-1(Hondalus *et al.*, 1993). Since this receptor is present primarily on mammalian monocytes and macrophages, it follows that disease is characterized by *R. equi* found within macrophages. *R. equi* is opsonized by the alternative complement pathway (ACP), as demonstrated by fully effective binding to Mac-1 when incubated with C2- and C4- deficient serum, and loss of binding with C3-depleted serum (Hondalus *et al.*, 1993).

R. equi's persistence in the vesicles of macrophages has lead some researchers to speculate that the bacteria prevent fusion of the phagolysosome. Fernandez-Mora *et al*. (2005) showed that *R. equi* containing vacuoles completed early endosome stage but did not progress to a fully mature late endosome. In the same study, the fusion of the phagolysosome did not occur in murine macrophages infected with plasmid-containing *R. equi*. However, another study has demonstrated that *R. equi* survival in murine J774 macrophages, which occurs in plasmid- containing isolates but not plasmid- cured isolates, was not due to failure of the phagolysosome, but by suppression of acidification of the phagolysosome (Toyooka *et al.*, 2005).

Immunology

Crucial to understanding why foals are the susceptible population of animals is knowledge of unique characteristics of the neonatal foal immune system. First, however, we will examine normal effective adult immune responses. Adult horses are not susceptible to *R. equi* pneumonia, even when inoculated with relatively large numbers of bacteria. T lymphocytes are absolutely required for protection from disease

caused by virulent, plasmid- containing *Rhodococcus equi*, as illustrated by studies performed in mice (Kanaly *et al.*, 1995;Madarame *et al.*, 1997). Both CD4+ and CD8+ T cell subsets are required for the generation of full protective immunity and will be discussed separately.

Role of CD4+ T Lymphocytes in Immunity

CD4+ T lymphocytes, also called T helper cells, can be divided into two main subsets based on their pattern of cytokine secretion and effector functions. The subsets are termed T helper 1 (Th1) and T helper 2 (Th2) subsets. CD4+ Th1 lymphocytes play a critical role in protection against *R. equi* infection in mice by secreting IFN-γ (IFN-gamma) (Kanaly *et al.*, 1993). The production of IFN-γ is both driven by, and helps to sustain, the production of interleukin (IL)- 12 by activated antigen- presenting cells (APCs) such as dendritic cells and macrophages. Th1- type responses are required for clearance of *R. equi*. Conversely, the development of Th2 type cytokine responses, characterized by the production of IL-4, IL-5, and IL-10 promoting humoral immunity, is associated with development of pneumonic lesions following experimental infection of mice with *R. equi* (Kanaly *et al.*, 1995).

The cascade of events leading to the development of adequate Th1 type responses must be initiated by appropriate stimulation of the innate immune system, which then influences cytokine and co- stimulatory molecule production by antigen-presenting cells. Exact specifications for stimulating the innate immune system to support Th1 type responses against *R. equi* are not yet fully understood. As the link between innate and acquired immune responses, dendritic cells likely play a role in the development of Th1 versus Th2 type responses. It has recently been shown that there

are differences in both phenotypic characteristics and cytokine expression of monocyte-derived macrophages from foals as compared to adult horses (Merant, *et al.*, 2009). Adult cells had higher numbers of $CD1w2^{+}CD86^{+}$ cells in their CD14- monocyte- derived macrophages as compared to newborn foals. In the same study, foal cells had lower expression of TNF-α, IL-10, MCP-1 and TGF-β than adult cells. Stimulation of these cells with LPS resulted in elevation of TNF-α and IL-10. In studies performed in murine models, the toll like receptor (TLR) 2 pathway was shown to be crucial for the initiation of intracellular signaling resulting in increased co- stimulatory molecule expression and increased cytokine responses (Darrah *et al.*, 2004). Specifically, up-regulation of CD40 molecules on dendritic cells and increased levels of INF-γ and TNF-α production by macrophages occurred after exposure of murine cells to VapA, but did not occur in mouse cell lines with knockout TLR 2 receptor. Similarly TLR2 knockout mice failed to clear the bacteria in an *in vivo* challenge. Based on these findings, activation of the innate immune system via TLR 2 and subsequent up- regulation of cytokine response led to increased macrophage killing of bacteria.

Role of CD8+ T Lymphocytes in Immunity

CD8+ T lymphocyte subsets also appear to play the major role in immunity through cell mediated defenses (Nordmann *et al.*, 1992). The killing of *Rhodococcus equi*- infected equine peripheral blood monocyte- derived macrophages has been investigated (Patton *et al.*, 2004). In this study, *R. equi*- specific CD8+ cytotoxic T lymphocyte activity was present in adult immunocompetent horses, and was carried out in a MHC class I- unrestricted fashion. Notably, in a similar study assessing the development of CTL activity in foals, *R. equi*- stimulated PBMCs from foals were unable

to lyse *R. equi* specific target cells at three weeks of age, but gained the ability to lyse by six weeks of age (Patton *et al.*, 2005). Cell- mediated killing of *R. equi* is crucial for clearance of the bacteria and prevention of disease; an inefficacy of CTL response in foals may help explain their unique susceptibility to *R. equi* pneumonia.

Neutrophils

It has been documented that neutrophils from foals exert age- related changes in their phagocytic and oxidative burst activity (McTaggart *et al.*, 2001;Witchel *et al*., 1991) Comparison of neutrophil function between foals and adults has given contradictory results with some studies showing equal and others showing decreased neutrophil function. Both adult horse and foal neutrophils are fully capable of killing ingested *Rhodococcus equi*, although a subset of neonatal foals may have decreased killing capabilities (Martens *et al.*, 1988;Takai *et al.*, 1986a;Takai *et al.*, 1986b). Antibody specific opsonization by neutrophils enhances phagocytosis and killing of the bacteria. In adult horses, stimulation of neutrophils with *R. equi* leads to increased expression of the pro- inflammatory cytokines TNF-α, IL-12p40, IL-6, IL-8, and IL-23p19 (Nerren *et al.*, 2009b). Similarly, foal neutrophils stimulated with *R. equi* also express pro-inflammatory cytokines TNF-α, IL-12p35, IL-12p40, IL-6, IL-8, and IL-23p19, as well as IFN-γ (Nerren *et al.*, 2009a). In the aforementioned study, expression of IL-6, IL-8, IL-12p40 and IL-23p19 increased with age.

Ontogeny of the Equine Immune System

Development of the equine immune system occurs relatively early during fetal life. Lymphocytes are present in the peripheral blood of the equine fetus by day 120 of gestation and they proliferate in response to mitogens by day 140 (Perryman *et al.*, 1980). Specific antibody responses to *in utero* vaccination with coliphage T2 have been

detected in equine fetuses as early as day 200 of gestation (Martin & Larson, 1973). In other studies, administration of a Venezuelian equine encephalomyelitis antigen to equine fetuses between 232 and 283 days of gestational age resulted in higher serum neutralization titers than that elicited by the same preparation in adult horses (Mock *et al*., 1978; Morgan *et al*., 1975). Recent work supports these findings, showing that active B cell development and immunoglobulin isotype switching occur during equine gestation and the neonatal period (Tallmadge *et al*., 2009). Proliferation of peripheral blood lymphocytes in response to mitogens is slightly reduced at birth but rapidly increases to adult levels (Flaminio *et al*., 2000;Sanada *et al*., 1992). Foals also have normal lymphokine activated killing (LAK) cell activity of peripheral blood lymphocytes at birth and during early life (Flaminio *et al.*, 2000).

The Neonatal Immune System

For neonates, exposure to a large number of infectious organisms occurs after birth. For the first few months, the maternal transfer of antibodies to the newborn may protect to some degree against potential pathogens until immune responses can be initiated. Intracellular organisms, such as *Rhodococcus equi*, present a particular challenge to foals because of their exposure at a very young age, and because passive immunization does not completely control infection. In addition, the neonatal immune system has many differences compared with the adult immune system that may make control of viral and intracellular bacterial infection more difficult. Differences in the neonatal immune system of mice and human neonates versus adults have been well-documented (Adkins *et al.*, 2004;Siegrist, 2001). Neonatal responses to agents that normally provoke strong immune responses in adults are in many cases dampened, which may lead to lack of protection from pathogens. Until fairly recently, neonatal

immune responses were considered to be deficient or immature. However, it is becoming apparent that the immune system of neonates is competent, but that their immune cells are under different regulation than adult immune cells on many levels.

Many studies have illustrated these differences of the neonatal immune system. For example neonatal mice have been found to produce less IL- 2 and have less T- cell proliferation in response to certain stimuli when compared to adult murine responses. Inoculation of neonatal mice results in a Th2- biased response, as opposed to the Th1 type response that adult mice develop. However, this Th2 bias is not absolute; neonatal mice will respond with “normal, fully mature” responses when exposed to some specific agents that are able to promote strong Th1 responses. Examples of these agents include some DNA vaccines, bacillus Calmette-Guerin (BCG), and oligonucleotides containing CPG motifs (Hussey *et al.*, 2002;Ito *et al.*, 2005). Additionally, other factors such as the dose of antigen and the pre- existing cytokine/environmental milleu, may have an effect on the neonate’s immune system responsiveness to stimuli (Power *et al.*, 1998). For example, exposure to a relatively low inoculum of BCG in neonatal mice leads to development of an almost exclusively Th1 type response and cell mediated immunity, whereas a higher inoculum of BCG resulted in a mixed Th1/Th2 response (Power *et al.*, 1998). This stratification of responses based on dose of antigen exposure is also important in foals and will be discussed below.

Expression of cell surface receptors also varies between neonates and adults. T Cell Receptor (TCR) complex density is lower on human and mouse neonatal cells versus adults (Harris, 1992), which may suggest a requirement for higher levels of receptor agonist to initiate intracellular signaling in neonatal cells. Additionally, the

level of expression of CD40- ligand, which assists in antibody production, class-switching of antibodies by produced by B cells, and memory B cell responses, by neonatal T lymphocytes is controversial. One study indicated that CD40- ligand expression in T cells stimulated by anti- CD3+ antibody isolated from human cord blood was similar to that of adults (Splawski *et al.*, 1996). In the aforementioned study, it was noted that up- regulation of expression was decreased from adult levels when exposed to the phorbol myristate acetate (PMA) and ionomycin. However, other studies have reported a consistent and substantial deficiency in expression of CD40- ligand by neonatal T cells after stimulation with ionomycin, PMA, or by engagement of the TCR receptor (Jullien *et al.*, 2003).

Human neonatal T cells have a variety of mechanisms for altered responsiveness when compared to adults which may explain their tendency away from developing Th1 responses. The expression of IFN-γ by CD4+ T lymphocytes is decreased in neonates. This has been associated with hypermethylation of CpG and non-CpG sites in or proximal to the IFN promoter region (White *et al.*, 2002). Hypermethylation of IFN-γ promoter sites in disease states such as infection with HIV has been shown to decreased expression of IFN-γ, illustrating hypermethylation's effect on cytokine production (Mikovits *et al.*, 1998). One study investigating other mechanisms of control over cell responsiveness described decreased levels of nuclear factors of activated transcription (NFAT) in un- stimulated human stem cells versus adult cells (Kadereit *et al.*, 1999;Kadereit *et al.*, 2003). In the study, decreased NFAT1 levels correlated well with decreased expression of cytokines TNF-α and IFN-γ. In a follow- up to this study, the same investigators were able to increase the production of

IFN-γ by stem cells. This was accomplished by exposure to IFN-γ and antigen presenting cells, which in turn causes positive feedback and up- regulation of NFATc2 driven IFN-γ production (Kadereit *et al.*, 2003). Two important concepts are highlighted here. The essential requirement for initial stimulation of the innate immune system is highlighted because, in vitro, the production of IFN-γ begins with recognition of a "danger" signal by innate immune cells (NK cells, dendritic cells, and macrophages). Adequate stimulation of these cells is mandatory for mounting Th1 responses; they create the environment that lymphocytes reside in and respond to. Secondly, under the correct environmental conditions (which are not yet fully defined but include certain cytokines and co- stimulatory factors), neonatal T cells are fully capable of producing cytokines that can lead to an effective Th1 type immune response. Once established, the specific T helper profile of cytokines tends to persist because of positive feedback and inhibition of the opposite type response. These principals become important when manipulations of the neonatal immune system, in the form of immunomodulators and vaccines, are attempted.

Foal Immune Responses

The immune defenses of neonatal foals are similar in many ways to that of mice and humans. In contrast to adults, neonatal foals are considered to have poor ability to mount Th1 responses, and are generally considered inefficient killers of intracellular organisms. The production of IFN-γ by equine neonatal cells in response to PMA and ionomycin stimulation has been shown to be markedly decreased in the first several weeks of life (Breathnach *et al.*, 2006). A study by Boyd *et al*. (2003) identified an increase in the production of RNA expression of IFN-γ, TGF-β1, and IL-1α by PBMCs

over the first 4 weeks of life. In contrast, under different conditions, the foal immune system can be stimulated to produce levels of Th1 cytokines comparable to that of adults. Infection of neonatal foals with a low dose of virulent *R. equi* results in higher production of IFN-γ mRNA than adult horses receiving the same inoculum (Jacks *et al.*, 2007a). In another study, the size of the *R. equi* inoculum was shown to modulate the IgG subisotype response and possibly the cytokine profile of foals. A recent study by Liu *et al*. (2009) shows that foal PBMCs stimulated with *R. equi* or CpG-ODN have increased expression of IL-6 and IL-8, and that the magnitude of this increased expression was greater in older foals versus newborn foals. In the same study, foals had a differential response to the stimulus used; stimulation with *R. equi* led to increased expression of IL-23p19/p40, whereas stimulation with CpG-OPN led to increased expression of IL-12p35/p40. Taken cumulatively, studies of the equine neonatal immune system indicate that there are difference between neonates and adults, with neonates being deficient in their ability to produce IFN-γ. However, when exposed to the right environment, foals appear capable of mounting Th1 responses of the same magnitude as adults.

Prevention of Disease

Several strategies have been employed in an attempt to prevent or decrease the severity of *R. equi* pneumonia in foals. Humoral immunity appears to play a role in host defense against the organism as indicated by results of passive immunization studies. In an effort to prevent disease, hyperimmune plasma has been administered to foals on farms with endemic *R. equi* pneumonia. There has been a range of effect, but on some farms with endemic *R. equi* pneumonia there was a significant decrease in incidence of

disease when foals received hyperimmune plasma (Madigan *et al.*, 1991;Martens *et al.*, 1989; Prescott *et al.*, 1997a). Other studies have been inconsistent in identifying significant effects of passive immunization (Giguère *et al.*, 2002; Hurley & Begg, 1995). These studies indicate that antibody plays a partial role in clearance but is not completely protective.

Foals that are present on farms that have endemic *Rhodococcus equi* are exposed to high concentrations of bacteria from very early on in life. However, most foals on endemic farms do not develop severe pneumonia. Also, previous studies on the effect of oral inoculation with live virulent *R. equi* indicate that oral immunization provides protection against heavy challenge in foals (Chirinotrejo *et al.*, 1987;Hooper-McGrevy *et al.*, 2005). These findings illustrate the potential utility in vaccinating foals against key antigens of *Rhodococcus equi*.

Several active vaccination strategies have been investigated in mice, and both foals and their dams, and have been met with varying degrees of success. In 1991 studies by Martens and Madigan investigated the utility of vaccinating mares to enhance colostral *R. equi*- specific antibody and increase passive immunity. In both studies, vaccination of the mares was non- protective. Vaccination of mares with VapA in a water- base nanoparticle adjuvant may have provided a degree of protection against a small number of naturally challenged foals (Cauchard *et al.*, 2004). Recently, a DNA vaccine expressing VapA was used successfully in mice to enhance clearance of virulent *R. equi* (Haghighi & Prescott, 2005). Concomitant increases in IgGa, indicative of a type 1- based immune response, were also noted. In the same study, the addition of IL- 12 to the VapA DNA vaccine resulted in even more marked effect on clearance of

R. equi by mice. However, a similar DNA vaccine induced strong cell mediated responses in adult horses but poor responses in immunized foals (Lopez *et al.*, 2003). Although success has not yet been achieved, active immunization is still likely to become an important part of *R. equi* prevention and further studies are ongoing.

Immunostimulants

An immunostimulant can be defined as an agent that "stimulates the response of effector cells such as macrophages, lymphocytes, and neutrophils, which subsequently activate one or more terminal immune responses such as antigen uptake, cytotoxicity, phagocytosis, cytokine release, and antibody response" (Flaminio *et al.*, 1998). Several immunostimulants are commercially available for use in equids. One product, EqStim® (Neogen Corporation), is composed of inactivated *Propionibacterium acnes*. It has been used for the treatment of non- specific respiratory disease in horses. This preparation has been used with favorable results in clinical trials for prevention of stress- induced respiratory infection in adult horses (Nestved, 1996). It has also been investigated as an adjunct to conventional therapy in the treatment of horses with equine respiratory disease complex (Vail *et al.*, 1990). Results showed that 96% of horses treated with traditional therapy in combination with *P. acnes* recovered from disease in 14 days versus only 35% recovery of horses treated with traditional therapy alone. Proposed mechanism of action is activation of macrophages, which contributes to enhanced pathogen killing. In horses, Davis *et al*. (2003) identified increased gene expression of the type- 1 cytokines IFN-γ and NK-lysin as determined by RT-PCR in adult horses treated with inactivated *P. acnes*. Flaminio *et al.* (1998) showed that administration of inactivated *P. acnes* to healthy weanling (aged 6-8 months) horses

resulted in increased CD4+ T lymphocytes and lymphocyte activated killer activity in peripheral blood and BAL fluid, increased nonopsonized phagocytosis in peripheral blood leukocytes, and decreased pulmonary cellularity. Administration of EqStim® to foals starting at age 2-3 days of age failed to have an effect on the production of IFN-γ by PMA stimulated mononuclear cells, as determined by flow cytometric analysis, from PBMC or BAL samples (Sturgill, 2006).

Another immunomodulator available for use in horses is inactivated paprapoxvirus (Orf virus). This viral product is able to stimulate the innate immune system. It has been shown to cause an up- regulation in human immune cells of both pro- inflammatory (Th1- type) cytokines such as IFN-γ and TNF-α, followed by anti-inflammatory (Th2- type) cytokines such as IL-10 and IL-4 (Friebe *et al.*, 2004a). In porcine leukocytes it has increased the release of IFN-γ, IFN-α, and IL-2, and has resulted in increased proliferation, while failing to increase phagocytosis, oxidative burst, or NK activity (Fachinger *et al.*, 2000). Inactivated parapoxvirus induces some protection against the intracellular viral pathogens hepatitis B and herpes simplex virus infections in mice (Weber *et al.*, 2003). In this study there was an initial up- regulation of the production of Th1 helper type cytokines IL-12, IL-18, and IFN-γ, which was followed by a down- regulation of the same cytokines and production of IL-4. IL-10 expression was also up- regulated in livers of hepatitis B virus- infected mice, which may have prevented excessive tissue damage.

CHAPTER 3
EFFECT OF AGE AND MITOGEN ON THE FREQUENCY OF INTERLEUKIN-4 AND INTERFERON GAMMA SECRETING CELLS IN FOALS AND ADULT HORSES AS ASSESSED BY AN EQUINE-SPECIFIC ELISPOT ASSAY[1]

Abstract

Peripheral blood mononuclear cells (PBMCs) were obtained from 6 foals < 1 week of age, 6 foals between 3 and 4 months of age, and 10 adult horses. PBMC were stimulated with concanavalin A (ConA) or calcium ionomycin-phorbol myristate acetate (CaI-PMA) and the frequency of interferon IFN-γ and IL-4-secreting cells was measured using an equine-specific ELISPOT assay. The number of IFN-γ -secreting cells was significantly lower in both groups of foals than in adult horses regardless of the mitogen used for stimulation. The number of IFN-γ-secreting cells was significantly higher in cells stimulated with CaI-PMA than in cells stimulated with ConA. In cells stimulated with CaI-PMA, the number of IL-4-secreting cells was significantly lower in both groups of foals compared to adult horses. In adult horses only, CaI-PMA stimulation resulted in significantly more IL-4-secreting cells than ConA stimulation. Regardless of age, the ratio of IFN-γ/IL-4 spot forming cells (SFC) was significantly higher in cells stimulated with CaI-PMA than in cells stimulated with ConA. These findings indicate that the frequency of IFN-γ and IL-4-secreting cells is lower in foals than in adult horses and that the type of mitogen used has a profound effect on the relative production of both cytokines.

[1] Reprinted with permission from Ryan, C., Giguère, S., Hagen, J., Hartnett, C. & Kalyuzhny, A. E. (2010).Effect of age and mitogen on the frequency of interleukin-4 and interferon gamma secreting cells in foals and adult horses as assessed by an equine-specific ELISPOT assay. Vet Immunol Immunopathol 133, 66-71.

Introduction

Neonates are particularly susceptible to infectious agents which rarely affect or only cause mild disease in adults. The susceptibility of newborns to infectious diseases may be partly explained by their lack of pre-existing immunological memory as well as

by the relatively small number of immune cells in peripheral lymphoid tissues in early life (Fadel & Sarzotti, 2000). Additionally, many studies in both humans and mice have demonstrated that lymphocytes from neonates are qualitatively distinct from adult cells. Neonatal responses in both humans and mice are often deficient in their ability to mount protective Th1 responses characterized by interferon (IFN)-γ production. While T-cell responses in neonatal mice are typically biased towards the Th2-cell lineage distinguished by producing primarily interleukin (IL)-4, this clear skewing is not always readily apparent in newborn humans (Adkins *et al.*, 2004). In human neonates, a Th2 bias has been demonstrated in some studies (Prescott *et al.*, 1998;Ribeiro-do-Couto *et al.*, 2001) while other studies have shown that both Th1 and Th2 responses are decreased in magnitude compared to the responses of adults (Adkins *et al.*, 2004;Xainli *et al.*, 2002).

Newborn foals are also deficient in their ability to produce IFN-γ in response to stimulation with mitogens (Boyd *et al.*, 2003;Breathnach *et al.*, 2006). These findings, along the peculiar susceptibility of foals to infection with *Rhodococcus equi*, a facultative intracellular pathogen known to only cause disease in immunocompetent mice when a Th2 response is experimentally induced (Kanaly *et al.*, 1995), have led to the hypothesis that T cell responses from newborn foals may be biased toward a Th2 cytokine profile. However, in recent studies, IL-4 mRNA expression in response to

stimulation of bronchial lymph node mononuclear cells with various *R. equi* antigens was significantly lower in foals than in adult horses (Jacks *et al.*, 2007a;Jacks *et al.*, 2007b). Collectively, the studies summarized above do not support the theory of a Th2 bias and suggest that young foals may be deficient in their intrinsic ability to produce both IL-4 and IFN-γ.

Studies performed in humans indicate that the type of mitogen used for *in vitro* stimulation of lymphocytes may have a profound effect on the relative production of IL-4 and IFN-γ (Brown *et al.*, 2003;Gonzalez *et al.*, 1994;Stolzenburg *et al.*, 1988). A variety of mitogens have been used to stimulate cytokine production by equine lymphocytes *in vitro*. To the authors' knowledge, there are no studies investigating the relative effect of the type of mitogen used on IFN-γ and IL-4 secretion in foals. Until fairly recently, most studies evaluating cytokine induction in horses have relied on bioassays or methods assessing mRNA expression. The development of immunoassays has been delayed by the lack of species-specific antibodies and cytokine standards. Recently, measurement of equine IFN-γ and IL-4 by flow cytometric analysis, enzyme-linked immunosorbent assay (ELISA) and enzyme linked immune spot (ELISPOT) assay has been reported using mouse anti-bovine antibodies that cross react with equine IFN-γ and IL-4 (Breathnach *et al.*, 2006;Hamza *et al.*, 2007;Paillot *et al.*, 2005;Pedersen *et al.*, 2002). The ELISPOT assay offers several advantages over other immunoassays. It is approximately 10-200 times more sensitive than ELISA, allowing detection of cytokines that are released at low concentrations or by a small frequency of cells (Tanguay & Killion, 1994).

Thus, the objectives of this study were to compare the frequency of IFN-γ and IL-4 secreting cells of newborn foals to that of older foals and adult horses, and to determine the effect of the type of mitogen used for *in vitro* stimulation on the relative frequency of cells secreting these cytokines. To achieve these objectives, we developed a ready-to-use ELISPOT assay using polyclonal antibodies against equine IFN-γ and IL4.

Materials and Methods

Animals

Twelve healthy Thoroughbred or Thoroughbred-Quarter Horse crossed foals, and ten Thoroughbred or Quarter Horse adult horses (seven mares and three geldings) were used in this study. Mares and foals were housed together on pasture with *ad libitum* access to grass hay and water, and supplemented with sweet feed twice daily. Animals were considered healthy on the basis of physical examinations and daily observation. Adequate passive transfer of immunoglobulin was confirmed in foals 12 to 24 h after birth using an immunoassay for measurement of total IgG (DVM Stat, VDx Inc, Belgium, WI). Additionally, all animals had normal CBC and plasma chemistry profile values at time of sampling. Blood samples from adult horses were collected at one time point. Blood from foals was collected at between day 1 and day 5 of age (n=6) or between 3 and 4 months of age (n=6). All procedures were approved by the University of Florida Institutional Animal Care and Use Committee.

Blood Collection and PBMC Isolation

Heparinized blood (15 ml) was collected by jugular venipuncture and stored at room temperature until processing. Peripheral blood mononuclear cells (PBMC) were separated by density gradient centrifugation using endotoxin-free Ficoll-Paque (Amersham Biosciences, Pittsburgh, PA). PBMCs were washed twice with PBS and

live cells were counted using trypan blue exclusion. The cells were resuspended at low (2×10^5 cells/ml) and high (1×10^6 cells/ml) concentrations in RPMI 1640 (Gibco BRL, Grand-Island, NY) supplemented with 10% fetal bovine serum, 25 mM HEPES, 55 mM 2-mercaptoethanol, and gentamicin (0.5 µg per ml).

ELISPOT Assay

Ready-to-use ELISPOT kits for the detection of equine IL-4 and IFN-γ were developed (R&D Systems, Minneapolis, MN) for this project. Both assays were optimized according to R&D Systems guidelines and performed as recommended by the manufacturer. Each kit included a dry polyvinylidene difluoride-backed 96-well plate pre-coated with the respective capture antibody (goat anti-horse specific for equine IFN-γ or IL-4), a concentrated solution of detection antibodies, a concentrated solution of streptavidin-conjugated alkaline phosphatase, BCIP/NBT substrate, as well as wash and dilution buffers. Antibodies were raised against recombinant equine IL-4 and IFN-γ proteins and then affinity purified using recombinant equine IFN-γ and IL-4 immunogens, respectively. Assays were performed according to the protocols included with each ELISPOT kit. Briefly, plates were saturated with 200 µl of RPMI 1640 and incubated for 20 min at room temperature. Culture media was aspirated and 100 µl of low (2×10^5 cells/ml) or high (1×10^6 cells/ml) concentration PBMCs was added to triplicate wells. Cells were stimulated with concanavalin A (ConA; 4 µg/ml or with phorbol 12-myristate 13-acetate (PMA; 0.05 µg/ml) and calcium ionomycin (CaI; 0.5 µg/ml) for 24 h at 37ºC in the presence of 5% CO_2. The ideal concentration of each mitogen was established based a dose response curve in preliminary experiments. Recombinant equine IL-4 or IFN-γ was used in triplicate wells as positive control

whereas unstimulated cells and sterile culture medium were used as negative control or background control, respectively. After incubation, PBMCs were removed from wells by washing the plates 4 times. Diluted biotinylated goat anti-horse IFN-γ or IL-4 antibody was added (100 μl) and the plates were incubated at 4ºC overnight. After 4 washes, 100 μl of diluted (1:120) alkaline phosphatase conjugated to streptavidin was added to each well and the plates were incubated at room temperature for 2 hours. Unbound enzyme was removed with 3 successive washes and 100 μl of BCIP/NBT chromogen solution was added to each. Plates were incubated in the dark at room temperature for 1 h. Wells were then rinsed with distilled water and dried before employing image analysis to quantify spots on the membranes. Aluminum foil was used as previously described to reduce background staining and minimize well-to-well variation (Kalyuzhny & Stark, 2001).

Collection of ELISPOT Images and Quantification of Spot-forming Cells

The cytokine-releasing activity of PBMCs was evaluated by quantifying spot-forming cells (SFC). SFC were determined by counting colored spots distributed over the entire area of the membrane backing each well assuming that one cell will produce one spot. Images from the developed ELISPOT plates were collected and quantified using semi-automated QHub ELISPOT reader (MVS Pacific, St. Paul, MN).

Western Blotting of Equine IFN-γ and IL-4 Capture Antibodies

Specificity of capture antibodies used in IFN-γ and IL-4 ELISPOT assays was tested on lysates of PBMCs from adult horses. PBMCs were stimulated with CaI-PMA as described above, lysed in RIPA buffer (Thermo Scientific, Waltham, MA) and 25 ng of protein from lysed cells per lane was run on 5-20% gradient Tris–HCl SDS–

polyacrylamide mini gels (Bio-Rad Laboratories, Hercules, CA). The proteins were then transferred to a nitrocellulose membrane in a semi-dry blot chamber (Bio-Rad Laboratories). After transfer, the membranes were blocked with 5% non-fat milk in PBS with 0.1% Tween 20 (TTBS) for 1 h at 4ºC. After 3 washes with TTBS, membranes were incubated with either 0.7 μg/ml of anti-IFN-γ or 0.3 μg/ml anti-IL-4 antibodies overnight on a plate shaker at 4ºC. Membranes were washed 3 times in TTBS and then incubated at room temperature for 1 h with HRP-conjugated donkey anti-goat secondary antibodies diluted 1:1000 in TTBS containing 2% non-fat milk. Membranes were washed in TTBS and then incubated with ECL reagent for 2 min and developed using Konica SRX-101A radiograph processor (Konica Minolta Medical Imaging, Wayne, NJ). Western blot absorption controls were done by incubating blots with the solution of primary antibodies mixed with their cognate recombinant protein at 1:10 molar ratio.

Statistical Analysis

Normality and equality of variance of the data were assessed using the Kolmogorov-Smirnov and Levene's tests, respectively. All data were log-transformed to meet the assumptions of the ANOVA. A 2-way ANOVA for repeated measurements was used to determine the effects of age (neonates, older foals, adult horses), type of mitogen (ConA, CaI/PMA), and the interaction between age and type of mitogen on the frequency of cells secreting IFN-γ or IL-4. When appropriate, multiple pairwise comparisons were done using the Student-Newman-Keuls test. For all analyses, a value of $P < 0.05$ was considered significant.

Results

The IFN-γ and IL-4 capture antibodies used in the ELISPOT assays appeared to be specific, producing single bands of expected (~10-14 kDa) sizes (Figure 3-1) based on previous reports (Dohmann *et al.*, 2000;Wu *et al.*, 2002). SFC were not detected in unstimulated cells. Wells containing mitogen-stimulated PBMCs from adult horses at a concentration of 1×10^6 cells/ml had too many merged spots for accurate quantification. As a result, only data from wells containing 2×10^5 cells/ml were analyzed statistically. There was a visible difference in the number of SFC between foals and adult horses for both IL-4 and IFN-γ (Figure 3-2).

There was a significant effect of age ($P < 0.001$) and type of mitogen ($P < 0.001$) on the number of IFN-γ SFC. The number of IFN-γ SFC was significantly lower in both groups of foals than in the adult horse group regardless of the mitogen used for stimulation (Figure 3-3). The number of IFN-γ SFC was significantly higher in cells stimulated with CaI-PMA than in cells stimulated with ConA, regardless of age (Figure 3-3). A significant effect of age ($P = 0.004$) and a significant interaction between age and type of mitogen ($P = 0.026$) was seen on the number of IL-4 SFC. In cells stimulated with CaI-PMA, the number of IL-4 SFC was significantly lower in both groups of foals compared to adult horse group (Figure 3-3). In cells stimulated with ConA, the number of IL-4 SFC was significantly lower in 3 to 4 month-old foals compared to newborn foals or adult horses (Figure 3-3). In adult horses only, CaI-PMA stimulation resulted in significantly more IL-4 SFC than ConA. There was a significant ($P < 0.001$) effect of the type of mitogen on the ratio of IFN-γ/IL-4 SFC. Regardless of age, the ratio of IFN-γ/IL-4 SFC was significantly higher in cells stimulated with CaI-PMA than in cells

stimulated with ConA (Figure 3-3). There was no effect of age (P = 0.299) on the IFN-γ/IL-4 ratio regardless of the type of mitogen used.

Discussion

Previous studies have documented the decreased ability of foals' PBMCs to produce IFN-γ in response to stimulation with mitogens (Breathnach *et al.*, 2006;Boyd *et al.*, 2003). However, assessment of the Th1/Th2 polarization of the foals' immune responses also necessitates measurement of Th2 cytokines such as IL-4. The present study is the first to document a significantly lower frequency of IL-4 secreting cells in foals compared to adults following stimulation with CaI-PMA and to document a significant effect of the type of mitogen used on the Th1/Th2 cytokine balance both in foals and in adult horses.

In one study, mRNA expression of IFN-γ by foals' PBMCs following stimulation with ConA increased by an estimated 2.5-fold during the first month of life (Boyd *et al.*, 2003). In another study, peripheral blood and bronchoalveolar lavage (BAL) mononuclear cells from newborn foals were deficient in their ability to produce IFN-γ following stimulation with CaI-PMA (Breathnach *et al.*, 2006). Similarly, the present study documented a significantly lower frequency of IFN-γ secreting cells in foals less than one week of age compared to that of adult horses regardless of the mitogen used for stimulation. However, as opposed to the results of Breathnach *et al*. (2006), the frequency of IFN-γ secreting cells in 3 to 4 month-old foals in the present study was not significantly different from that of newborn foals. This may be a reflection of the environment of the foals and exposure to different antigenic stimuli. Alternatively,

differences in methodologies and the small sample sizes in both studies may be the origin of this inconsistency.

Collectively, the two studies referenced above and the results of the present study clearly indicate that young foals, like neonatal mice and humans, are deficient in their baseline ability to produce IFN-γ. However, multiple studies have shown that, although their default response is of the Th2 phenotype, murine neonates can mount Th1 responses providing the right antigen, dose of antigen, costimulatory signal, or type of adjuvant (Adkins, 2005;Barrios *et al.*, 1996;Forsthuber *et al.*, 1996;Martinez *et al.*, 1997;Sarzotti *et al.*, 1996). Human neonates also have the ability to mount strong Th1 responses providing the right circumstances. For example, vaccination of infants with *Mycobacterium bovis* BCG, a microorganism closely related to *R. equi*, induces IFN-γ production of a similar magnitude to that produced by adults (Marchant *et al.*, 1999;Ota *et al.*, 2002;Vekemans *et al.*, 2001). In a recent study, young foals experimentally infected with *R. equi* were shown to mount strong Th1-based immune responses as evidenced by their significantly higher IFN-γ mRNA expression in bronchial lymph nodes cells following stimulation with *R. equi* antigens and significantly higher IFN-γ/IL-4 ratio compared to that of adult horses (Jacks *et al.*, 2007a;Jacks *et al.*, 2007b). These findings suggest that, like human and murine neonates, foals have the ability to mount adult-like Th1-based responses providing an appropriate stimulus.

Much less is known regarding the ability of foals to produce IL-4. In one study, mRNA expression of IL-4 by foals' PBMCs following stimulation with ConA did not change significantly during the first month of life (Boyd *et al.*, 2003). In another study, IL-4 mRNA expression was significantly lower in healthy or *R. equi*-infected foals

compared to normal or *R. equi*-infected adult horses (Jacks *et al.*, 2007a). Similar to results obtained with IFN-γ, the frequency of IL-4 secreting cells in response to stimulation with CaI-PMA in the present study was significantly lower in both groups of foals than in adults. The difference in the number of IL-4-secreting cells between newborn foals and adult horses following stimulation with ConA was not significant. This was because significantly lower numbers of IL-4-producing cells in adult horses responded to stimulation with ConA compared to CaI-PMA, not because a greater number of ConA- stimulated foal PBMCs produced IL-4.

T-cell mitogens such as PMA, ConA, phytohemagglutinin, and pokeweed mitogen are commonly used in immunological assays to induce cell proliferation and cytokine production. Studies performed in humans indicate that the type of mitogen used for *in vitro* stimulation of lymphocytes may have a profound effect on the relative production of IL-4 and IFN-γ (Brown *et al.*, 2003;Gonzalez *et al.*, 1994;Stolzenburg *et al.*, 1988). In one study, stimulation of human PBMCs with CaI-PMA, phytohemagglutinin, and ConA resulted in IFN-γ/IL-4 ratios of 58, 14 and 6, respectively (Gonzalez *et al.*, 1994). The present study indicates that the type of mitogen used has a profound effect on the relative frequency of IFN-γ and IL-4 secretion in horses also. CaI-PMA induced primarily a “Th1-like” profile (positive IFN/IL-4 ratio) whereas ConA induced primarily a “Th2-like” pattern (negative IFN-γ/IL-4 ratio). As a result, general conclusions regarding Th1 or Th2 bias in horses should not be made based on stimulation with a single mitogen. In the present study, there was no significant effect of age on the IFN-γ/IL-4 ratio. This would contradict the theory of a Th2 bias in newborn foals. However, these results

must be interpreted with caution because the small sample size resulted in a low statistical power for the effect of age.

Flow cytometry had been used previously to detect intracytoplasmic equine IFN-γ in response to antigenic or mitogenic stimulation (Breathnach *et al.*, 2006;Hines *et al.*, 2003;Paillot *et al.*, 2005). The disadvantage of flow cytometry is that it only measures intracytoplasmic cytokines produced over a small window of time. Studies assessing the kinetics of intracytoplasmic Th1 and Th2 cytokines in humans have shown that flow cytometry is a useful tool providing that multiple time points are examined. For example, intracytoplasmic IFN-γ peaks at 12 h, decreases around 24 h, and peaks again after 36 h of culture following *in vitro* cell stimulation with CaI-PMA (Rostaing *et al*., 1999). In contrast, IL-4 peaks at 4 and 36 h (Rostaing *et al*., 1999). The ELISPOT assay used in the present study provides the advantage of detecting cytokines produced during the entire incubation period, hence eliminating the need for sampling at multiple time points (Kalyuzhny, 2005). ELISPOT assays can detect cytokine release by as little as 10-100 cells per well compared to the minimum of 10,000 needed in an ELISA (Tanguay & Killion, 1994). The ELISPOT assays for the detection of equine IFN-γ and IL-4 described in the present study can now be applied to investigate both pathogen-specific immune responses and immune responses following vaccination.

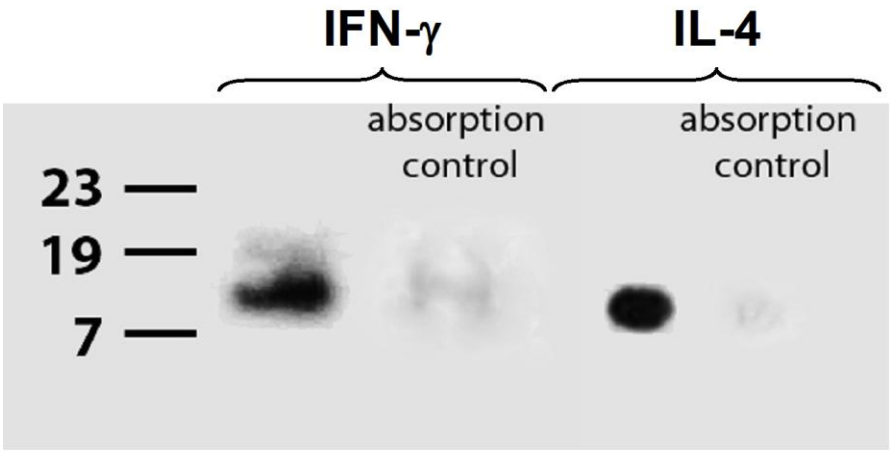

Figure 3-1. Western blot of cellular extract from equine PBMC stimulated for 24 h with CaI-PMA.

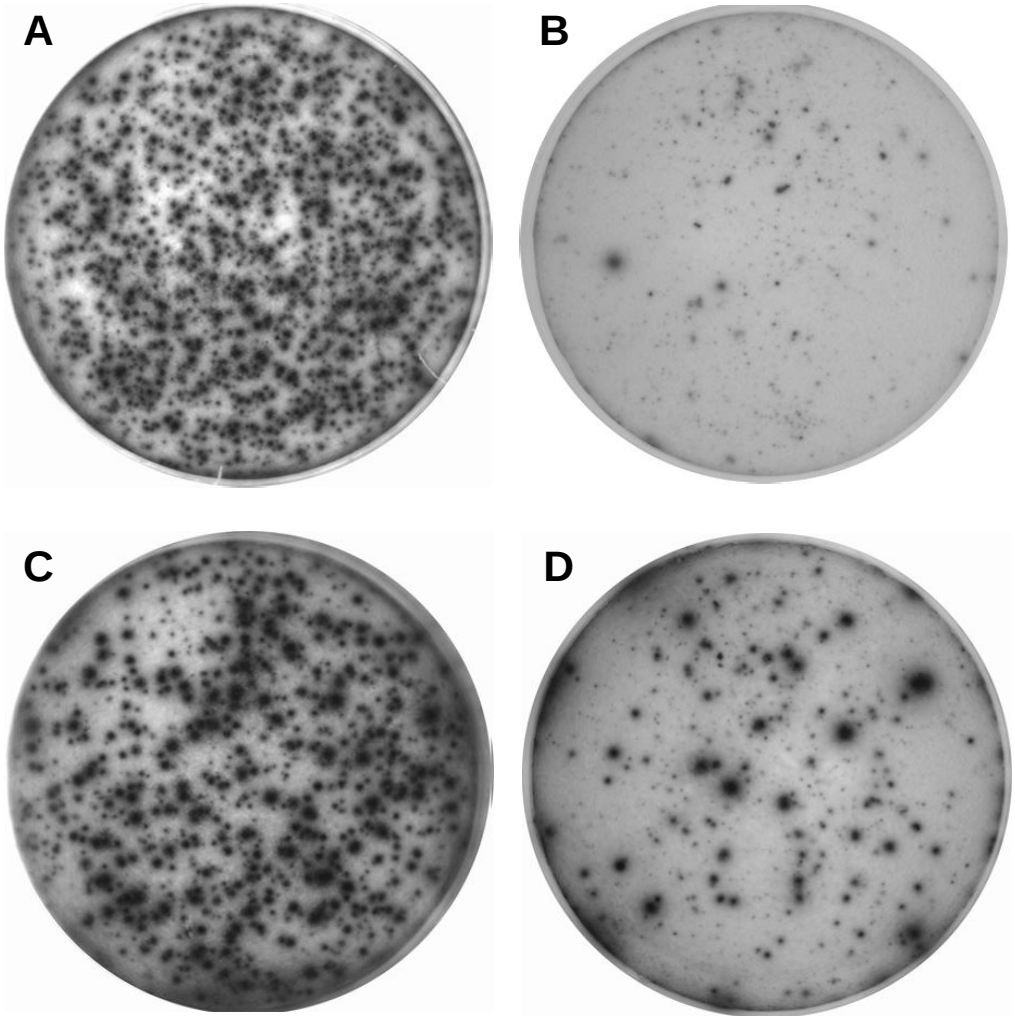

Figure 3-2. ELISPOT assay for the quantification of IL-4 and IFN-γ-producing cells in equine PBMCs after stimulation for 24 h with calcium ionomycin-phorbol-myristate acetate (CaI-PMA). (A) IL-4 SFC in an adult horse. (B) IL-4 SFC in a 2-day-old foal. (C) IFN-γ SFC in an adult horse. (D) IFN-γ SFC in a 5-day-old foal.

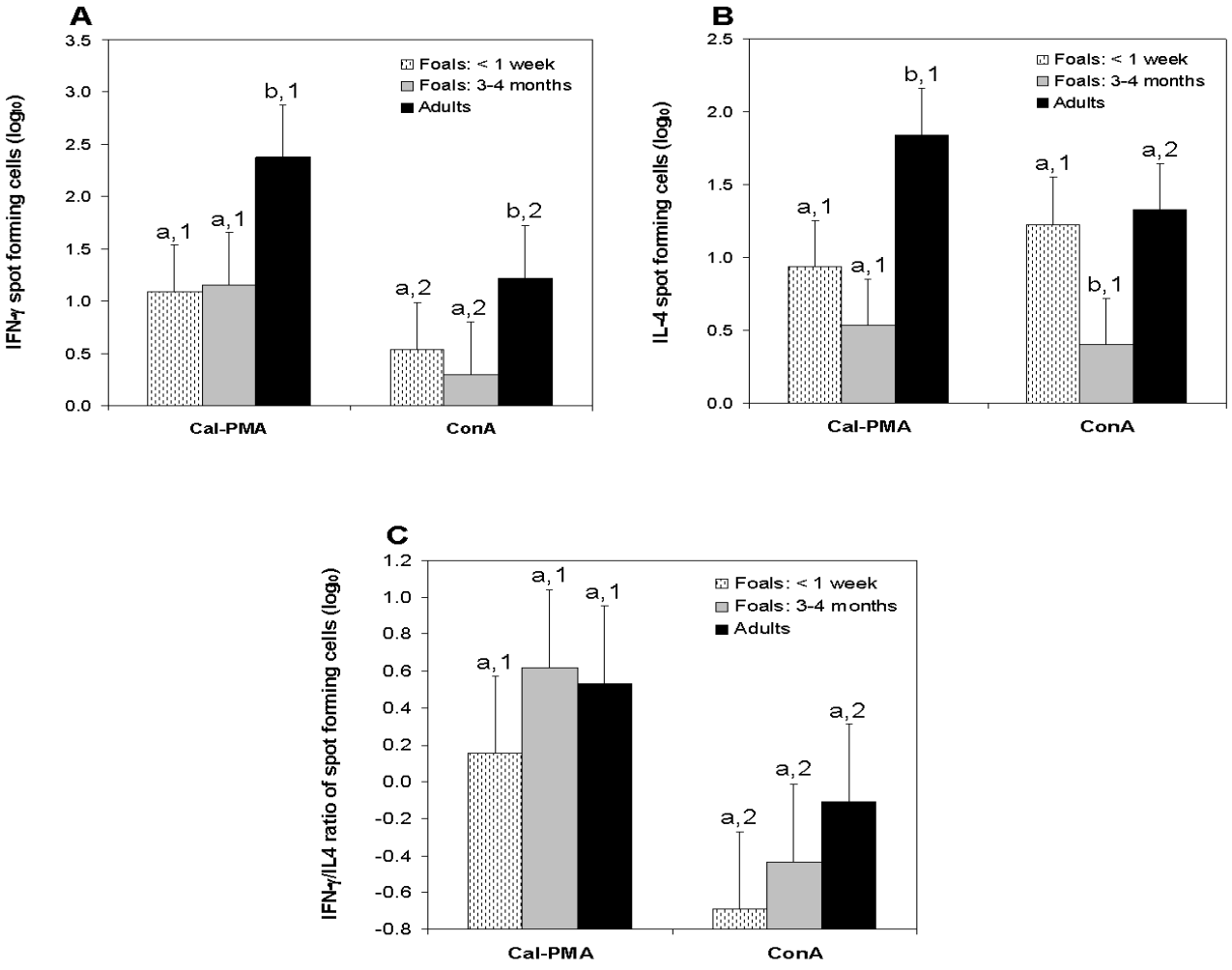

Figure 3-3. Least square mean IFN-γ (A), IL-4 (B), and IFN-γ/IL-4 ratio (C) spot forming cells (Log10 ± SD) in mononuclear blood cells of 6 foals < 1 week of age, 6 foals between 3 and 4 months of age, and 10 adult horses following in vitro stimulation with concanavallin A (ConA) or calcium ionomycin-phorbol-myristate acetate (CaI-PMA). [1,2]Different letters indicate a statistically significant difference between the 2 mitogens within a given age group ($P < 0.05$). [a,b]Indicates a statistically significant difference between foals and adult horses within a given mitogen ($P < 0.05$).

CHAPTER 4
EFFECTS OF TWO COMMERCIALLY AVAILABLE IMMUNOSTIMULANTS ON LEUKOCYTE FUNCTION OF FOALS FOLLOWING EX VIVO EXPOSURE TO *RHODOCOCCUS EQUI*

Abstract

The objective of this study was to determine the effect of immunostimulants on neutrophil, macrophage, and lymphocyte function following *ex vivo* exposure to *R. equi*. Eighteen foals were randomly assigned to one of 3 treatment groups. Treatment consisted of inactivated *Propionibacterium acnes* (PA), inactivated parapoxvirus ovis virus (PPVO), or saline (control) administered on days 0 (7 days of age), 2, and 8. Bronchoalveolar lavage (BAL) fluid and blood were collected on days 0 (baseline), 12, 24 and 36. Intracellular replication of *R. equi* in macrophages, cytokine induction by *R. equi*-infected macrophages, phagocytic and oxidative burst activity of neutrophils, lymphoproliferative responses, and cytokine induction of proliferating lymphocytes were measured. Neutrophils from foals treated with PPVO had significantly greater ability to phagocytize *R. equi* and undergo oxidative burst on day 12 and day 24 compared to baseline values. On day 24, foals treated with PPVO had significantly greater phagocytosis and oxidative burst than foals treated with PA. Treatment with PA resulted in significantly less intracellular proliferation of *R. equi* within monocyte-derived macrophages on day 12 compared to control foals. The ability of *R. equi* to replicate in BAL macrophages decreased significantly with time with lower replication in BAL macrophages of older foals compared to younger foals, regardless of treatment. On day 12, TNF-α induction in monocyte-derived macrophages and IL-12 p40 induction in BAL macrophages infected with *R. equi* was significantly higher in foals treated with

PPVO than in controls. Lymphoproliferative responses and IFN-γ induction were not significantly different between groups.

Introduction

Rhodococcus equi, a facultative intracellular pathogen that replicates in macrophages, is one of the most important causes of pneumonia in foals and has a major financial impact on the equine industry. Control of *R. equi* infections on farms where the disease is endemic currently relies on early detection of disease using thoracic ultrasonography and initiation of treatment with antimicrobial agents prior to development of clinical signs. Although this approach has decreased mortality (Slovis *et al.*, 2005) mass antimicrobial treatment is not without risking reported sequelae of macrolide-associated hyperthermia and diarrhea in treated animals, and may lead to development of antimicrobial resistance. Previous immunoprophylactic strategies for the prevention of *R. equi* pneumonia by vaccination of foals, vaccination of the dam, or administration of hyper-immune plasma have not consistently decreased the incidence of disease (Giguère *et al.*, 2002b;Hurley & Begg, 1995;Martens *et al.*, 1991;Prescott *et al.*, 1997b).

Epidemiological evidence suggests that foals become infected with *R. equi* early in life (Horowitz *et al.*, 2001) while adult horses are typically resistant to *R. equi* infections. Neutrophils are critical for host resistance against infection with virulent *R. equi* in mice (Martens *et al.*, 2005). In addition, studies in adult horses and mice have clearly shown that a T lymphocyte helper type 1 (Th1) response characterized by IFN-γ induction is essential for macrophage activation and protection against infection with *R. equi* whereas IL-4 is detrimental (Darrah *et al.*, 2000;Kanaly *et al.*, 1996;Lopez *et al.*,

2002). Neonatal foals show a marked decrease in their ability to produce IFN-γ in response to mitogens in the first few weeks of life (Boyd *et al.*, 2003;Breathnach *et al.*, 2006;Ryan *et al.*, 2010). Recent data demonstrate that experimental infection of neonatal foals with a low inoculum of virulent *R. equi* triggers induction of IFN-γ mRNA transcription in a manner that is similar to that of adult horses (Jacks *et al.*, 2007a;Jacks *et al.*, 2007b). These findings indicate that foals are capable of inducing IFN-γ providing the proper stimulus. A product capable of increasing IFN-γ induction and activating neutrophils and macrophages in neonatal foals prior to natural infection may be effective in preventing, or at least curtailing, infection with *R. equi* during the narrow window of susceptibility to this pathogen.

Immunostimulants are products that induce non-antigen specific enhancement of innate or adaptive defense mechanisms. Most commercially available veterinary immunostimulants are relatively crude preparations derived from bacteria, plants, or viruses. Data in humans, laboratory animals, and adult horses suggest that commercially available immunostimulants such as inactivated *Propionibacterium acnes* (PA) or parapoxvirus ovis (PPVO) are potent inducers of IFN-γ and contribute to neutrophil and macrophage activation (Davis *et al.*, 2003b;Flaminio *et al.*, 1998b;Friebe *et al.*, 2004b;Weber *et al.*, 2003). Randomized clinical trials in adult horses and weanling foals indicate that these immunostimulants significantly decrease the incidence, duration and severity of undifferentiated respiratory disease (Cormack *et al.*, 1991;Nestved, 1996;Vail *et al.*, 1990;Ziebell *et al.*, 1997). However, the exact effects of these products on foals in relation to host defense against *R. equi* have not been studied.

As a basis for this study, it was hypothesized that commercially available immunostimulants enhance equine phagocytic cell function against *ex vivo* exposure to *R. equi*. The objectives of this study were to determine the effect of immunostimulants on intracellular survival and replication of *R. equi* in foal macrophages, cytokine induction by *R. equi*-infected macrophages, phagocytic activity and oxidative burst of blood neutrophils, and the lymphoproliferative responses and cytokine induction in foals.

Materials and Methods

Animals

Eighteen healthy Thoroughbred or Thoroughbred-cross foals were used. The foals were considered healthy on the basis of physical examinations, adequate transfer of passive immunity, and complete blood cell counts on day one of life. Foals were randomly assigned to one of 3 treatment groups and treatments began at one week of age (day 0). Six foals received 1 ml of inactivated PA (EqStim, Neogen Corporation, Lexington, KY) intravenously on days 0, 2, and 8 of the study. Six foals received 2 ml of PPVO (Zylexis, Pfizer Animal Health, New York, NY) intramuscularly on days 0, 2, and 8. Six foals served as controls and received 2 ml of physiological saline (0.9% NaCl) intramuscularly on day 0, 2 and 8 of the study. Foals were managed under normal pasture conditions and overall health monitored daily for the duration of the project. Starting at day 0 (prior to treatment), and following the initial injection of immunostimulant or placebo, rectal temperatures were recorded twice daily for 14 days. Blood and bronchoalveolar lavage (BAL) fluid samples were collected from each foal on day 0 (prior to treatment) and again on days 12 (4 days after the last treatment), 24, and 36 of the study. The study was approved by the Institutional Animal Care and Use Committee of the University of Florida.

Blood and BAL Sampling

A 16 gauge teflon catheter (Abbocath, Abbott Labs, Abbott Park, IL) was placed in a jugular vein and 250 mL of blood was collected into a glass collection bottle that contained sodium heparin as the anticoagulant. Foals were sedated intravenously with xylazine (0.5 mg/kg) and butorphanol (0.04 mg/kg) prior to BAL fluid collection. A 1.8 m bronchoalveolar lavage catheter (Jorvet, Loveland, CO) was passed via nasal approach until wedged into a bronchus. The lavage solution consisted of 4 aliquots of 50 mL physiologic saline (0.9% NaCl) solution infused and aspirated immediately.

Cell Separation

Mononuclear cells were harvested from both blood and BAL samples by density gradient centrifugation (Ficoll-Paque, Amersham Biosciences, Pittsburgh, PA), washed 3 times with phosphate buffered saline (PBS), and counted using a hemacytometer. Aliquots of peripheral blood mononuclear cells (PBMCs) were used to generate monocyte-derived macrophages (see below). The resulting monocyte-derived and BAL macrophages were used for infection with virulent *R. equi*. Aliquots of 3×10^7 PBMCs or BAL mononuclear cells were cryogenically preserved in 90% fetal bovine serum and 10% DMSO in liquid nitrogen until used for lymphocyte proliferation assays (see below).

For the neutrophil phagocytosis and oxidative burst assay, freshly drawn whole blood was aliquoted into 50 mL conical tubes and allowed to settle for 30-40 min until a visual separation of plasma and the erythrocytes occurred without formation of an obvious buffy coat. This leukocyte-rich plasma was removed without disruption of the erythrocyte layer, placed in a clean centrifuge tube, and centrifuged at 150 × g for 10 min at room temperature to pellet the cells. After centrifugation, the plasma was decanted from the pellet, the cells were washed twice by resuspending in PBS and

counted to determine the number of *R. equi* to add in the phagocytosis assay (see below).

Monocyte-Derived Macrophage Isolation

Monocyte- derived macrophages were obtained using the procedure previously described by Raabe *et al*. (1998). Gelatin-coated plates were incubated for 1 hour at 37°C in 6% CO_2 with 15 ml of donor horse serum, and washed 3 times with PBS prior to plating the blood mononuclear cells. Briefly, PBMCs were suspended in Minimum Essential Medium-alpha (MEMα) containing 10% horse serum (HS) at a concentration of 4 $\times 10^6$ cells/ml and incubated for 18 hours at 37ºC in 6% CO_2. After incubation, non-adherent and loosely adherent cells were removed by a series of washes and the remaining cells were harvested by eluting with a 1:1 mixture of 10 mM EDTA and MEM*α* + 10% HS medium for 5–10 min at 37°C. Cells were pelleted by centrifugation at 200 × *g* for 10 min at 4°C. Cells were then resuspended in media for infection with virulent *R. equi*.

Infection of BAL and Monocyte-derived Macrophages

Monocyte-derived and BAL macrophages were incubated in two- chamber glass slides (Nalgene Nunc International, Rochester, NY) at a concentration of 10^5 cells/ml in one ml of culture media (MEM*α* with 10% HS and 2 mM glutamine). Cells were incubated at 37ºC in a humidified atmosphere containing 6% CO_2 for four hours to allow macrophages to adhere to the glass slide. Media was removed and macrophages were then infected with virulent *R. equi* (ATCC #33701, Rockville, MD).

Bacterial cultures were resuspended in MEMα supplemented with 10% HS as a source of complement. The bacterial suspension was added to the monolayers at a ratio of five bacteria to one macrophage. The slides were incubated for 40 minutes at

37ºC to allow phagocytosis. Noninfected macrophage monolayers cultured under the same conditions were used as controls. The slides were washed 3 times and incubated in MEMα supplemented with 10% HS, 2mM glutamine, and 8 µg of gentamicin sulfate per ml to kill remaining extracellular bacteria and to prevent extracellular growth with continuous re-infection of macrophages (Hondalus & Mosser, 1994). At time 0 (immediately post-infection) and 24 h post-infection, monolayers were fixed with methanol and stained with Wright-Giemsa stain to enumerate *R. equi* (Giguère & Prescott, 1998). The number of bacteria associated with 200 macrophages was determined using light microscopy. Because of the difficulty in reliably counting large numbers of bacteria in a macrophage, any cell containing 10 or more bacteria was scored as having 10 bacteria (Hondalus & Mosser, 1994). Each macrophage infection assay was performed in triplicate. In parallel monolayers containing 1×10^6 cells/well, the supernatants were removed and the cells were lysed in denaturing solution (RNeasy kit, QIAGEN Inc., Valencia, CA) 4 h post-infection for mRNA extraction and quantification of IL-1β, IL-6, IL-8, IL-10, IL-12 p35, IL-12 p40, and TNF-α mRNA expression by real time PCR as described below. For each cytokine in a given animal the results were reported as the ratio of mRNA expression in infected macrophages to that of uninfected control monolayers.

Flow Cytometric Analysis of Neutrophil Phagocytosis and Oxidative Burst in Response to *R. equi*

Flow cytometric analysis of neutrophil phagocytosis and oxidative burst activity was performed as previously reported except that *R. equi* (ATCC 33701) was used as the test microorganism instead of *Staphylococcus aureus* (Flaminio *et al.*, 2002). Bacteria were enumerated by colony forming unit (CFU) counting and subsequently

suspended in PBS at a concentration of 7×10^9 CFU/ml. A suspension of killed *R. equi* was prepared by exposure to heat (90°C for 45 min) prior to the assay. Heat killed *R. equi* was then added at a 1:10 ratio to a propidium iodide (PI) working solution (200 mg/ml in 0.1M carbonate buffer). The bacteria and PI dye were incubated at room temperature overnight, in the dark, on a rocking plate to ensure mixing. The stained bacteria were then centrifuged at 14,000 × *g* for 20 min, the supernatant was removed, and the pellet was resuspended in PBS to maintain a concentration of 7×10^9 CFU/ml. The solution was stored in the dark until the assay was performed. On the day of blood collection, the heat-killed, PI-labeled *R. equi* was opsonized by incubating 1 part of bacterial suspension with 1 part of commercial donor horse serum (Invitrogen, Eugene, OR) for 30 min at 37°C in the dark on a rocking plate. The same source of adult horse serum was used on all samples to eliminate the possibility of variations in serum opsonic activity.

After processing, 1 ml of the neutrophil suspension containing 1×10^7 cells/ml was added to a 5 ml Falcon tube (Becton Dickinson, Franklin Lakes, NJ). Twenty-eight µl of the dihydrorhodamine (DHR) working solution was added to each tube, gently mixed and then incubated with rotation at 37°C for 10 min in the dark. Heat- killed PI-labeled *R. equi* were added to each sample at a ratio of 10 bacteria per cell. Phorbol myristate acetate (PMA [1 mg/ml], Sigma-Aldrich Biochemika, St. Louis, MO) was used as a positive control to stimulate neutrophil oxidative burst. Non-opsonized *R. equi* was used as a negative control. The samples were then incubated at 37°C for 40 min in the dark, with constant rotation. The tubes were then immediately placed on ice to stop

phagocytosis and oxidative burst. Ten µl of 0.4% trypan blue was added to each tube to quench extracellular fluorescence.

The sample was analyzed for phagocytosis and oxidative burst activity using a flow cytometer (FacScan, Becton Dickenson, San Jose, CA). Forward scatter and side scatter were used to identify the granulocyte population and to gate out other cells and debris as previously described (Richardson *et al.*, 1998). Twenty-thousand events were counted per sample. Mean fluorescence intensity of PI and DHR were used to quantify the phagocytic and oxidative burst responses, respectively as previously described (Flaminio *et al.*, 2002).

R. equi Antigen Production

The antigen used for stimulation of cells in proliferation assays was prepared as previously described (Lopez *et al.*, 2002). Briefly, *R. equi* ATCC 33701 was grown in brain heart infusion (BHI) for 48 h at 37ºC with agitation. The bacteria were harvested by centrifugation at 3,840 × *g* for 10 min and washed with sterile PBS. Two ml of the bacterial pellet were resuspended in 10 ml of PBS and the bacteria were disrupted by three cycles of freezing at -20°C and thawing in a water bath at 37°C. The sample was centrifuged at 12,000 × *g* for 15 min at 4°C to separate the pellet of intact bacteria and debris. The resulting supernatant was further centrifuged at 25,000 × *g* for 20 min at 4°C to obtain the soluble antigens. The supernatant was tested for protein concentration using a BCA protein assay kit (Thermo Scientific Pierce, Rockford, IL) according to the manufacturer's instructions.

Proliferation and Cytokine mRNA Expression of PBMC

Immediately after thawing, PBMCs were washed twice and placed in MEM*α* supplemented with 10% HS, 2 mM glutamine, and penicillin-streptomycin (100 U and

100 µg per ml, respectively). More than 80% of the cells were viable after thawing as assessed by trypan blue (Mediatech , Herndon, VA) exclusion. Proliferative responses were assessed using a non-radioactive colorimetric assay. This assay has been shown to correlate closely with conventional radioactive [^{3}H] thymidine incorporation in many species, including the horse (Ahmed *et al.*, 1994;Witonsky *et al.*, 2003), and has been used previously in *R. equi* experiments (Jacks *et al.*, 2007a;Jacks *et al.*, 2007b). Aliquots (100 µl) of cells (1 x 10^6 cells/ml) were placed in triplicate wells of 96-well black plates with flat, clear-bottom wells (Corning Inc., Corning, NY). Cells were separately incubated either without antigen (blank), 2 µg/ml of Concanavalin A (ConA, positive control), or 10 µg/ml of soluble *R. equi* antigen. Optimal concentrations of antigens and mitogen were determined in previous studies (Jacks *et al.*, 2007a;Jacks *et al.*, 2007b). The cells were stimulated at 37ºC for 72 h in 6% CO_2. Eighteen hours before the end of the assay, 20 µl of alamar blue (Accumed International Inc, Westlake, OH) was added to each well and fluorescence was determined with a fluorometer (Synergy HT, BioTek Instruments Inc., Winooski, VT) using an excitation wavelength of 530 nm and emission was measured at 590 nm. Change in fluorescence was calculated as the mean of the stimulated cells minus the mean of the cells without antigen or mitogen (blank).

Blood mononuclear cells used for quantification of mRNA expression were prepared as described previously with the exception that the cells were stimulated with the soluble *R. equi* antigen or with PMA (0.05 µg/ml) and calcium ionomycin (CaI; 0.5 µg/ml) for 24 h. Expression of mRNA for IL-2, IL-4, IL-10, and IFN-γ was quantified by real time RT-PCR as described below.

RNA Isolation, cDNA Synthesis, and Quantification of Cytokine mRNA Expression by Real-time PCR

Isolation of total RNA was performed with the RNeasy kit (QIAGEN Inc., Valencia, CA) according to the manufacturer's instructions. The RNA concentration was measured by optical density at 260 nm (OD_{260}). All RNA samples were treated with amplification-grade DNase I (Gibco BRL, Rockville, MD) to remove any trace of genomic DNA contamination. Briefly, 1 U of DNase I and 1 µl of 10x DNase I reaction buffer were mixed with 1 µg of total RNA for a total volume of 10 µl. The mixture was incubated for 10 min at room temperature and then inactivated by the addition of 1 µl of 25 mM of EDTA and incubating at 65°C for 10 min.

A commercial kit was used to synthesize cDNA (Advantage RT-for-PCR kit, Clontech, Palo Alto, CA) according to the protocol of the manufacturer. Briefly, 1 µg of DNase-treated total RNA was mixed with 1 µl of oligo$(dT)_{18}$ primer (20 µM) and heated at 70°C for 2 min. After cooling to room temperature, the following reagents were added: 4 µl of 5x reaction buffer, 1 µl of deoxynucleoside triphosphates (10 mM each), 0.5 µl of RNase inhibitor, (40 U/µl) and 1 µl of Moloney murine leukemia virus reverse transcriptase (200 U/µl). The mixture was incubated at 42°C for 1 h, heated at 94°C for 5 min, diluted to a final volume of 100 µl, and stored at –70°C until being used for PCR analysis.

Gene-specific primers and internal oligonucleotide probes for equine G3PDH (glyceraldehyde-3-phosphate dehydrogenase), IL-1β, IL-2, IL-4, IL-8, IL-10, IL-12p35, IL-12p40, and IFN-γ have been previously reported (Ainsworth *et al.*, 2003;Garton *et al.*, 2002b). The internal probes were labeled at the 5' end with the reporter dye 6-carboxyfluorescein and at the 3' end with the quencher dye 6-carboxytetramethyl-

rhodamine. Amplification of 2 µl of cDNA was performed in a 25-µl PCR mixture containing 900 nM concentrations of each primer, 250 nM TaqMan probe, and 12 µl of TaqMan Universal PCR Mastermix (Applied Biosystems). Amplification and detection were performed with the ABI Prism 7900 Sequence Detection System (Applied Biosystems) with initial incubation steps at 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Serial dilutions of cDNA from equine blood mononuclear cells stimulated for 24 h with ConA were used to generate a standard curve for relative quantification of each gene of interest. Each sample was assayed in triplicate, and the mean value was used for comparison. Samples without cDNA were included in the amplification reactions to determine background fluorescence and to check for contamination. To account for variations in the amount and quality of the starting material, all results were normalized to G3PDH expression.

IL-4 and IFN-γ ELISA

Supernatant of PBMC stimulated with PMA-CaI for 72 h was concentrated using a centrifugal filter device (Amicon® Ultra, Millipore, Billerica, MA) according to the manufacturer's instruction. The concentration of IL-4 and IFN-© proteins in the concentrated supernatant was measured using a commercially available equine cytokine ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. For each assay, a seven point standard curve using 2-fold serial dilutions of recombinant equine IFN-γ or IL-4 (starting at 1000 pg/ml) was used for quantification and as positive controls. The lower detection limits were 30 and 16 pg/ml for IFN-γ and IL-4, respectively.

Statistical Analyses

Normality of the data and equality of variances were analyzed using the Kolmogorov-Smirnov and Levene's tests, respectively. A 2-way ANOVA for repeated measures was used to evaluate the effect of treatment (control, PPVO, PA), time (0, 12, 24, and 36 days), and the interaction between treatment and time on neutrophil phagocytosis and oxidative burst, survival of *R. equi* into monocyte-derived and BAL macrophages, lymphoproliferative responses, and cytokine induction by PBMCs in response to stimulation by PMA-CaI or *R. equi*. Variables that did not meet the assumptions of the ANOVA were log-transformed prior to analysis. Cytokine mRNA expression in monocyte-derived and BAL macrophages infected with *R. equi* as well as IFN-γ and IL-4 in the supernatant of proliferating PBMC were compared between treatment groups at each time point using a 1-way ANOVA or a Kruskal-Wallis ANOVA on ranks. When indicated, multiple pairwise comparisons were done using the Student-Newman-Keuls test. Significance was set at $P < 0.05$.

Results

Clinical Data and Flow Cytometric Analysis of Neutrophil Phagocytosis and Oxidative Burst in Response to *R. equi*

One foal randomly assigned to the PPVO group was removed from the study because of an illness unrelated to the study protocol. No adverse effects of immunostimulant administration were noted. Rectal temperature of foals receiving PPVO or PA was not significantly different from that of controls. Neutrophils from foals treated with PPVO had significantly greater ability to phagocytize opsonized *R. equi* and undergo oxidative burst on day 12 and day 24 compared to baseline values (Figure 4-1). On day 24, foals treated with PPVO had significantly greater phagocytosis and

oxidative burst than foals treated with PA (Figure 4-1). There was no significant effect of time on phagocytosis or oxidative burst in control foals and in foals treated with PA. The effect of PPVO on neutrophil function was no longer detectable on day 36.

Intracellular Survival and Cytokine mRNA Expression in Monocyte-Derived and BAL Macrophages Infected with *R. equi*

Treatment with PA resulted in significantly less intracellular proliferation of *R. equi* within monocyte-derived macrophages on day 12 compared to control foals but not compared to foals treated with PPVO (Figure 4-2A). There was no significant effect of treatment on day 24 or day 36 of the study. There was no effect of treatment on intracellular proliferation of *R. equi* within BAL macrophages at any time point. However, the ability of *R. equi* to replicate in BAL macrophages decreased significantly ($P = 0.005$) with time (Figure 4-2B) with lower replication in BAL macrophages of older foals (day 24 and 36 of the study) compared to younger foals (day 0), regardless of treatment. Expression of IL-1β, IL-6, IL-8, IL-10, and IL-12 p35, IL-12 p40, and TNF-α mRNA by both monocyte-derived and BAL macrophages infected with *R. equi* was not significantly different between groups at time 0 (data not shown). On day 12 only, TNF-α mRNA expression in monocyte-derived macrophages and IL-12 p40 mRNA expression in BAL macrophages infected with *R. equi* was significantly higher in foals treated with PPVO than in controls (Figure 4-3). There was no significant difference in cytokine mRNA expression between groups on days 24 and 36 of the study (data not shown).

Proliferation and Cytokine mRNA Expression of PBMC Stimulated with Mitogens or *R. equi*

There was no significant difference in lymphoproliferative responses to ConA or *R. equi* between groups at any time (data not shown). Treatment with PA significantly increased expression of IL-10 mRNA on day 24 in PBMCs stimulated with *R. equi* antigens compared to that of control foals or foals treated with PPVO (Figure 4-4). Expression of mRNA for IL-2, IL-4, and IFN-γ in PBMCs stimulated with *R. equi* was not significantly different between experimental groups (data not shown). Similarly, expression of mRNA for IL-2, IL-4, IL-10 and IFN-γ following stimulation with ConA was not significantly different between experimental groups regardless of the time point (data not shown).

IL-4 and IFN-γ in the Supernatants of PBMC Stimulated with CaI-PMA

Concentration of IFN-γ secreted by *R. equi*- or CaI-PMA-stimulated PBMCs was below the limit of detection for the assay (30 pg/ml) at all time points. Concentration of IL-4 in the supernatant of PBMCs stimulated with CaI-PMA was significantly higher in foals treated with PPVO compared to controls and foals treated with PA on day 12 only (Figure 4-5).

Discussion

Immunostimulants have been defined as agents that stimulate the response of effector cells such as macrophages, lymphocytes, and neutrophils, which subsequently activate one or more terminal immune responses such as antigen uptake, cytotoxicity, phagocytosis, cytokine release, and antibody response (Rush & Flaminio, 2000). The present study was the first to assess the effect of commercially available

immunostimulants on immune function of foals following *ex vivo* stimulation of effector cell populations with *R. equi*.

Neutrophils are absolutely essential in host resistance against infection with virulent *R. equi* in mice (Martens *et al.*, 2005). In addition to their direct microbicidal effects, equine neutrophils are capable of producing pro- inflammatory cytokines such as IFN-γ, TNF-α, IL-12, IL-6, IL-8 and IL-23p19 when stimulated with *R. equi* (Nerren *et al.*, 2009b). Decreased neutrophil function against *R. equi* has been documented in some neonatal foals and this has been proposed as one of the possible mechanisms for the peculiar susceptibility of foals to this pathogen (Martens *et al.*, 1988). The significant enhancement of phagocytosis and oxidative burst activity of neutrophils in foals pre-treated with PPVO (inactivated parapoxvirus ovis) was not unexpected in the present study. In one study, inactivated parapoxvirus ovis stimulation enhanced the phagocytotic activity of canine neutrophils and monocytes in a dose-dependent manner (Schutze *et al.*, 2010)**.** Similarly, data in humans have demonstrated enhancement of both phagocytosis and oxidative burst following *in vitro* exposure to inactivated parapoxvirus ovis(Forster *et al.*, 1994). In one study, administration of PA (inactivated *Propionibacterium acnes*) to healthy weanlings horses resulted in significantly increased non-opsonized phagocytosis and oxidative burst in peripheral blood leukocytes following *ex vivo* exposure to *Staphylococcus aureus* (Flaminio *et al.*, 1998b). In the present study, treatment of neonatal foals with PA had no significant effect on neutrophil phagocytosis and oxidative burst. These different findings might be related to time of sampling, the age of the animals, or the different bacterial species used to measure phagocytosis (*R. equi* versus *S. aureus*). Given the importance of neutrophils in early

host defense against infection with *R. equi*, a product capable of directly or indirectly enhancing neutrophil function might be useful in preventing disease associated with *R. equi* during the window of susceptibility to this pathogen. Additional studies will be necessary to determine if the positive effects of PPVO observed *ex vivo* will result in enhanced host defense against *R. equi* under natural challenge in a field situation.

Several significant effects of immunostimulant on macrophage function were demonstrated in the present study. Treatment with PA resulted in significantly less intracellular proliferation of *R. equi* within monocyte-derived macrophages on day 12 compared to control foals but not compared to foals treated with PPVO. However, this effect could not be documented in alveolar macrophages. Interestingly, replication of virulent *R. equi* in BAL macrophages was significantly higher on day 0 of the study compared to day 24 or 36, regardless of treatment. These results suggest that *R. equi* replicates to a greater extent in the BAL macrophages of 1-week-old foals compared to that of older foals. To the authors' knowledge, this is the first study documenting a defect in BAL macrophage function of neonatal foals following infection with *R. equi*. In one study, the migrational activity of alveolar macrophages was significantly impaired in foals 2-3 days of age compared to 2-week-old foals and adult horses (Liu *et al.*, 1987). In another study, newborn foal alveolar macrophages had a lesser ability to phagocytize and kill *S. aureus* than peripheral blood neutrophils from the same animals (Fogarty & Leadon, 1987). In the present study, TNF-α mRNA expression in monocyte-derived macrophages and IL-12 p40 mRNA expression in BAL macrophages infected with *R. equi* were significantly higher in foals treated with PPVO than in controls on study day 12 only (4 days following the last treatment). Interleukin 12 is essential for development

of the Th1 responses necessary for clearance of *R. equi* (Kanaly *et al.*, 1996;Trinchieri & Gerosa, 1996). Similarly, TNF-α is also essential for host defense against infection caused by *R. equi* in mice (Kasuga-Aoki *et al*., 1999).

Previous studies have documented the ability of immunostimulants at inducing IFN-γ expression in adult horses. In one study, administration of PA resulted in a significant increase in mRNA expression of the Th1 cytokines IL-2 and IFN-γ in PBMCs 1 week after administration (Davis *et al.*, 2003). Similarly, administration of PPVO to adult horses in another study resulted in a significant increase in IFN-γ mRNA expression (Horohov *et al*., 2008). In the present study, there was no difference between treatment groups in the induction of IFN-γ mRNA following *ex vivo* stimulation with *R. equi* or a mitogen. Consistent with our findings, administration of PA to foals starting at 2-3 days of age failed to increase production of IFN-γ by PMA-stimulated mononuclear cells (Sturgill & Horohov, 2006). These finding might be due to the fact that neonatal foals typically have lower IFN-γ induction than adult horses in response to mitogens (Boyd *et al.*, 2003;Breathnach *et al.*, 2006;Ryan *et al.*, 2010). Alternatively, the lack of IFN-γ induction in the present study may have been influenced by the sampling time selected. In one study, peak IFN-γ induction following administration of PPVO to adult horses occurred 24 h following administration (Horohov *et al*., 2008). The first sampling time in the present study (study day 12) was 4 days after administration of the last dose. This time point was selected based on prior studies demonstrating enhancement of various measures of immune function in adult horses and weanlings following administration of immunostimulants (Davis *et al.*, 2003;Flaminio *et al.*, 1998). A transient increase in cytokine mRNA expression might have been

missed. Additional studies including more time points would be needed to characterize the kinetics of cytokine mRNA expression following administration of immunostimulants.

In the present study, administration of PPVO resulted in significantly increased concentration of IL-4 in the supernatant of PBMCs stimulated with PMA on day 12 of the study. This effect is not completely unexpected based on studies in other species. In humans, inactivated parapoxvirus ovis has been shown to cause up-regulation of pro-inflammatory (Th1- type) cytokines such as IFN-γ and TNF-α, followed by anti-inflammatory (Th2- type) cytokines such as IL-10 and IL-4 (Friebe *et al.*, 2004). Inactivated parapoxvirus ovis has been shown to induce some protection against hepatitis B and herpes simplex virus infections in mice (Weber *et al.*, 2003). In the aforementioned study, there was initial up-regulation of the production of Th1 cytokines such as IL-12, IL-18, and IFN-γ, followed by a down-regulation of the same cytokines and production of IL-4 (Weber *et al.*, 2003). Neither PA nor PPVO increased relative expression of IFN-γ in PBMCs from neonatal foals in this study. IFN-γ concentrations in the supernatant of PBMCs were below the threshold of detection in this study, despite concentrating the supernatant samples. To address this issue of relatively low IFN-γ levels in PBMC supernatants, increased numbers and concentrations of cells should be used for stimulation in future studies.

In conclusion, the two commercially immunostimulants evaluated in the present study significantly enhanced phagocytic cell function upon *ex vivo* exposure to virulent *R. equi*. The clinical relevance of these findings in the prevention of *R. equi* infections on endemic farms remains to be determined in a prospective randomized clinical trial.

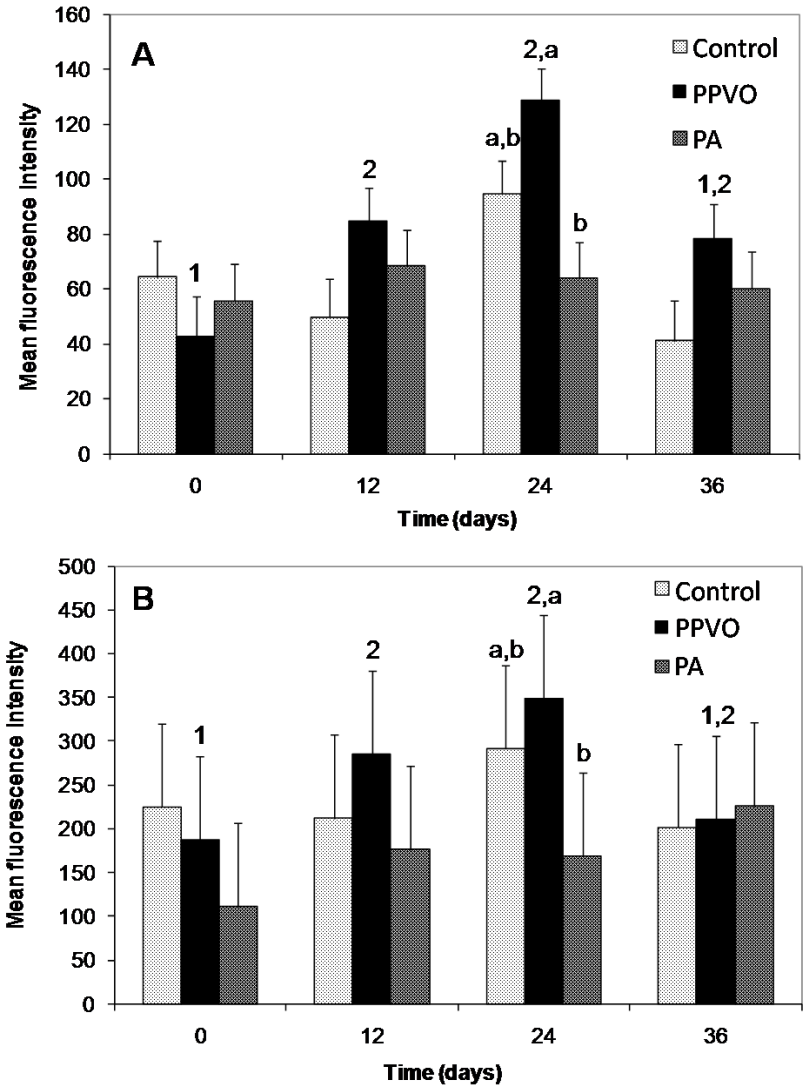

Figure 4-1 . Phagocytosis (A) and oxidative burst activity (B) of blood neutrophils after *ex vivo* exposure to *R. equi*. Neutrophils were collected for baseline (day 0) neutrophil function testing prior to administration of a placebo (control; n=6), PPVO (n=5), or PA (n=6). Neutrophil function was reassessed on day 12, 24, and 36 of the study. The results are displayed as least square mean fluorescence intensity ± SD. [1,2,3] Different numbers within a given treatment indicate a significant difference in neutrophil function between sampling days ($P < 0.05$). [a,b] Different letters between experimental groups within a given day indicate a statistically significant difference in neutrophil function ($P < 0.05$).

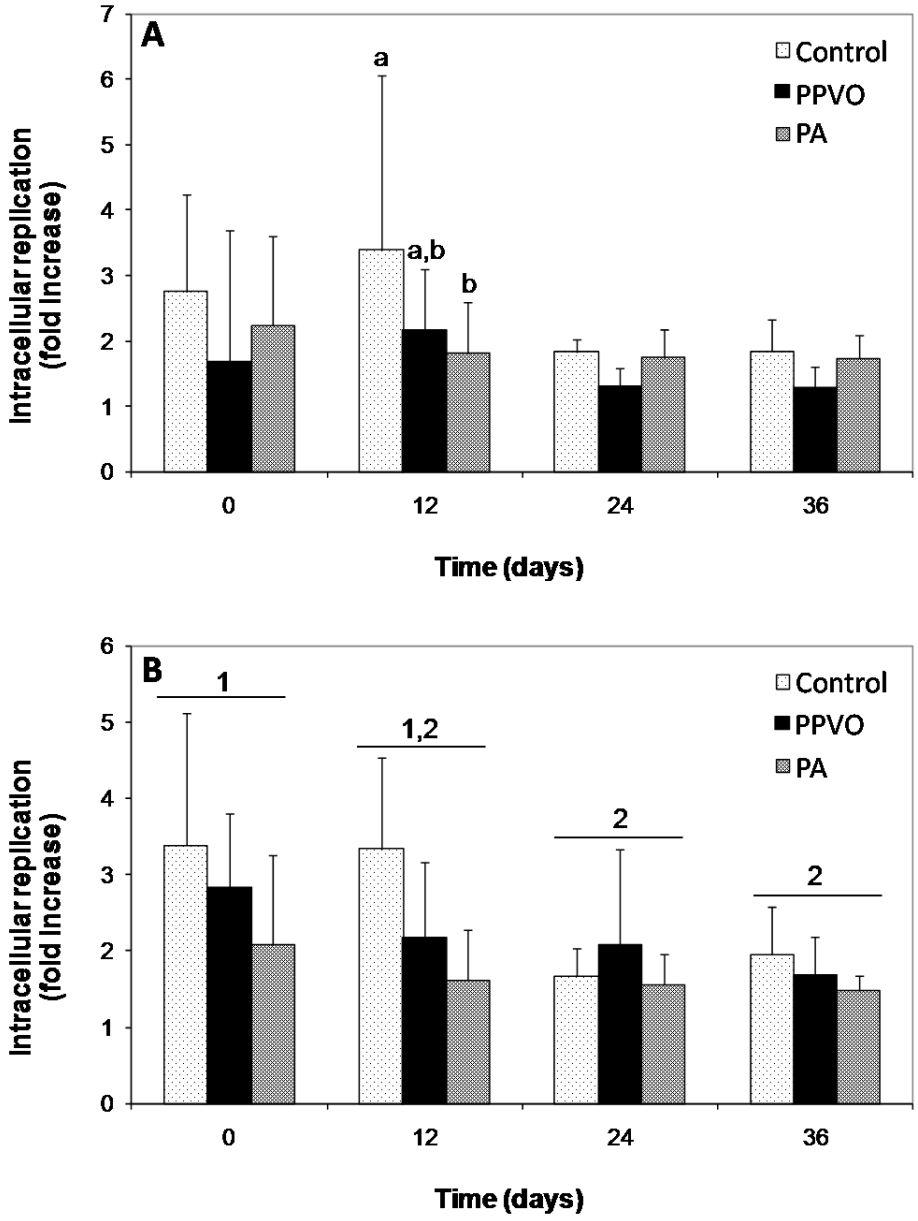

Figure 4-2. Intracellular proliferation of *R. equi* in PBMC-derived macrophages (A) and BAL macrophages (B) of foals prior to (day 0) or after (days 12, 24, 36) administration of a placebo (control; n=6), PPVO (n=5), or PA (n=6). The results are displayed as mean fold change (± SD) in intracellular bacterial number over a 24 h period. [a,b] Different letters between experimental groups within a given day indicate a statistically significant difference ($P < 0.05$). [1,2,3] Different numbers within a given treatment indicate a significant difference in neutrophil function between sampling days ($P < 0.05$).

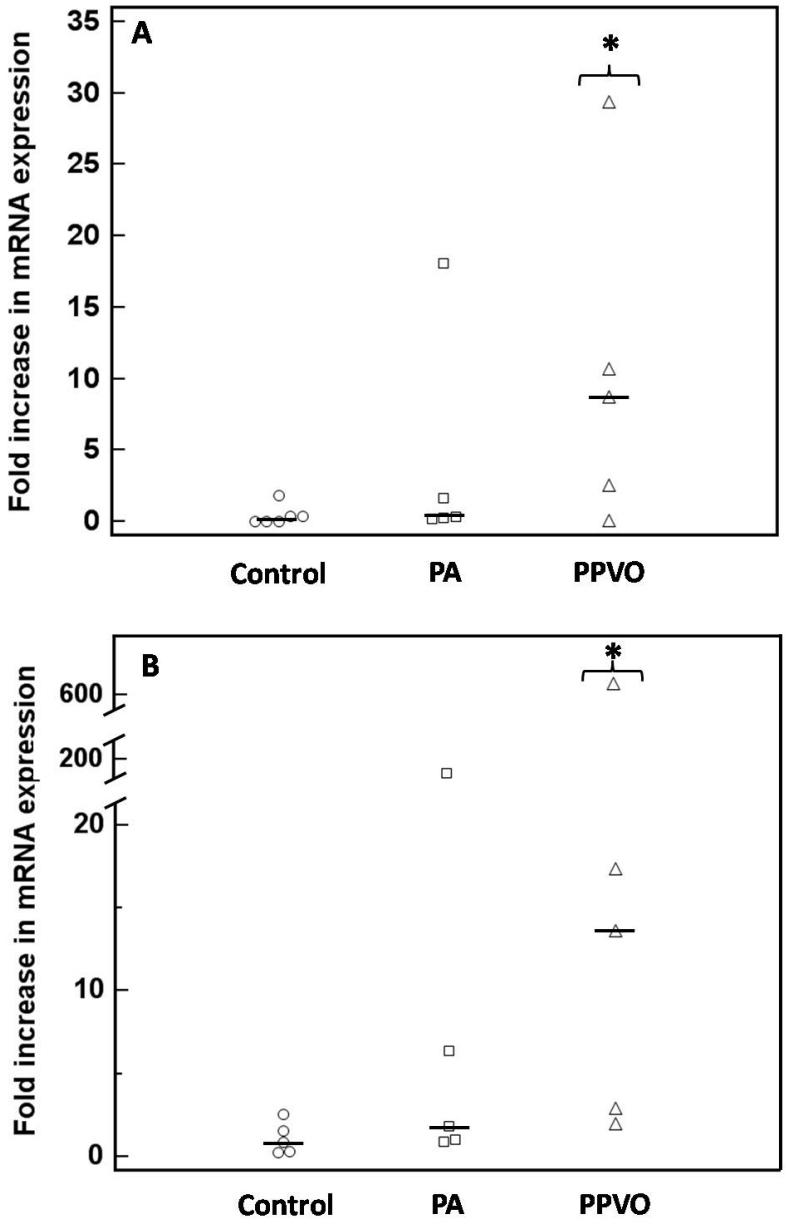

Figure 4-3. Fold increase in relative mRNA expression of IL-12p40 in BAL macrophages (A) and of TNF-α in monocyte-derived macrophages (B) 4 h following infection with virulent *R. equi*. The cells were collected on day 12 of the study. Foals were administered a placebo (control; n=6), PPVO (n=5), or PA (n=6). Each symbol represents an individual foal and the median value from each group is represented as a horizontal line. *Indicates a statistically significant difference in mRNA expression compared to controls ($P < 0.05$).

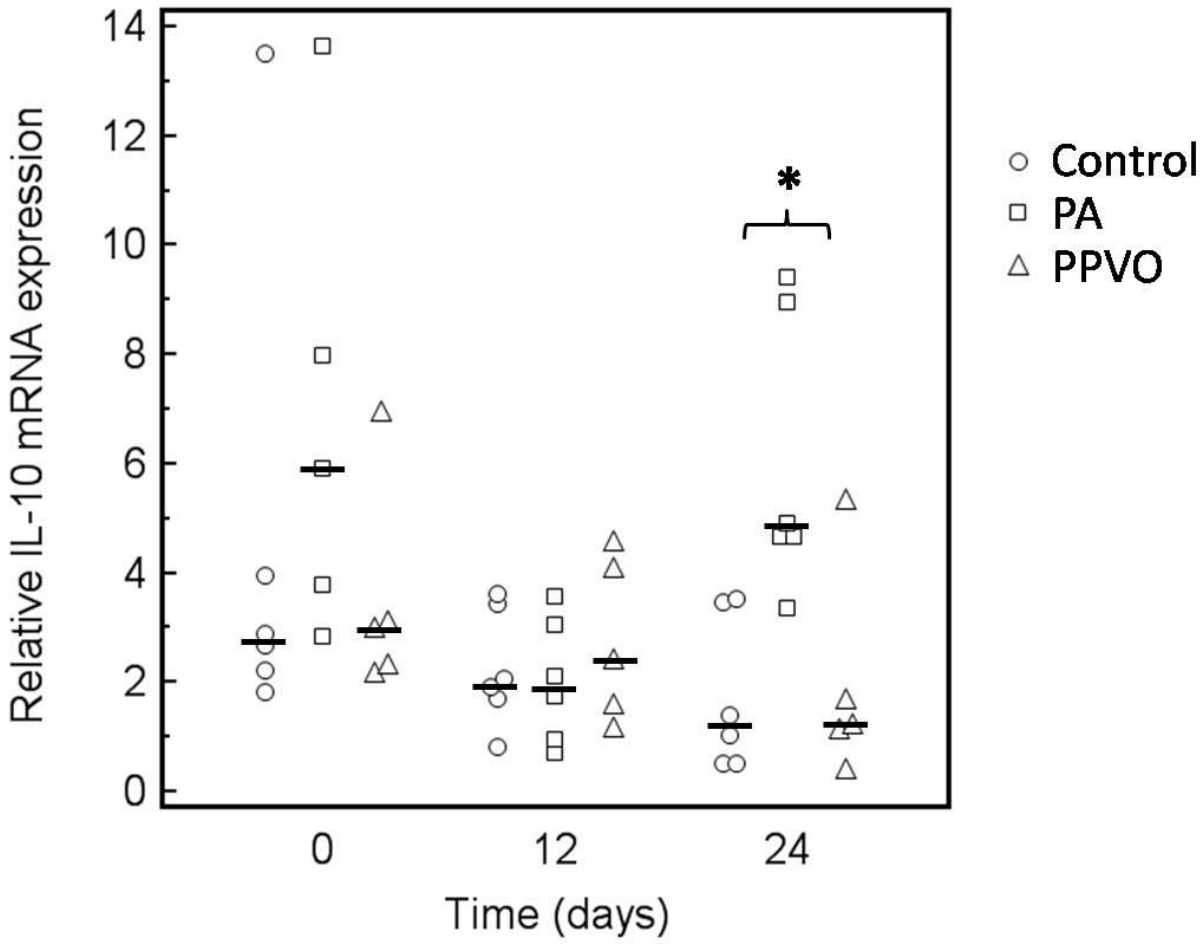

Figure 4-4. Relative IL-10 mRNA expression in PBMC stimulated with *R. equi* antigens. Foals were administered a placebo (control; n=6), PPVO (n=5), or PA (n=6). Each symbol represents an individual foal and the median value from each group is represented as a horizontal line. *Indicates a statistically significant difference in mRNA expression at 24 days compared to control foals and foals treated with PPVO ($P < 0.05$).

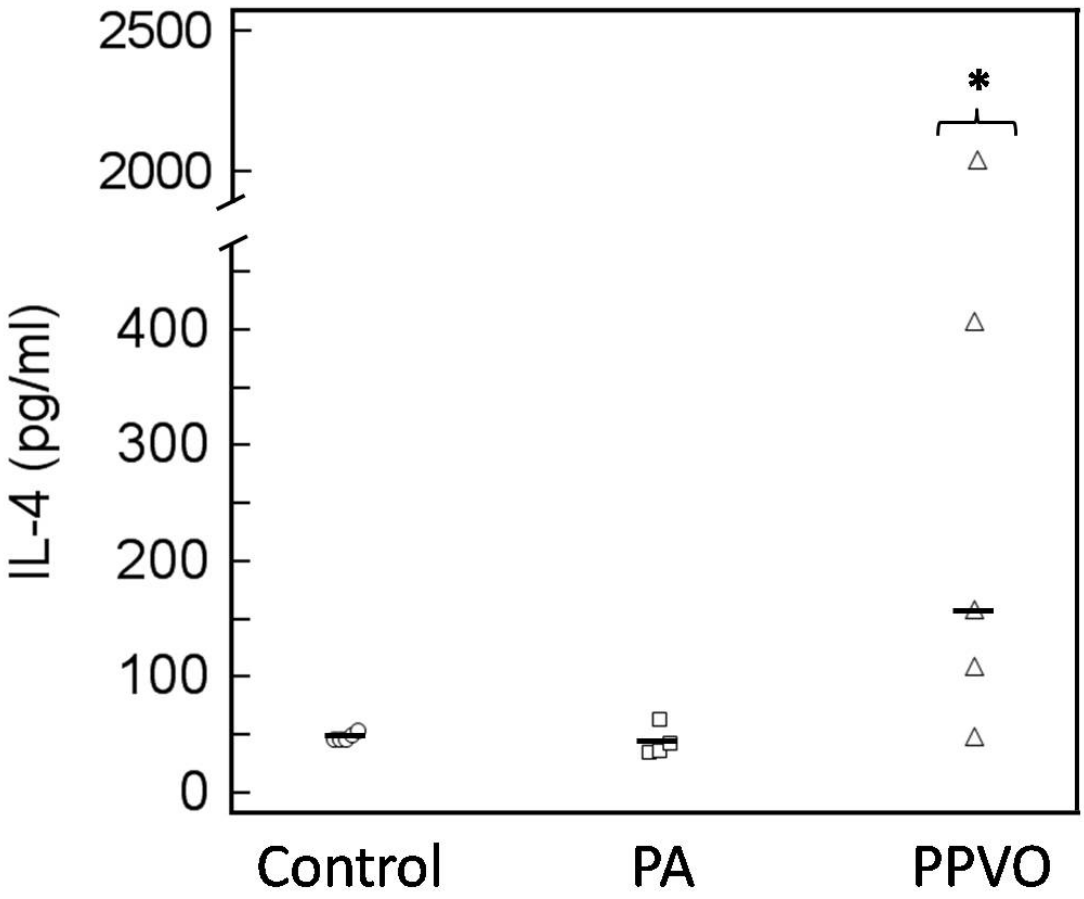

Figure 4-5. IL-4 concentration in the supernatants of PBMC stimulated with CaI-PMA. The cells were collected on day 12 of the study. Foals were administered a placebo (control; n=6), PPVO (n=5), or PA (n=6). Each symbol represents an individual foal and the median value from each group is represented as a horizontal line. *Indicates a statistically significant difference compared to controls ($P < 0.05$).

CHAPTER 5
EQUINE NEONATES HAVE ATTENUATED HUMORAL AND CELL-MEDIATED IMMUNE RESPONSES TO A KILLED ADJUVANTED VACCINE COMPARED TO ADULT HORSES

Abstract

The objectives of this study were to compare relative serum immunoglobulin concentrations, antigen-specific lymphoproliferative responses, and cytokine profile of proliferating lymphocytes between 3-day old foals, 3-month old foals, and adult horses following vaccination with a bovine killed adjuvanted vaccine. Horses were vaccinated intramuscularly twice at 3-week intervals with a vaccine containing antigens from bovine viral respiratory pathogens to avoid interference from maternal antibody. Both groups of foals and adult horses responded to the vaccine with a significant increase in relative vaccine-specific IgGa and IgG(T) concentrations. In contrast, only adult horses and 3-month old foals mounted significant total IgG, IgGb and IgM responses. Relative concentrations of IgM and IgG(T) were significantly different between all groups with the highest concentrations in adult horses followed by 3-month-old foals and finally 3-day old foals. Only the adult horses mounted significant vaccine-specific lymphoproliferative responses. Baseline IFN-γ and IL-4 concentrations were significantly lower in 3-day old foals than in adult horses. Vaccination resulted in a significant decrease in IFN-γ concentrations in adult horses and a significant decrease in IL-4 concentrations in 3-day old foals. After vaccination, the ratio of IFN-γ/IL-4 in both groups of foals was significantly higher than that of adult horses. The results of this study indicate that the humoral and lymphoproliferative immune responses to this killed adjuvanted vaccine are modest in newborn foals. Although immune responses

improve with age, 3-month old foals do not respond with the same magnitude as adult horses.

Introduction

Development of the equine immune system occurs relatively early during fetal life. Lymphocytes are present in the peripheral blood of the equine fetus by day 120 of gestation and they proliferate in response to mitogens by day 140 (Perryman *et al.*, 1980). Specific antibody responses to *in utero* vaccination with coliphage T2 have been detected in equine fetuses as early as day 200 of gestation (Martin & Larson, 1973). In other studies, administration of a Venezuelian equine encephalomyelitis antigen to equine fetuses between 232 and 283 days of gestational age resulted in higher serum neutralization titers than that elicited by the same preparation in adult horses (Mock *et al*., 1978;Morgan *et al*., 1975). Recent work supports these findings, showing that active B cell development and immunoglobulin isotype switching occur during equine gestation and the neonatal period (Tallmadge *et al*., 2009). Proliferation of peripheral blood lymphocytes in response to mitogens is slightly reduced at birth but rapidly increases to adult levels (Flaminio *et al*., 2000;Sanada *et al*., 1992). Foals also have normal lymphokine activated killing (LAK) cell activity of peripheral blood lymphocytes at birth and during early life (Flaminio *et al.*, 2000).

Although these findings suggest that newborn foals should be able to mount adequate immune responses at birth, maternal antibodies acquired through ingestion of colostrum have been shown to exert a considerable suppressive effect on antibody production (Jeffcott, 1974). In addition, the recognized Type 2 bias in immune responses of murine and human neonates, along with the recent finding that young foals are deficient in their ability to produce IFN-γ in response to stimulation with

mitogens, has led to the widespread hypothesis that foals are born with an inherent inability to mount strong cell-mediated immune response (Boyd *et al.*, 2003;Breathnach *et al.*, 2006). Recently we have demonstrated that newborn foals can produce robust IFN-γ responses and high concentrations of IgG subclasses when challenged with intrabronchially with virulent *R. equi* (Jacks *et al.*, 2007a;Jacks & Giguère , 2010). However, there are no studies comparing primary humoral and cell-mediated immune responses of newborn foals to that of older foals and adult horses following vaccination in the absence of vaccine-specific maternal antibody interference. A thorough understanding of immune responses of newborn foals following vaccination would be essential for the future development of rational vaccination strategies against pathogens likely to infect foals early in life.

The objectives of this study were to compare serum IgM and IgG subclass concentrations, antigen-specific lymphoproliferative responses, and cytokine profile of proliferating lymphocytes of newborn foals, older foals, and adult horses following vaccination with a killed adjuvanted vaccine. A killed adjuvanted vaccine was selected because most vaccines commercially available for use in horses currently are of this type. The central hypothesis for the present study was that newborn foals mount inferior immune responses to a killed vaccine compared to adult horses.

Materials and Methods

Animals and Experimental Design

Thirty-two healthy Thoroughbred or Thoroughbred-cross foals were used. The foals were considered healthy on the basis of adequate transfer of passive immunity, and complete blood cell counts on day one of life, physical examinations, and daily monitoring. Healthy adult horses (n=6) were also used. Foals were randomly assigned

to one of 2 age groups; 3-day old (n=11) and 3-month old (n=15). Each animal received a series of 2 intramuscular injections of a killed adjuvanted cattle vaccine (Triangle®4, Fort Dodge Animal Health, Fort Dodge IA) at 3-week intervals. This vaccine included antigens from type II bovine viral diarrhea virus, infectious bovine rhinotracheitis virus, parainfluenza-3 virus, and bovine respiratory syncytial virus. The vaccine was selected based on the lack of detectable serum antibody to these antigens in horses as well as demonstrated safety and immunogenicity in horses (Slack *et al*., 2000). Blood was collected from each animal on day 0 (prior to vaccination), 21 (dose 1; prior to administration of the second dose), and 42 days (dose 2) after initiation of immunization.

Blood Collection and Cell Separation

One hundred ml of blood was collected by jugular venipuncture using heparin as the anticoagulant. Blood (10 ml) was collected without anticoagulant for separation of serum. Peripheral blood mononuclear cells (PBMCs) were harvested from blood samples by density gradient centrifugation (Ficoll-Paque, Amersham Biosciences, Pittsburgh, PA), washed 3 times with phosphate buffered saline (PBS), and counted using a hemacytometer. Aliquots of 3×10^7 PBMCs were cryogenically preserved in 90% fetal bovine serum and 10% DMSO in liquid nitrogen until used for lymphocyte proliferation and cytokine assays (see below). Serum was stored at -80°C until used for measurement of vaccine-specific immunoglobulin concentrations.

Vaccine- specific Serum Immunoglobulin Concentrations

Vaccine-specific relative IgM, total IgG, IgGa, IgGb, and IgG(T) concentrations in serum were determined by ELISA as previously described (Jacks *et al.*, 2007a). Optimal dilutions of reagents were determined by checkerboard titration. Briefly, wells

in 96-well microtiter plates (Immulon II, Thermo Fisher Scientific, Waltham, MA) were coated at 4ºC overnight with whole vaccine (Triangle®4, Fort Dodge Animal Health) diluted 1:250 in carbonate-bicarbonate buffer (pH 9.6; total volume 100 μl/well). Plates were washed 4 times with PBS-0.05% Tween 20 between each of the following incubations. Plates were blocked with PBS-1% BSA for 1 h at room temperature. Serum from each experimental animal was diluted 1:100 and 100 μl were added to each well for 1 h of incubation at room temperature. To determine isotype-specific responses, 100 μl of peroxidase-conjugated goat anti-equine IgGa (1:5000), IgGb (1:5000), IgG(T) (1:1000), and IgM (1:2500) (Serotec, Raleigh, NC) were added to the wells for 1 h incubation at room temperature. After addition of substrate (ABTS, Roche Diagnostics, Indianapolis, IN), plates were incubated for 45 min in the dark at room temperature and the OD was measured at 405 nm. For each immunoglobulin subisotype measured, serum from a high responder was serially diluted to generate a standard curve for relative quantification of immunoglobulin concentration in the experimental animals. The standard curve was run on each plate to correct for interplate variability. Wells incubated without serum were used as blank to subtract out the background absorbance. Each sample was run in triplicate and the mean OD was used.

Vaccine-specific Lymphocyte Proliferations

Immediately after thawing, PBMCs were washed twice and placed in MEMα supplemented with 10% horse serum, 2 mM glutamine, and penicillin-streptomycin (100 U and 100 μg per ml, respectively). More than 80 % of the cells were viable after thawing as assessed by trypan blue (Mediatech , Herndon, VA) exclusion.

Lymphoproliferative responses were assessed using a non-radioactive colorimetric assay. This assay has been shown to correlate closely with conventional radioactive [3H] thymidine incorporation in many species, including the horse (Ahmed *et al*., 1994; Witonsky *et al*., 2003). In preliminary experiments, the adjuvant of the killed vaccine was found to exert mitogenic effects on equine lymphocytes, thereby preventing our ability to detect antigen-specific lymphoproliferative responses. A modified live vaccine (Pyramid®5, Fort Dodge Animal Health) containing the same viral agents as the killed adjuvanted vaccine was used as a source of antigen for the lymphoprolifertive assay. The advantage of the modified-live vaccine was that antigen and adjuvants were provided in separate vials. Vaccine antigen was diluted in MEMα and inactivated by heating at 60°C for 1 h. The optimal concentration of antigen (1:7000) was determined in preliminary experiments. Aliquots (100 µl) of cells (1×10^6 cells/ml) were placed in triplicate wells of 96-well black plates with flat, clear-bottom wells (Corning Inc., Corning, NY). Cells were separately incubated either without antigen (blank), 5 µg/ml of pokeweed mitogen (positive control), or vaccine antigen. The cells were stimulated at 37ºC for 72 h in 6% CO_2. Eighteen hours before the end of the assay, 20 µl of alamar blue (Accumed International Inc, Westlake, OH) was added to each well and fluorescence was determined with a fluorometer (Synergy HT, BioTek Instruments Inc., Winooski, VT) using an excitation wavelength of 530 nm and emission was measured at 590 nm. Change in fluorescence was calculated as the mean of the stimulated cells minus the mean of the cells without antigen or mitogen (blank).

Cytokine mRNA Expression by Real-time PCR

PBMCs were cultured in triplicate wells for 24 h in the presence of the vaccine antigen as described above. Time of stimulation (24 h) was selected based on peak

mRNA expression in preliminary experiments with adult horse PBMCs (Giguère & Prescott, 1999b;Jacks *et al.*, 2007b). Isolation of total RNA was performed with the RNeasy kit (QIAGEN Inc., Valencia, CA) according to the manufacturer's instructions. The RNA concentration was measured by optical density at 260 nm (OD260). All RNA samples were treated with amplification-grade DNase I (Gibco BRL, Rockville, MD) to remove any trace of genomic DNA contamination. Briefly, 1 U of DNase I and 1 µl of 10x DNase I reaction buffer were mixed with 1 µg of total RNA for a total volume of 10 µl. The mixture was incubated for 10 min at room temperature and then inactivated by the addition of 1 µl of 25 mM of EDTA and incubating at 65°C for 10 min.

A commercial kit was used to synthesize cDNA (Advantage RT-for-PCR kit, Clontech, Palo Alto, CA) according to the protocol of the manufacturer. Briefly, 1 µg of DNase-treated total RNA was mixed with 1 µl of oligo(dT)18 primer (20 µM) and heated at 70°C for 2 min. After cooling to room temperature, the following reagents were added: 4 µl of 5x reaction buffer, 1 µl of deoxynucleoside triphosphates (10 mM each), 0.5 µl of RNase inhibitor, (40 U/µl) and 1 µl of Moloney murine leukemia virus reverse transcriptase (200 U/µl). The mixture was incubated at 42°C for 1 h, heated at 94°C for 5 min, diluted to a final volume of 100 µl, and stored at –70°C until being used for PCR analysis.

Gene-specific primers and internal oligonucleotide probes for equine G3PDH (glyceraldehyde-3-phosphate dehydrogenase), IL-2, and IL-10 have been previously reported (Garton *et al*., 2002). The internal probes were labeled at the 5' end with the reporter dye 6-carboxyfluorescein and at the 3' end with the quencher dye 6-carboxytetramethyl-rhodamine. Amplification of 2 µl of cDNA was performed in a 25-µl

PCR mixture containing 900 nM concentrations of each primer, 250 nM TaqMan probe, and 12 µl of TaqMan Universal PCR Mastermix (Applied Biosystems). Amplification and detection were performed with the ABI Prism 7900 Sequence Detection System (Applied Biosystems) with initial incubation steps at 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. cDNA from equine blood mononuclear cells stimulated for 24 h with ConA was used as positive control and run on each plate as a calibrator sample. Each sample was assayed in triplicate, and the mean value was used for comparison. Samples without cDNA were included in the amplification reactions to determine background fluorescence and to check for contamination. DNase-treated RNA samples were subjected to PCR using the G3PDH primers to confirm the absence of genomic DNA contamination. Relative gene expression was calculated using the method described by Pfaffl *et al.* (2001).

IFN-γ and IL-4 Concentrations

Supernatants of PBMCs stimulated as described above for 72 h were collected and stored at -80ºC until use. Supernatants were concentrated using a centrifugal filter device (Amicon Ultra, Millipore, Billerica, MA) according to the manufacturer's instruction. The concentration of IL-4 and IFN-γ proteins in the concentrated supernatant was measured using commercially available equine cytokine ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. For each assay, a seven point standard curve using 2-fold serial dilutions of recombinant equine IFN-γ or IL-4 (starting at 1000 pg/ml) was used for quantification and as positive controls. The lower detection limits were 30 and 16 pg/ml for IFN-γ and IL-4, respectively.

Statistical Analysis

Normality and equality of variance of the data were assessed using the Kolmogorov-Smirnov and Levene's tests, respectively. Variables that were not normally distributed were log- or rank-transformed prior to analysis. A two-way ANOVA for repeated measurements was used to determine the effects of vaccination (baseline, dose 1, and dose 2), experimental group (3-day old, 3-month old, and adult horses), and the interactions between vaccination and group on antibody concentration, lymphoproliferative responses, and cytokine induction. When appropriate, multiple pairwise comparisons were done using the Holm-Sidak test. A value of $P < 0.05$ was considered significant.

Results

Vaccine-specific Immunoglobulin Concentrations

Both groups of foals and adult horses responded to the vaccine with a significant increase in relative IgGa and IgG(T) concentrations (Figure 5-1). In contrast, only adult horses and 3-month old foals mounted significant total IgG, IgGb and IgM responses. Relative concentrations of IgGb were significantly higher in adult horses than in both groups of foals. Relative concentrations of IgM and IgG(T) were significantly different between all groups with the highest relative concentrations in adult horses followed by 3-month old foals and finally 3-day old foals (Figure 5-1). Relative concentrations of IgGa were not significantly different between groups.

Vaccine- specific Lymphoproliferative Responses and Cytokine Induction

Adult horses had significantly greater lymphoproliferative responses to the vaccine antigen after the second dose of vaccine compared to that measured at baseline or after a single dose (Figure 5-2). In both groups of foals, vaccine- induced

lymphoproliferative responses after vaccination were not significantly different from baseline values (Figure 5-2). Lymphoproliferative responses of adult horses after administration of 2 doses of vaccine were significantly higher than that of either group of foals (Figure 5-2). Three-month-old foals had significantly greater lymphoprolifertive responses after 2 doses of vaccine than 3-day old foals. There was no significant effect of vaccination on lymphoproliferative responses to pokeweed mitogen but there was a significant effect of group (P = 0.016). Lymphoproliferative responses to pokeweed mitogen in adult horses (1,470 ± 1058) were significantly higher than that of 3-day old foals (387 ± 533) and 3-month old foals (806 ± 1608). Lymphoproliferative responses to pokeweed mitogen were not significantly different between the 2 groups of foals.

Cytokine Induction in Vaccine-stimulated PBMCs

Baseline IFN-γ concentrations were significantly lower in 3-day old foals than in 3-month old foals and adult horses (Figure 5-3A). Vaccination resulted in a significant decrease in IFN-γ concentrations after the first dose of the vaccine in 3-day old foals and after both doses in 3-month old foals and adult horses. After 2 doses of the vaccine, IFN-γ concentrations were significantly lower in adult horses than in both groups of foals (Figure 5-3A). Baseline IL-4 concentrations were significantly lower in both groups of foals compared to adult horses (Figure 5-3B). Concentrations of IL-4 significantly decreased after the second vaccination compared to baseline in 3-day old foals. Concentrations of IL-4 after the second vaccination were significantly lower in 3-day old foals than in 3-month old foals and adult horses (Figure 5-3B). Baseline IFN-γ/IL-4 ratio was significantly higher in 3-month old foals than in 3-day old foals (Figure 5-3C). There was a significant increase in the IFN-γ/IL-4 ratio after the second dose of

vaccine in 3-day old foals and a significant decrease in the same ratio in adult horses. The IFN-γ/IL-4 ratio of both groups of foals was significantly higher than that of adult horses after the second dose of the vaccine (Figure 5-3C).

Baseline relative IL-10 mRNA expression was not significantly different between groups. There was a significant increase in relative IL-10 mRNA expression after the first dose of the vaccine in 3-day old foals only but expression returned to baseline after the second dose (Figure 5-4B). Relative IL-10 mRNA expression after the first dose of the vaccine was significantly higher in 3-day old foals than in adults (Figure 5-4B). Relative IL-10 mRNA expression did not change significantly with vaccination in 3-month old foals and in adults. There was a significant effect of group ($P = 0.033$) on relative IL-2 mRNA expression but the effect of vaccination ($P = 0.17$) and the interactions between group and vaccination ($P = 0.97$) were not statistically significant. Relative IL-2 mRNA expression in adult horses was significantly higher than that of both groups of foals (Figure 5-4A).

Discussion

Although vaccination of horses is widely practiced and forms an important part of infectious disease control programs, very little is known regarding development of immune responses following vaccination in newborn foals. Response to vaccination is generally assessed in a population of healthy adult horses. However, age has a profound effect on immune responses as evidenced by the fact that old horses have decreased antibody production and lymphoproliferative responses to some vaccines (Horohov *et al.*, 2010). Current equine vaccination guidelines state that vaccination of foals should begin between 3 and 6 months of age, depending on the specific vaccine.

This is because maternal antibody acquired through ingestion of colostrum has been shown to exert a considerable suppressive effect on antibody production. This is substantiated by the fact that the onset of antibody production is advanced in colostrum-deprived foals compared to foals with adequate transfer of passive immunity (Jeffcott, 1974). The rate of decline of maternal antibodies varies depending on the nature of the antigen. For many pathogens, the concentration of maternal antibody in foals falls to non-protective levels by 2-3 months of age. For equine influenza and tetanus, maternal antibodies in foals born from mares vaccinated in the last 2 months of pregnancy can persist until approximately 6 months of age and prevent adequate immune responses in foals vaccinated prior to reaching that age (Wilson *et al.*, 2001). There are no studies comparing primary humoral and cell-mediated immune responses of newborn foals to that of older foals and adult horses following vaccination in the absence of antigen-specific maternal antibody interference. A thorough understanding of the default immune response of newborn foals following vaccination is essential for the future development of rational vaccination strategies for pathogens expected to cause disease early in life.

The killed adjuvanted vaccine selected for use in the present study was well tolerated and invoked robust humoral and lymphoproliferative responses in adult horses. Relative serum concentrations of IgM, IgG(T), IgG(a), IgG(b) and total IgG all increased following vaccination. Total IgG, IgM, IgG(T), and IgG(b) concentrations in adult horses were highest after the second vaccination. In contrast, IgGa concentrations increased after the first vaccination but a considerable decrease in IgGa concentrations was observed after the second vaccination in most horses. These

results are in accordance with those of Slack *et al*. (2000) who reported a peak in IgGa concentrations 2 weeks following administration of the second dose of the same vaccine to adult horses with a substantial decrease in IgGa concentrations at 3 weeks. Although newborn foals mounted statistically significant IgGa and IgG(T) responses, the magnitude of these responses was modest compared to that of older foals and adult horses. In one study, vaccination of 8- to 15-day old foals with 2 doses of a DNA vaccine expressing the vapA gene of *Rhodococcus equi* failed to elicit a measurable antibody response while the same vaccine elicited robust antigen-specific IgG responses in adults (Lopez, *et al.*, 2003). In contrast, infection of 7-day old foals with live virulent *R. equi* resulted in a significant increase in IgGa, IgGb, IgGc, and IgM concentrations compared to pre-infection values (Jacks & Giguère, 2010;Jacks *et al.*, 2007a). In the same study, post-infection IgGa and IgGb concentrations in infected foals were significantly higher than those achieved following administration of the same inoculum to adult horses. (Jacks *et al*., 2007a) Administration of a higher inoculum of the same virulent *R. equi* resulted in significantly higher IgG(T) and IgM responses(Jacks & Giguère, 2010). Collectively, these findings indicate that newborn foals can mount adequate humoral immune responses providing the right stimulus. However, the nature and dose of antigen and possibly the type of adjuvant have a profound effect on the magnitude and IgG subclass of the response in newborn foals. In this study, relative immunoglobulin concentrations were measured by an ELISA technique utilizing the whole vaccine. Therefore, immunoglobulin responses to the entire vaccine, not just the viral antigens, were measured. Immunoglobulin responses

to other components of the vaccine such as adjuvant or additives (as opposed to vaccine antigen- specific responses) cannot be ruled out.

The present study showed that the antigen-specific lymphoproliferative responses of adult horses are substantially greater than those of foals. Similarly, 7-day old foals infected with virulent *R. equi* had decreased lymphoproliferative responses to *R. equi* antigens when compared to adult horses (Jacks *et al.*, 2007a). Limited antigen-specific lymphoproliferative responses in foals are unlikely to be a result of impaired proliferative ability of neonatal lymphocytes because several studies have indicated that neonatal foals and adult horses have similar lymphoproliferative responses in response to stimulation with various mitogens (Flaminio *et al.*, 2000;Jacks & Giguère , 2010;Sanada *et al.*, 1996). The decreased antigen-specific lymphoproliferative responses in neonatal foals may be the result of impaired or immature function of antigen presenting cells. Recent studies have shown that monocyte-derived dendritic cells from foals are phenotypically different from that of adult horses having decreased MHC class II and CD86 expression (Flaminio *et al.*, 2009;Merant *et al.*, 2009).

Cell-mediated immune responses of murine and human neonates are generally thought to be biased toward a Th2 response (Adkins, 2000). Several studies have documented that newborn foals are deficient in their ability to induce IFN-γ in response to stimulation with mitogens (Boyd *et al.*, 2003;Breathnach *et al.*, 2006). These findings, along with the peculiar susceptibility of foals to infection with *R. equi*, a facultative intracellular pathogen known to only cause disease in immunocompetent mice when a Th2 response is experimentally induced (Kanaly *et al.*, 1995), have led to the hypothesis that T cell responses from newborn foals may be biased toward a Th2

cytokine profile. However, experimental infection of neonatal foals with virulent *R. equi* triggers induction of IFN-γ mRNA transcription in a manner that is similar to that of adult horses, indicating that foals can mount adequate IFN-γ responses providing the proper stimulus (Jacks *et al*., 2007a;Jacks *et al*., 2007b). Thorough assessment of the Th1/Th2 polarization of the foals' immune responses also necessitates measurement of Th2 cytokines such as IL-4. Recent data demonstrate that foals are also deficient in their ability to produce IL-4 in response to stimulations with mitogens, suggesting that a clear cut polarization towards a Th2 response in unlikely in neonatal foals.(Ryan *et al.*, 2010;Wagner *et al.*, 2010). The relative Th1/Th2 polarization of equine neonatal immune responses would be better assessed by measuring antigen-specific responses after vaccination rather than after stimulation with mitogens. To the authors' knowledge, the present study is the first to measure Th1 and Th2 cytokines in response to vaccination of newborn foals with a killed adjuvanted vaccine. Consistent with studies using mitogens, baseline IFN-γ and IL-4 concentrations in the present study were significantly lower in 3-day old foals compared to that measured in adult horses. However, the IFN-γ/IL-4 ratio after vaccination was significantly higher in both groups of foals compared to adult horses. These results indicate that, although basal cytokine secretion on neonatal foals may be considerably dampened, there is not a clear bias towards a Th2 response to the vaccine used in the present study.

In the current study, we found that foal and adult horse PBMCs produced IFN-γ and IL-4 when cultured in the presence of vaccine antigen. Surprisingly, the amount of IFN-γ produced by these antigen- stimulated 3-month old foal and adult PBMCs decreased after vaccination, while production from 3-day old foals was unchanged. It is

possible the use of a killed (rather than live) vaccine led to a diversion away from an IFN-γ dominated response, towards a more prominent Th2 type response. Although this theory is cannot be supported by a simultaneous rise in IL-4 production, the increased mRNA expression of IL-10 in 3-month old foals could fit with this scenario.

In conclusion, the present study demonstrated considerably decreased humoral and cell-mediated immune responses in newborn foals after vaccination with a killed vaccine compared to that of adult horses even in the absence of vaccine-specific maternal antibody interference. Although immune responses to the vaccine improved with age, 3-month old foals did not respond with the same magnitude as adult horses. However, newborn foals do not have a bias toward a Th2 response in response to vaccination. Additional studies are needed to determine the effects of type of antigen, dose of antigen, and form of adjuvant on induction of robust humoral and cell-mediated immune responses in foals.

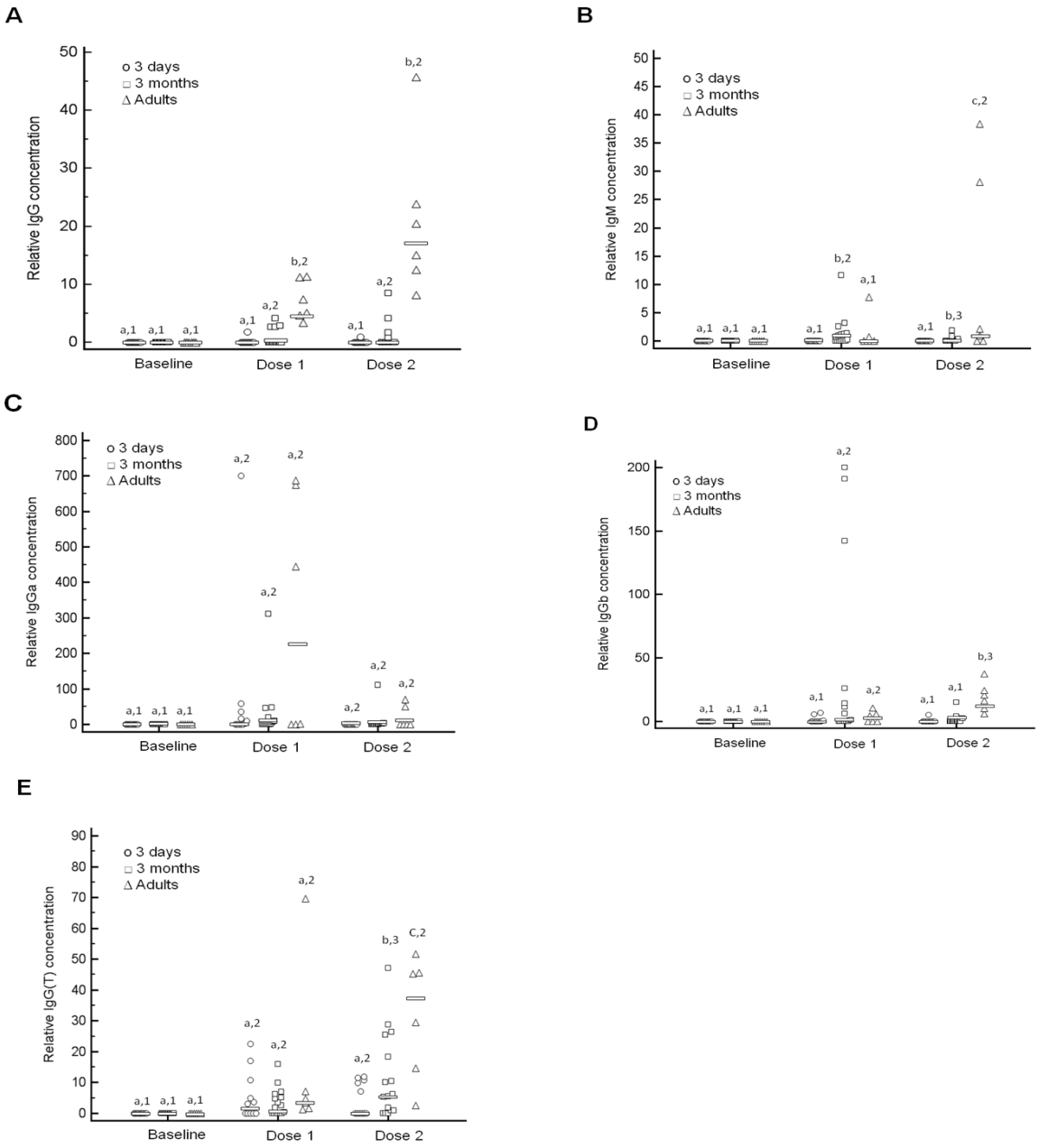

Figure 5-1. Relative vaccine-specific serum total IgG (A), IgM (B), IgGa (C), IgGb (D), and IgG(T) (E) concentrations as determined by capture ELISA. Adult horses, 3-day old foals, and 3-month old foals were vaccinated with a killed adjuvanted vaccine twice with 3 weeks between administrations. Serum was collected prior to vaccination as well as 3 weeks after administration of each dose of the vaccine. Symbols represent individual data points. Horizontal bars indicate median values. [1,2,3]Different numbers within an age group indicate significant differences between sample time points. [a,b,c]Different letters within a time point indicate significant differences between age groups. Significance was set at $P < 0.05$.

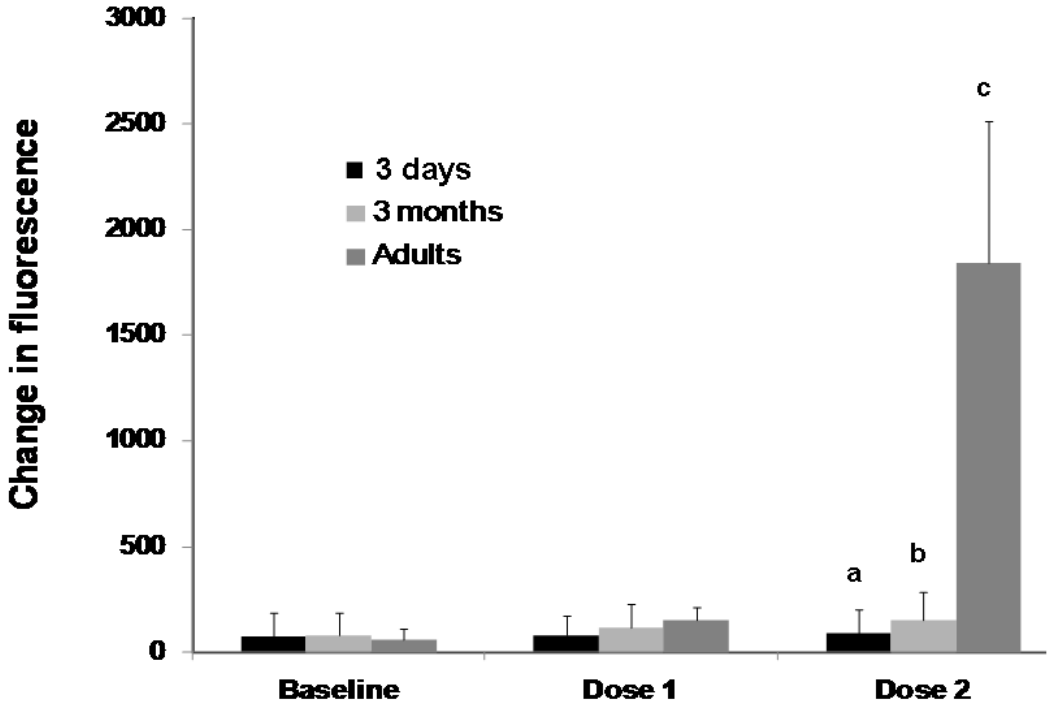

Figure 5-2. Mean (±SD) vaccine-specific lymphoproliferative responses as determined by a colorimetric lymphocyte proliferation assay. Adult horses, 3-day old foals, and 3-month old foals were vaccinated with a killed adjuvanted vaccine twice with 3 weeks between administrations. PBMCs were collected prior to vaccination as well as 3 weeks after administration of each dose of the vaccine. [a,b,c] Different letters within a time point indicate significant differences between age groups ($P < 0.05$).

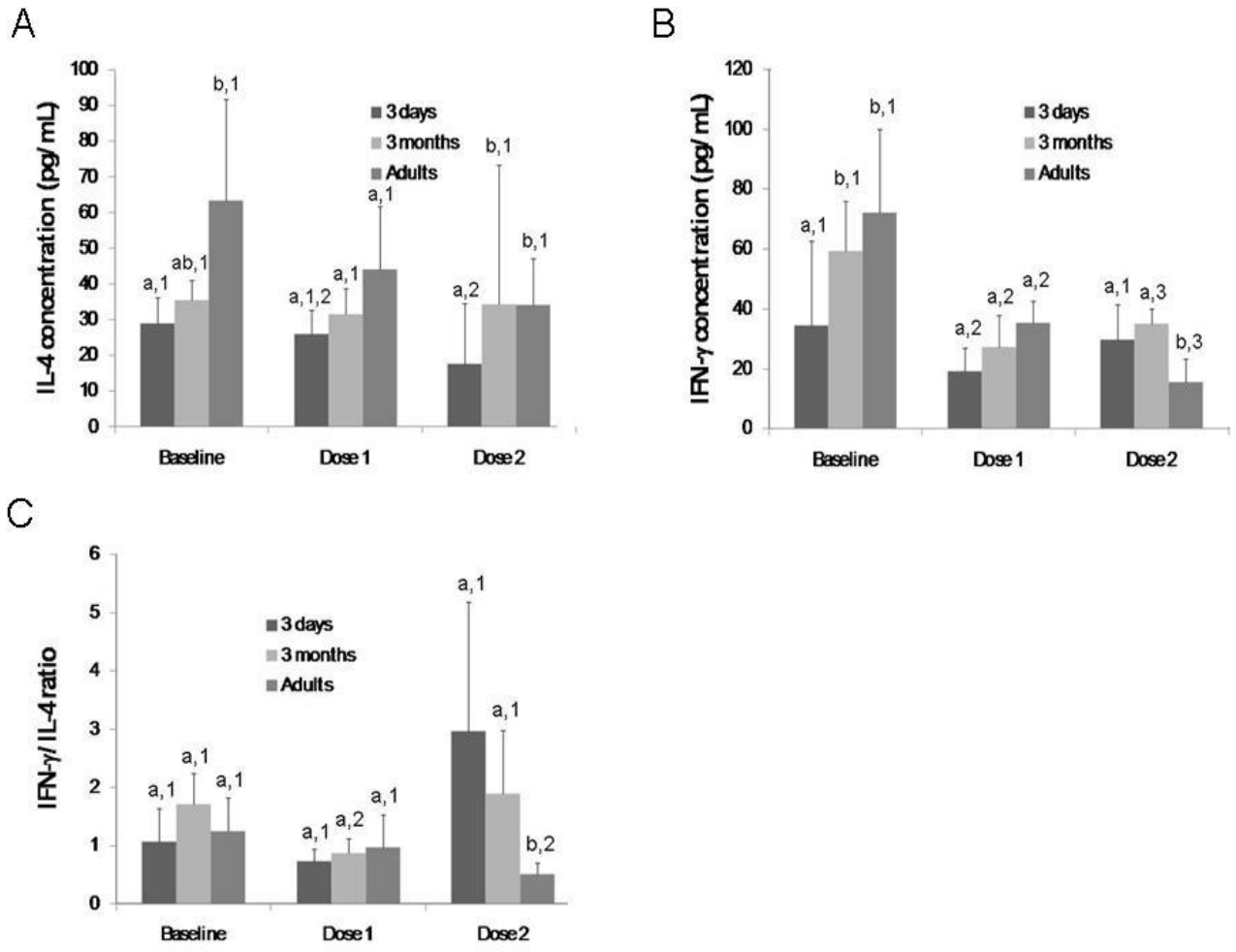

Figure 5-3. Mean (±SD) concentrations of IFN-γ (A) and IL-4 (B), and IFN-γ/IL-4 ratio (C) in the supernatants of PBMCs stimulated with vaccine antigens as determined by ELISA. Adult horses, 3-day old foals, and 3-month old foals were vaccinated with a killed adjuvanted vaccine twice with 3 weeks between administrations. PBMCs were collected prior to vaccination as well as 3 weeks after administration of each dose of the vaccine. [1,2,3]Different numbers within an age group indicate significant differences between sample time points. [a,b,c] Different letters within a time point indicate significant differences between age groups. Significance was set at $P < 0.05$.

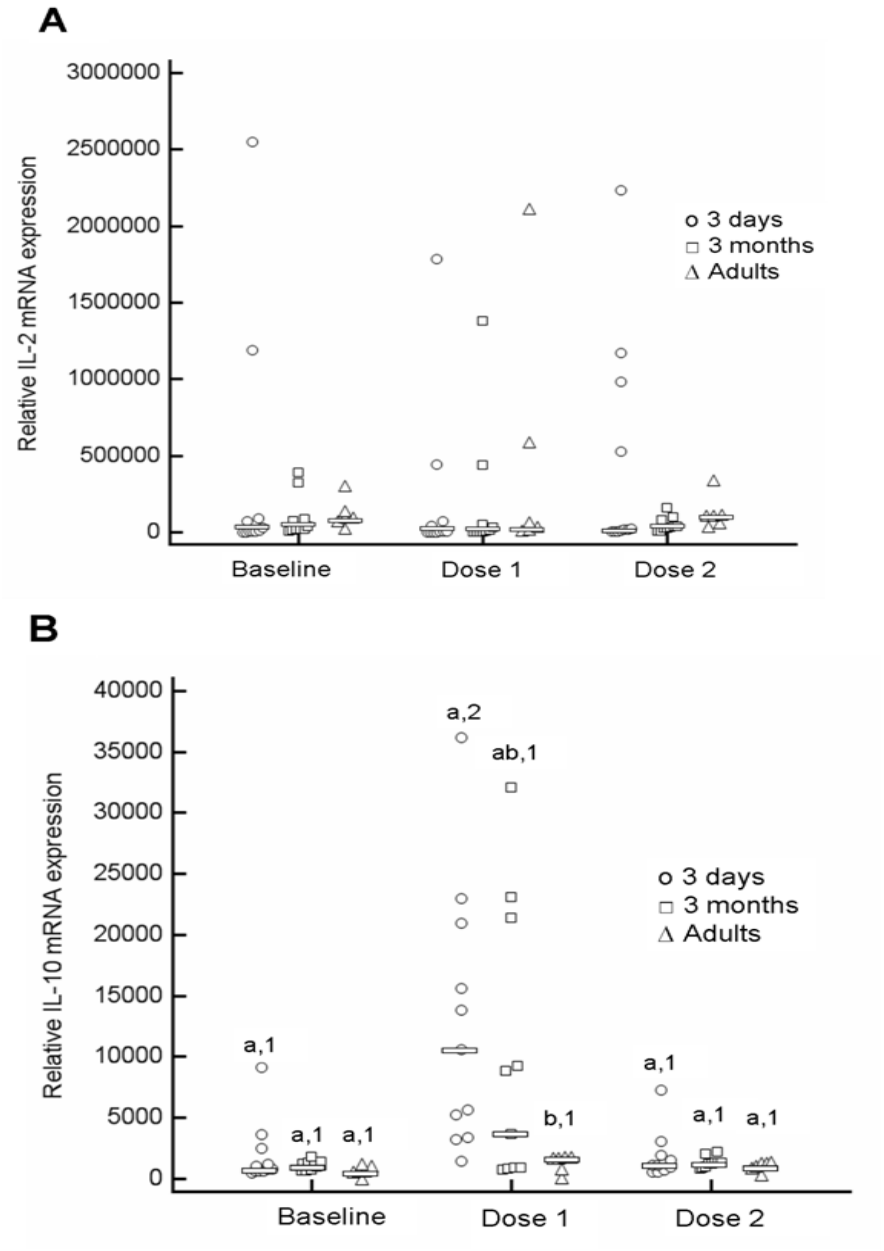

Figure 5-4. Relative IL-2 (A) and IL-10 (B) mRNA expression in PBMCs stimulated with vaccine antigens. Adult horses, 3-day old foals, and 3-month old foals were vaccinated with a killed adjuvanted vaccine twice with 3 weeks between administrations. PBMCs were collected prior to vaccination as well as 3 weeks after administration of each dose of the vaccine. Symbols represent individual data points. Horizontal bars indicate median values. [1,2,3]Different numbers within an age group indicate significant differences between sample time points. [a,b,c] Different letters within a time point indicate significant differences between age groups. Significance was set at $P < 0.05$.

CHAPTER 6
SUMMARY AND CONCLUSIONS

The research presented in this body of work focused on examining the differences in immune system function between newborn foals, older foals, and adult horses. The importance of this work lies in our ability to use this information to design future preventative and treatment strategies for foal diseases, such as *R. equi* pneumonia. It has become clear in recent years that neonates of many species, including equids, do not mount immune responses in the exact same way or magnitude as compared to adults. Although foal responses seem to "mature" over time and reach adult levels at about 1 year of age, there is still a large window of time during which foals are susceptible to infections that are easily controlled by adults. For these reasons, foals have been historically been considered to have "immature" immune systems. However, instead of viewing their responses as "immature", it is probably more appropriate to view their responses as naïve. Some authors have suggested that a predisposition of foals to develop Th2 type responses may be responsible for their susceptibility to intracellular infections such as *R. equi*. However, only a small percentage of foals exposed to *R. equi* actually develop the disease, indicating that foal's development of protective Th1 responses can and does occur.

Our research is focused on the unique aspects of the foal immune system which might allow them to become infected with an organism that is non- pathogenic to adults. The main objectives of this project were threefold. First, the effect of age and different mitogens on the ability of PBMCs to secrete cytokines was determined. It was shown that foals are not just deficient in their ability to produce IFN-γ. They are also deficient in their ability to produce IL-4. As a result, it may not be appropriate to state that the

immune system of equine neonates is biased towards a Th2 response. In addition, the present study demonstrated that regardless of age, the type of mitogen used for *in vitro* stimulation had a significant effect on the IFN-γ/IL-4 ratio. Therefore, it will be important to take the type of cellular stimulation in consideration in future studies aiming at assessing regulation of immune responses in horses.

Second, the effects of two commercially available immunostimulants on phagocytic cell function after *ex vivo* exposure to *R. equi* were assessed. The two commercially immunostimulants evaluated significantly enhanced phagocytic cell function upon *ex vivo* exposure to virulent *R. equi*. The clinical relevance of these findings in the prevention of *R. equi* infections on endemic farms remains to be determined in a prospective randomized clinical trial.

Finally, the effects of age on humoral and cell- mediated immune responses to a killed adjuvanted vaccine were examined. Humoral and lymphoproliferative immune responses to the killed adjuvanted vaccine used were modest in newborn foals, consisting mainly of a weak IgGa and IgG(T) response. Although immune responses improved with age, 3-month-old foals did not respond with the same magnitude as adult horses. The post-vaccination IFN-γ/IL-4 ratio of both groups of foals was significantly higher than that of adult horses. Collectively, the work presented in this dissertation indicate that, although basal cytokine secretion in neonatal foals may be considerably dampened, newborn foals do not exhibit clear bias towards a Th2 response

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BIOGRAPHICAL SKETCH

Clare Ryan attended the University of Wisconsin- Madison where she majored in biochemistry. She received her Doctor of Veterinary Medicine from the University of Wisconsin- Madison School of Veterinary Medicine in 2002. She completed a large animal medicine and surgery internship at the Ontario Veterinary College in Ontario, Canada in 2003. She came to the University of Florida's College of Veterinary Medicine for a residency in large animal internal medicine, which she completed in 2006. She also completed her requirements to become a diplomate of the American College of Veterinary Medicine at this time. She then began her graduate studies at the University of Florida. She received her Ph.D. from the University of Florida in the summer of 2010.