THE ROLE OF AGE-ASSOCIATED CHANGES IN VITAMIN D, CORTISOL, AND CYTOKINES IN MODULATING THE EQUINE IMMUNE RESPONSE TO *RHODOCOCCUS EQUI*

By

LONDA JEAN BERGHAUS

(Under the Direction of Kelsey A. Hart)

ABSTRACT

Bronchopneumonia caused by *Rhodococcus equi* (*R. equi*) occurs in a narrow age window in foals. On farms endemic for *R. equi* pneumonia, individual responses to the bacteria are inconsistent. Currently, a biomarker to predict disease susceptibility and severity has not yet been identified.

Steroid hormones, like cortisol and vitamin D, modulate immune responses. Age-related differences in cortisol and vitamin D predispose the neonatal foal to the risk of developing severe disease in other bacterial infections. Steroid hormone-regulated immune responses, in other species, are vital, for the clearance of intracellular bacteria. Immunoregulation by vitamin D and cortisol may play a role in foal *R. equi* susceptibility.

The aim of this work was to examine the role of age-associated changes in vitamin D, cortisol, and the inflammatory cytokines they regulate in relation to *R. equi* disease. First, we characterized vitamin D receptor (VDR) expression within equine alveolar macrophages (AMϕ), the cell involved in primary *R. equi* respiratory infection and showed age-associated differences in VDR expression between foals and adults.

Second, we characterized VDR expression and cytokine protein production in response to *ex vivo R.equi* infection of AMϕ. VDR expression was downregulated in response to *R. equi* in AMϕ from adult horses but unchanged in AMϕ cells from young foals. Age- and *R. equi*-associated cytokine production in AMϕ cells from four-week-old foals was equivalent to responses from adult horse cells. These findings support our hypothesis that the vitamin D/VDR pathway is involved in immune regulation within equine leukocytes.

Lastly, we wanted to determine if an age- and disease-associated effect on vitamin D and cortisol concentrations is present in foals naturally exposed to *R. equi* on an endemic farm. Furthermore, we hoped that concentration differences in cytokines and steroid hormones would predict disease severity in pneumonic foals. This study elucidated age-associated differences in steroid hormones and cytokines, but these differences did not predict disease susceptibility and severity in pneumonic foals. However, these age-associated differences provide information to better understand foal immunity. Disease-associated differences in interferon (IFN)-γ concentrations indicate that the mechanisms and kinetics of cytokine production may play a role in disease susceptibility.

INDEX WORDS: *Rhodococcus equi*, pneumonia, immunomodulatory, equine, foal, vitamin D, cortisol, cytokines

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DEDICATION

To Steeve

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When you begin a PhD at 50 years old, there are plenty of people to thank, and I would be remiss if I did not start with two men who changed the trajectory of my professional career. First is the late Dr. Mark Estes, who hired me as a lab technician, 25 years ago, in spite of my interview confession that I had no idea what the word immunology meant. Mark took a chance hiring me and as a result I discovered a love for the immune system and became enthralled with the little miracles that, constantly, take place within those little cells. Ten years later, Dr. Steeve Giguère, hired me to be his lab manager, after I confessed to him that I did not know one end of a horse from the other. While in his laboratory I learned to understand why people love horses and found a passion for equine research. Dr. Giguère challenged me every day to be a better person and scientist. He treated me as a colleague, always valuing my contributions and perspective. He was exceptionally brilliant. He was extraordinarily kind. And I miss him every day. Working as Steeve's lab manager was my professional "Plan A".

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the challenge of mentoring me through this process. During these past few years, we have literally and figuratively crossed oceans, climbed mountains, and stared in the face of lions, and most importantly remained friends. Kelsey, I love you dearly and am so very grateful for everything you have done for me.

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This journey has been hard and while it represents a great achievement for me, the goal of my life will continue to love others well and reflecting God's deep grace and mercy wherever the road takes me. Plan C….hmmmm….I can't wait!

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CHAPTER 1

INTRODUCTION

This dissertation and the chapters contained herein aim to explore how age-associated differences in immunomodulators affect susceptibility to and severity of *Rhodococcus equi* (*R. equi*) pneumonia. *R. equi* pneumonia is a leading cause of morbidity and mortality in young foals, yet older foals and adult horses are rarely affected. The immune response to *R. equi* exposure in foals ranges from individuals that appear to be naturally resistant to the disease to others that manifest severe clinical signs and may or may not survive the infection. The mechanisms resulting in this variability in immune response and limited age range of susceptibility are not well understood. Steroid hormones like vitamin D and cortisol regulate the immune response to pathogens and involve both up- and down-regulation of a diverse array of inflammatory cytokines. Age-associated differences between newborn foals and adult horses for vitamin D, cortisol, and cytokine concentrations have been reported. In these studies, we aimed to understand if vitamin D and cortisol modulate the immune response during *R. equi* exposure. Specifically, we sought to determine if the vitamin D/vitamin D receptor (VDR) pathway is present in equine alveolar macrophages (the cell type primarily infected by *R. equi*). We measured expression of VDR in alveolar macrophages (AMϕ) from foals at different ages and adult horses and evaluated

changes in VDR expression following *ex vivo R. equi* infection. We also examined whether vitamin D, cortisol, and *R. equi*-associated/steroid hormone-regulated cytokine concentrations are associated with susceptibility to pneumonia following *R. equi* exposure on a farm endemic for disease and could serve as useful predictors of disease severity among individual foals.

Chapter 2 is a literature review and is divided into five sections. Section I provides an overview of the *R. equi* bacterium and includes a subsection describing important similarities between *R. equi* and *Mycobacterium tuberculosis* (*Mtb*). Section II reviews the clinical presentation of *R. equi* pneumonia in foals and disease susceptibility. Section III covers the immune system of the foal, including transfer of passive maternal immunity, an overview of age-associated differences in both the innate and adaptive immune systems, and cytokine immune signaling. Section IV provides an overview of the steroid hormones cortisol and vitamin D, including their potential interactions during the immune response. Finally, Section V provides the justification for the studies contained in subsequent chapters.

Chapters 3 through 5 present a series of manuscripts detailing the results conducted for this dissertation. The objective of the first study (Chapter 3) was to determine if the vitamin D receptor (VDR), the nuclear receptor that modulates the molecular effects of vitamin D, is expressed in equine leukocytes and whether expression varies with age. We hypothesized that the VDR would be expressed in equine alveolar macrophages (AMϕ) and expression would differ in an age-dependent fashion.

The objective of the second study (Chapter 4) was to determine if VDR expression is affected by *R. equi* infection in AMϕ, and if VDR expression, VDR synthesis, and vitamin D/VDR pathway-associated cytokine production was impacted by age in an *ex vivo* model of *R. equi* infection within AMϕ from healthy growing foals compared to AMϕ from healthy adult horses. The specific hypothesis was that infection with *R. equi* would affect VDR expression, active vitamin D metabolite synthesis, and cytokine production in an age-dependent manner in AMϕ isolated from healthy foals and adult horses. Chapter 5 presents the results of a prospective cohort study in which we measured concentrations of circulating vitamin D, cortisol, and cytokines in foals from birth to weaning on a large breeding farm endemic for *R. equi* pneumonia. Additionally, we evaluated association among these parameters and disease occurrence and severity. We hypothesized that there would be a significant effect of age and respiratory disease on steroid hormone concentrations, and that variations in cytokines and steroid hormone concentrations would predict the incidence and severity of disease in foals.

Chapter 6 summarizes the findings of the studies contained in this dissertation and proposes that age-associated differences in VDR expression are evident in equine AMϕ, with a response in infection observed in adult horses but not foals. Age-associated differences in circulating concentrations of vitamin D, cortisol, and cytokines from birth to weaning occur, but only IFN-γ concentration is associated with respiratory disease in foals naturally exposed to *R. equi*. The disease-associated changes in IFN-γ reported

herein suggest that further investigation into the mechanisms and kinetics of IFN-γ production by leukocytes in young foals is warranted.

CHAPTER 2

LITERATURE REVIEW

SECTION I: *RHODOCOCCUS EQUI* BACTERIA: AN OVERVIEW

Rhodococcus equi (*R. equi*) is a coccobacillus within the class Actinomycetia and is considered the most pathogenic bacterium within the *Rhodococcus* genus (Karlson et al., 1940; Magnusson, 1923). The bacterium was first isolated in 1923 from the lungs of a foal with pyogranulomatous pneumonia and identified as the causative agent (Magnusson, 1923). Disease caused by *R. equi* is discussed in Section II. The name *Rhodococcus* was coined by Goodfellow and Alderson in 1977 as an ode to the y-carotene that results in the classic pink color of the bacteria in culture and the bacterial shape (Goodfellow & Alderson, 1977). *R. equi* is a gram-positive, aerobic, and non-motile soil saprophyte that prefers dry, dusty soil but can also be found in feces of herbivores. Survival in soil and feces is attributed to a capsule that helps prevent dehydration and may also aid in virulence and immune system evasion (Prescott & Hoffman, 1993; Sydor et al., 2008). Virulent and avirulent isolates can persist in the environment and are routinely shed in horse and foal feces.

The *R. equi* genome consists of over five million base pairs that are highly conserved between members of the Actinomycetia class (Letek et al., 2010). Much of

the genome is dedicated to proteins involved in secretion, regulation, and transport, which are necessary for bacterial survival in both extracellular and host-cell environments (Letek et al., 2010). Virulence of *R. equi* in equids is dependent on the presence of a large circular plasmid known as Virulence-Associated Plasmid A (pVAPA) which encodes for virulence associated protein A (VapA) (Letek et al., 2008; Takai et al., 2000). *R. equi* isolates without the *vapA* gene are considered avirulent (Takai et al., 1991). *R. equi* virulence is characterized by the ability of the bacteria to survive and replicate within phagocytic cells and is dependent upon *vapA* gene-regulated modifications within the cellular environment (Hondalus, 1997). *R. equi* survival within phagocytes can also be attributed to the mycolic-acid-containing glycolipid cell envelope which serves to protects the bacteria from destructive enzymes and reactive oxygen species (ROS) produced by infected host cells (Giguère et al., 2011a; Sutcliffe, 1998). Additionally, *R. equi*, like other actinobacteria, use steroids, like cholesterol, as their primary energy source (van der Geize et al., 2011; Yam et al., 2009). In fact, mutant *R. equi* strains (deleted of genes critical to cholesterol catabolism) were unable to infect and survive within macrophages *in vivo* and failed to induce disease in an experimental challenge model in foals (van der Geize et al., 2011). This study indicates that cholesterol catabolism is an important aspect of *R. equi* virulence.

Inhalation and ingestion of virulent *R. equi* isolates are the primary routes of infection in foals (Meijer & Prescott, 2004). *R. equi* is phagocytosed by macrophages, specifically alveolar macrophages following inhalation, using either the complement

receptor (CR3) or mannose receptors (Garton et al., 2002; Hondalus et al., 1993). Survival and replication within pulmonary macrophages are the basis of *R. equi* pathogenicity in the foal (Giguère et al., 1999). Once inside the macrophage, *R. equi* alters its gene expression patterns to facilitate survival within the intracellular environment (Ren & Prescott, 2003; Toyooka et al., 2005). The VapA protein prevents phagolysosome fusion (a key response of phagocytic cells for bacterial clearance and antigen presentation), allowing the bacteria to survive and replicate protected from host-defense mechanisms (von Bargen et al., 2009). Intracellular replication of *R. equi* within macrophages leads to necrotic cell death, reinfection of surrounding macrophages and subsequent formation of abscessing granulomas within lung tissue (Falcon et al., 1985). In contrast, macrophages activated by pro-inflammatory cytokine signals from adaptive immune cells (discussed in greater detail in Section III) are able to clear *R. equi* through an increase in a myriad of defense strategies, such as increased reactive oxygen intermediates and acidification of the phagolysosome (Kanaly et al., 1995; Weiss & Schaible, 2015). Many of the characteristics of *R. equi* described above are shared by other Actinomycetia bacterium (von Bargen & Haas, 2009).

Similarities to *Mycobacterium Tuberculosis*

Mycobacterium tuberculosis (*Mtb*) causes the human respiratory disease called tuberculosis and is a leading cause of death worldwide (Sotgiu et al., 2017). *Mtb* is a closely related bacteria within the same class as *R. equi* and many similarities exist

between the two organisms (von Bargen & Haas, 2009). Because of the importance of tuberculosis in human health, a large body of research on *Mtb* exists, and much of what we know about the pathogenicity and immunogenicity of *R. equi* has been birthed out of *Mtb* research (von Bargen & Haas, 2009).

Like *R. equi*, *Mtb* is characterized by a mycolic acid-containing cell envelope that contributes to its survival and pathogenesis (Daffe & Draper, 1998; Gurtler et al., 2004). *Mtb* are facultative intracellular bacteria that can survive and replicate within human macrophages and monocytes. Normal phagocytosis of bacteria by a macrophage can occur through several phagocytic receptors and results in an engulfed particle wrapped in a phagocytic membrane to form an organelle called a phagosome. Phagosomal maturation occurs in stages, with the end result being a highly acidic environment containing proteins and ROS metabolites, that destroy the pathogen (Akira, 2009; Gordon, 2016). *Mycobacterium tuberculosis* has evolved several mechanisms for intracellular survival including the ability to be phagocytosed without causing macrophage activation and prevention of phagosome maturation, phagolysosome fusion, and phagolysosome acidification (Anes et al., 2003; Liu & Modlin, 2008; Xu et al., 2014). Additionally, the persistence and pathogenicity of *Mtb* is dependent on its ability to utilize lipids, like cholesterol, within host cells as a growth substrate. Furthermore, *Mtb* can dysregulate the balance of lipids within the cell resulting in the formation of macrophages packed with lipid bodies referred to as foamy macrophages (Crowe et al., 2017; Lee & Bensinger, 2022; VanderVen et al., 2015). Similar survival mechanisms are

found in *R. equi*, as described above (van der Geize et al., 2011; von Bargen & Haas, 2009). The survival tactics of *Mtb* (and *R. equi*) are unsuccessful in macrophages that are activated by the pro-inflammatory cytokines interferon (IFN)-γ and tumor necrosis factor (TNF)-α (Hines et al., 2003; Kanaly et al., 1995; Sakamoto, 2012). Cytokines are a broad class of proteins involved in immune cell signaling and activation and are discussed in greater detail in Section III.

Phagocytosis of *Mtb* by activated macrophages results in a myriad of downstream responses. A key response is the production of cytokines that act in an autocrine fashion to induce apoptosis or a paracrine fashion to stimulate surrounding immune cells, including those involved in the formation of granulomas (Means et al., 1999; Sakamoto, 2012). Another response by the activated macrophage is to increase expression of nuclear receptors (Leopold Wager et al., 2019a). While there are several nuclear receptors within macrophages, the vitamin D receptor (VDR) (and its ligand the steroid hormone vitamin D, discussed in Section IV) has been associated with *Mtb* clearance in human macrophages (Leopold Wager et al., 2019a). During *Mtb* infection, the vitamin D/VDR pathway is responsible for regulating the production of antimicrobial peptides, pattern recognition receptors, and cytokines that are critical for bacterial clearance (Ismailova & White, 2022) (Figure 2.1). The discovery of the relationship between the vitamin D/VDR pathway and *Mtb* clearance provided scientific evidence to support the ancient therapeutic use of heliotherapy (AKA sunshine therapy) for the treatment of tuberculosis (Baeke et al., 2010). Consequently, the VDR has now become

a therapeutic target in the treatment of tuberculosis (Leopold Wager et al., 2019b). The vitamin D/VDR pathway is discussed in greater detail in Section IV. The vitamin D/VDR pathway is unexplored in terms of its role in equine macrophages and relevance in equine respiratory disease, such as *R. equi* pneumonia.

Infection with *Mtb* is primarily found in humans and is spread by aerosolized particles. Primary infection occurs in the lower respiratory tract with alveolar macrophages the specific niche for bacterial survival and replication (Bussi & Gutierrez, 2019). This slow growing infection is characterized by the formation of granulomas within the lungs (Ehlers & Schaible, 2012). Granulomas are considered a protective host-defense to contain the infection and are an aggregate of parasitized macrophages and other immune cells (Ramakrishnan, 2012). Granulomas formed by *Mtb* infection are highly organized with a caseating central area surrounded by macrophages, epithelial and Langerhans giant cells with an outer layer of lymphocytes that help contain the bacteria (Ehlers & Schaible, 2012). In contrast, the granuloma formed during *R. equi* infection is non-caseating and less well organized, containing a mix of neutrophils, macrophages, lymphocytes, and multinucleated giant cells (Ehlers & Schaible, 2012; Prescott, 1991). Infection with *Mtb* can result in either active or latent infection in people and patients with latent tuberculosis are at risk for reactivation later in life. Active *Mtb* infection more closely resembles *R. equ*i disease, (Le et al., 2015), as discussed in more detail in Section II.

SECTION II: RHODOCOCCUS EQUI PNEUMONIA

Bronchopneumonia caused by the bacterium *R. equi* is a leading cause of disease in young foals worldwide. At one point it was estimated that 17% of foals worldwide were affected by this disease each year (Elissalde et al., 1980). Some breeding farms are endemic for the disease with the majority of foals on these farms having some evidence of *R. equi* infection, as determined by physical exam and transthoracic ultrasonography, while other breeding farms have sporadic or no disease (Elissalde et al., 1980; Slovis, 2005; Venner et al., 2013). It is unclear why certain farms are endemic for the disease, but mare/foal density and housing in stalls versus paddocks are contributing factors for disease incidence and prevalence (Bordin et al., 2022; Muscatello, 2012). The financial repercussions of treating foals on endemic farms can be quite high (Giguère et al., 2011b; Slovis, 2005). The economic impact is attributed to the cost of the treatment itself, the farm-labor to treat the foal, the veterinary care, the potential loss of sale due to foal mortality, and potential for decreased athletic performance as a recovered adult (Ainsworth et al., 1998).

The disease is characterized by pyogranulomatous lesions in the lungs shown in Figure 2.2A. These lesions can often be imaged by radiology (Figure 2.2B) or ultrasonography (Figure 2.2C) in a live foal. The clinical signs usually develop in an insidious but progressive manner (Falcon et al., 1985; Giguère et al., 2011a). The most common clinical signs are fever, cough, lethargy, and increased respiratory effort (Giguère et al., 2011a). Extrapulmonary signs (diarrhea, polysynovitis, uveitis, synovitis,

etc.) can also occur and are often associated with poor outcome (Giguère et al., 2011a; Reuss et al., 2009). Rarely, but occasionally, foals may develop severe, acute respiratory distress that can result in sudden death (Giguère et al., 2011a). Conclusive diagnosis of *R. equi* pneumonia is based on consistent clinical signs (described above), and identification of the bacteria in culture and polymerase chain reaction (PCR) amplification of the *vapA* gene (discussed in Section I) from a transtracheal aspirate sample (Giguère et al., 2011b). PCR detection of the *vapA* gene in feces is not adequate for diagnosis. While feces are easier to obtain than a transtracheal aspirate, it could result in a false positive, as healthy foals and adults regularly shed virulent bacteria in their feces (Giguère et al., 2011b).

Currently, there are no effective vaccines to prevent *R. equi* pneumonia, and in their absence, early detection by ultrasonographic screening of lungs and early initiation of effective antimicrobial therapy have been the most effective strategy to reduce disease-associated morbidity and mortality. Environmental exposure to the bacteria occurs shortly after birth, and pulmonary lesions are detectable in affected foals as early as 4 weeks of age, usually prior to development of clinical signs (Cohen et al., 2013; McCracken et al., 2009; Slovis, 2005). Prophylactic, transthoracic ultrasound screening of apparently healthy foals for early detection of pulmonary abscesses has been recommended for all foals on farms endemic for *R. equi* pneumonia (McCracken et al., 2009; Slovis, 2005). Moreover, it was originally suggested that all foals should be treated with antimicrobials upon initial evidence of pulmonary lesions (regardless of lesion size

or lack of clinical signs), with the rationale that both disease-associated mortality and duration of antimicrobial therapy would be decreased by this strategy (McCracken et al., 2009; Slovis, 2005). This screening and treatment protocol has been a mainstay at endemic farms for over 20 years and has resulted in some farms treating 30 to 70% of their foals with antimicrobials (Venner et al., 2013). This mass antimicrobial treatment strategy may have decreased *R. equi*-associated mortality on endemic farms, but unfortunately this has also been associated with emergence of antimicrobial-resistant *R. equi* isolates (Burton et al., 2013; Giguère et al., 2010; Huber et al., 2021). New evidence suggests that only foals with large ($\geq$ 15 cm total) areas of ultrasonographic pulmonary consolidation should be treated with antimicrobials, as spontaneous recovery (without intervention) will occur in most other foals with smaller lesions (Arnold-Lehna et al., 2019; Giguère et al., 2011b; Venner et al., 2012). Treating only foals with large lesions could reduce antimicrobial usage on endemic farms by > 50%, and therefore decrease the high costs associated with treatment while subsequently lowering the environmental impact accompanying mass antimicrobial usage (Arnold-Lehna et al., 2019; Hildebrand et al., 2015).

The American College of Veterinary Internal Medicine (ACVIM) consensus statement on *R. equi* recommends combination therapy with a macrolide (azithromycin, erythromycin, or clarithromycin) and rifampin for the treatment of *R. equi* pneumonia in foals (Giguère et al., 2011b). Working synergistically, this combination reduces the likelihood of drug-resistant isolates developing (Nordmann et al., 1993). While this

combination therapy is usually effective in treating susceptible isolates, it is expensive and needs a long treatment duration from 2-12 weeks (Giguère et al., 2011b). Unfortunately, documentation of multi-drug resistant *R. equi* has increased since 2001, and treatment of resistant isolates represents a major challenge to veterinarians because effective alternatives to macrolides and rifampin are limited (Bordin et al., 2022; Cohen et al., 2016). Additionally, foals with pneumonia linked to antimicrobial-resistant isolates are less likely to survive (Giguère et al., 2010).

While *R. equi* pneumonia is primarily a disease in foals, it can be an opportunistic pathogen in immunocompromised humans and immunocompromised adult horses and is considered a zoonotic pathogen with human infections associated mostly with animal strains (Hondalus, 1997; Takai et al., 2020). The combination therapy of macrolide and rifampin is also the preferred treatment for human *R. equi* infection, and macrolide resistance is considered of critical importance by the World Health Organization because of the usefulness of these drugs in treating many bacterial infections in humans (Collignon et al., 2016; Stewart et al., 2019).

Disease Susceptibility

A unique and perplexing feature of *R. equi* bronchopneumonia in foals is the limited age-window of disease susceptibility. Unlike other bacterial causes of pneumonia, *R. equi* disease is almost exclusively diagnosed in foals between 2 to 6 months of age (Giguère et al., 2011a). Diagnosis in healthy older foals and adult horses

rarely occurs (Giguère et al., 2011a; Hondalus, 1997). The bacterium is a ubiquitous soil saprophyte, as noted in Section I, and epidemiological evidence indicates that foals are exposed shortly after birth (Cohen et al., 2013). Epidemiological evidence suggests that foals less than 1 month of age are highly susceptible to infection by *R. equi* and the insidious growth of the bacteria during natural infection results in a delay of visible lung lesions and clinical signs for several weeks after exposure in experimental infections (Bordin et al., 2022; Sanz et al., 2013). However, in naturally occurring *R. equi*, the exact incubation period between infection and clinical signs is unknown. It is assumed that this limited age-window of susceptibility is related to vulnerabilities in the foal's developing immune system, but years of research have not been able to pinpoint the specific deficiency or deficiencies that render foals vulnerable to *R. equi* disease during this time period (see Section III below).

Another interesting aspect of this disease is related to the vast array of individual responses to bacterial exposure. Foals under the same husbandry practices, and with the assumptive equivalent exposure on endemic farms will either be naturally resistant, have only subclinical indication of infection that resolves spontaneously, or will develop clinical manifestations of disease. Most foals (up to 70%) on endemic farms will have evidence of small (< 10 cm total) pulmonary abscesses, but the majority of these foals (up to 88%) will recover without veterinary intervention (McCracken et al., 2009; Slovis, 2005; Venner et al., 2013). Other foals, though, will require extensive antimicrobial therapy and a small proportion of foals will not survive the infection even with

aggressive treatment (Giguère et al., 2011a). Currently, it is not known why one foal appears to be naturally resistant while another succumbs to the disease. It is possible that distinct characteristics of an individual foal's immune response may enable the clearance of intracellular bacteria like *R. equi* resulting in natural resistance to disease. However, at this time, there is no reliable prognosticator of immune parameters that enables us to evaluate the potential outcome of the foal's response to bacterial exposure.

SECTION III: FOAL IMMUNITY

Successful clearance of intracellular bacteria, like *R. equi* and *Mtb*, in mammals depends on both the innate and adaptive immune response as shown in Figure 2.3 (Thakur et al., 2019). Skin, mucosa, and chemical barriers provide an initial blockade for pathogen invasion and are a vital component of the innate immune system. Once a pathogen has broken through this initial barrier, it will encounter phagocytic cells of the innate immune system, including neutrophils, monocytes, macrophages, and dendritic cells. Phagocytic cells recognize pathogens through characteristics referred to as pathogen-associated molecular patterns (PAMPs) displayed on the bacterial cell surface. Cell surface receptors, called pattern recognition receptors, found on phagocytic cells recognize PAMPs, and ligand binding of these receptors initiates the process of phagocytosis. Phagocytosis of bacteria by neutrophils results in production of reactive oxygen mediators, release of extracellular traps, histones, elastase, and

myeloperoxidase, resulting in both bacterial cell death and death of the neutrophil itself (Baz et al., 2024; Murphy et al., 2012). Phagocytosed bacteria within macrophages are contained within a compartment called the phagosome, which fuses with lysosomes inside the macrophage and the phagolysosome is able to digest the engulfed bacteria and process the digested components into innocuous waste products (Murphy et al., 2012; Weiss & Schaible, 2015). If macrophages and neutrophils are unable to adequately clear all of the bacteria, macrophages (and neutrophils to a lesser degree) will secrete proteins (cytokines and chemokines) to initiate a broader inflammatory response to signal cells of the adaptive immune system (Weiss & Schaible, 2015).

Dendritic cells are tissue-resident innate cells that specialize in presenting bacterial antigen to cells of the adaptive immune system (Murphy et al., 2012). Antigenic presentation by dendritic cells usually occurs in the lymph nodes and results in the activation of T or B cells that have specific surface receptors that recognize the presented antigen (Murphy et al., 2012). Once the antigen is recognized, rapid proliferation of cells of the adaptive immune response ensues. T helper (Th) cells initially proliferate and secrete cytokines that help other T and B cells to proliferate and respond to enhance bacterial cell clearance (Korn et al., 2007). Adaptive immune cell functions include differentiation of B cells, resulting in antibody production, differentiation of cytotoxic T cells that recognize and destroy infected cells, migration of Th cells to sites of infection to secrete cytokines that enhance the innate immune response, and differentiation of other specialized T cells including Th17 and T regulatory cells. Robust

adaptive immune responses usually result in bacterial clearance and regularly occur in mature individuals with a fully developed immune system (Simon et al., 2015).

It is generally accepted that mammalian neonates have immunological differences compared to adults of the same species, rendering them more susceptible to disease during the first few weeks or months of life (Butler et al., 2009; Chase et al., 2008; Ghazal et al., 2013; Hodgins & Shewen, 2012). The neonate is moving from a mostly sterile environment *in utero* to an environment with a plethora of both harmful and harmless antigens and the immune system must quickly learn to differentiate between "friend and foe." An additional challenge of the naïve immune system is to learn to develop an effective response to harmful antigens while limiting an overzealous response that might lead to tissue damage or decrease the rapid cellular proliferation needed in a growing animal (Perkins & Wagner, 2015). Therefore, immunological differences in neonates usually include attenuated antibody and pro-inflammatory cytokine production as well as a naïve and undeveloped adaptive immune response (Perkins & Wagner, 2015; Sedney & Harvill, 2023). Thus, the newly born animal must rely primarily on either acquired maternal immune factors or innate immune responses for protection from infection post parturition and through the first weeks (and sometimes months) of life (Ghazal et al., 2013).

Maternal Immune Transfer

Foals have an absence, or extremely low concentrations, of antibodies at birth because the anatomical structure of the equine placenta prevents maternal transfer of antibodies *in utero*. Newborn foals can, however, absorb antibodies found in colostrum for up to 36 hours after birth, through a process of pinocytosis in the digestive tract, referred to as transfer of passive immunity. Failure of transfer of passive immunity (FTPI) occurs when there is inadequate ingestion of colostral immunoglobulins, which can occur for reasons such as poor colostrum quality or inability of the newborn foal to nurse (Hart & Wong, 2024). Thus, FPTI in the foal results in the newborn foal being highly susceptible to infection and development of sepsis (Hart & Wong, 2024). Immunoglobulin G (IgG) is considered the most important antibody type passed to a foal via colostrum and concentrations of IgG $\geq$ 800 mg/dL render a foal protected from certain diseases (like influenza and rotavirus) until their immune system is more mature (Perkins & Wagner, 2015). Mares are often vaccinated during late pregnancy to enhance the transfer of these antigen-specific antibodies to the foal via colostrum (Wilson et al., 2001).

An effective *R. equi*-targeted maternal vaccine, to utilize this pathway of transfer of passive immunity has yet to be developed. Several candidate vaccines that have induced *R. equi*-specific antibodies in mares have been studied but none have successfully or consistently protected foals from disease (Madigan et al., 1991; Martens et al., 1989). Maternal vaccines targeted at specific antigenic components of the

bacteria, such as the virulence plasmid or surface polysaccharides, have also failed to offer protection in challenge models of the disease (Cohen et al., 2021; Cywes-Bentley et al., 2018). Immunization of plasma donors (adult horses vaccinated with killed *R. equi*) to create a plasma product containing high concentrations of *R. equi*-specific antibodies that can subsequently be transfused intravenously to foals shortly after birth (or later as maternal antibodies wane, as discussed in Section III) is utilized on many endemic farms, but has also not consistently protected foals from disease (Caston et al., 2006; Giguère et al., 2002; Higuchi et al., 1999; Hooper-McGrevy et al., 2001; Hurley & Begg, 1995; Muller, 1992; Perkins et al., 2002; Sanz et al., 2014). Differences in the hyperimmune product itself, timing of administration, and variations in the antigenic antibody target, may partly explain the differences in protective outcome with the use of these products (Bordin et al., 2022). *Rhodococcus equi*-specific antibodies produced in the adult horse may also be of a different isotype (and thus have different properties) than those that are necessary to protect the foal (Lopez et al., 2002). There are seven different known IgG isotypes that differ in their ability to opsonize pathogens, interact with immune cells, and bind to complement (Lopez et al., 2002). IgG4/7 is the most predominant isotype found in adult horses and colostrum, with other subtypes present to a lesser extent (Perkins & Wagner, 2015). Research suggests that IgG1 is more effective at neutralizing intracellular bacteria, but the evidence to support this for *R. equi* has not been confirmed either mechanistically or in experimental challenge models of infection (Cohen et al., 2021; Rocha et al., 2019). Vaccine candidates targeted to

induce *R. equi*-specific IgG1 antibodies in foals and hyperimmune plasma containing *R. equi*-specific IgG1 haves failed to protect foals from disease in experimental challenges (Lopez et al., 2002; Perkins & Wagner, 2015; Rocha et al., 2019). Consequently, the development of an effective antibody-specific preventative to protect the foal from *R. equi* pneumonia has remained elusive and may not be possible.

In mammalian species, colostrum is also a source of other immune factors, like complement, cytokines, and maternal leukocytes (Burton et al., 2009; Gardner et al., 2007; Le Jan, 1996). These immune components of colostrum provide the newborn with a degree of protection against many pathogens and potentially a priming mechanism to initiate development of the neonate's own adaptive immune system (Perkins et al., 2014). Maternal cells in human colostrum are mainly cells of the innate immune system (Peroni et al., 2013). However, mare colostral cells are mostly cells of the adaptive immune system, predominantly a T lymphocyte subgroup with a pro-inflammatory phenotype (Perkins et al., 2014; Perkins & Wagner, 2015). This means that these cells produce high concentrations of pro-inflammatory cytokines, like IFN-γ, upon antigen stimulation (Perkins et al., 2014). More research is needed to determine the role that these maternally-derived colostral leukocytes might play in guiding the development of the foal's immune system.

Innate Immunity

As stated previously, the innate immune system is critical for protecting the young foal from infection, since the adaptive immune response slowly matures over the first few months of life (Bordin et al., 2012). The innate immune response is the initial host response to antigen and is key for providing signals to initiate an effective adaptive immune response (Murphy et al., 2012). While functional at birth, the innate immune response of the neonatal foal still differs from that of older foals and is initially highly dependent on the maternally transferred immune factors (like antibodies and complement) to enhance opsonization and subsequent phagocytosis of pathogens (Bordin et al., 2012; Cohen, 2014; Cuenca et al., 2013; Gardner et al., 2007). Age-related differences in innate immune cell function in the foal include decreased cytokine gene expression, decreased phagocytosis and opsonic capacities, and altered cell receptor expression (Flaminio et al., 2000; Grondahl et al., 2001; Hoynowski et al., 2007; Ryan & Giguère, 2010). These differences (described in more detail below) have been considered immunologic weaknesses and are considered an important component of *R. equi* susceptibility (Giguère et al., 2011a).

Neutrophils are the most abundant cell type of the innate immune system and the initial responders to infection. Neutrophils have several antimicrobial actions to combat and clear pathogens, including bacterial phagocytosis, production of reactive oxygen species (ROS), cytokines, chemokines, production of antimicrobial peptides, and secretion of extracellular traps (Mantovani et al., 2011). These antimicrobial responses

can be damaging to host tissue, and therefore must be carefully regulated (Margraf et al., 2022; Sheats, 2019). Foal neutrophils have been described as functional at birth and do have a vital role in host defense against *R. equi*, but some studies have shown that functional responses, such as bactericidal activity and opsonic recognition, mature further as the foal ages (Bordin et al., 2012). Toll-like receptors (TLR) found on neutrophils (and on other leukocytes) are important for pathogen recognition and initiation of signaling cascades that activate the immune response. Currently, only TLR8 and TLR9 have been directly studied in foal neutrophils and it is only assumed that other TLRs are present and functional (Bordin et al., 2012; Harrington et al., 2012). Unmethylated short strands of DNA (referred to as CPG motifs) are present on *R. equi* (and other bacteria) and recognized as danger signals by TLR9 in endosomal membranes of mammalian neutrophils (Mutwiri et al., 2003). TLR9 expression in the neutrophils of newborn foals was shown to be equivalent to expression in adult horses, and CPG-induced cytokine expression was not age-dependent (Bordin et al., 2012; Felippe, 2016). These findings suggest neutrophils from newborn foals are capable of phagocytosing bacteria. Interestingly, in the presence of *R. equi*-specific antibodies, neutrophils from foals as young as 3 days of age can phagocytose and kill *R. equi* as effectively as neutrophils from adult horses (Martens et al., 1988; Takai et al., 1986; Yager et al., 1986; Zink et al., 1985). Moreover, neutrophils from foals less than one day of age increase expression of inflammatory cytokines in response to *ex vivo R. equi* exposure (Nerren et al., 2009). Together, these findings indicate that neutrophil function is not deficient in

response to *R. equi* but may still be inadequate to prevent disease without contribution from other aspects of the immune system.

The macrophage is another key innate immune cell. Macrophages, whose precursor is the circulating monocyte, are tissue-resident immune cells. Macrophage functions include clearance of debris and dead cells, engulfing and digesting harmful pathogens, and presenting antigens to cells of the adaptive immune system (Murphy et al., 2012). Macrophages that reside in the pulmonary tissue are called alveolar macrophages (AMϕ) and are of particular importance for the immune response to respiratory pathogens like *R. equi*. Inhalation of *R. equi* is considered the primary route of infection. Therefore, AMϕ are the primary cell type involved in the initial uptake of the bacteria in the lungs (Berghaus et al., 2014). *R. equi* has developed survival strategies that enable it to survive and replicate within macrophages, similar to *Mtb* survival strategies described in Section I. Functional immaturity of AMϕ from neonates in other species has been reported, and includes reduced phagocytosis, diminished oxidative burst, including ROS production, and reduced pathogen clearance (Bakker et al., 1998; Dickie et al., 2009).

In the equine, age-associated changes in macrophage function have been described to a lesser extent. In an *ex vivo* model of *R. equi* infection, macrophages (regardless of cellular lineage) from foals at 3 months of age had lower ability to prevent *R. equi* replication and survival when compared to cells from either younger foals (< 1 week of age, 2 weeks of age, or 1 month of age), older foals (5 months of age), or adult

horses (Berghaus et al., 2014). Additionally, *R.equi* replicated at significantly higher rates in AMϕ compared to peripheral blood monocyte-derived macrophages from foals at all ages and adult horses, but bacterial replication was highest in BAL macrophages in foals 3 to 5 months of age, indicating that age-related functional differences in AMϕ may play a role in *R. equi* susceptibility (Berghaus et al., 2014).

The mechanism behind these age-related functional macrophage differences is not clear. Decreased *R. equi*-associated expression of cluster of differentiation (CD)1 in infected macrophages from both horses and foals is described (Dascher & Brenner, 2003; Pargass et al., 2009). CD1 is a protein expressed on antigen-presenting cells and is known to present mycobacterial lipid antigens to T cells. Decreased CD1 expression on *Mtb*-infected macrophages has similarly been described; suppression of CD1 expression may represent another survival mechanism for this class of bacteria (Pargass et al., 2009). CD1 and major histocompatibility complex (MHC) class II expression is also lower on macrophages from very young foals compared to expression on cells from older foals and adult horses, which may impact the young foal's ability to effectively present antigen to adaptive immune cells (Flaminio et al., 2007; Pargass et al., 2009). More research is needed to determine other age-associated changes in functional mechanisms of macrophages that contribute to survivability and replication of *R. equi* within foal cells.

Dendritic cells are antigen-presenting cells that bridge the gap between the innate and adaptive arms of the immune system. Antigen presentation by dendritic cells

relies on the expression of MHC class II molecules. Antigens are loaded onto MHC class II molecules when dendritic cells phagocytose and digest pathogens. MHC class II then translocates to the surface of the cell and, acting in concert with secondary messenger signaling cascades, presents antigen to leukocytes for adaptive immune system activation. Expression of MHC class II on monocyte-derived dendritic cells from foals was diminished for the first 3 months of life when compared to expression on dendritic cells from adult horses (Flaminio et al., 2009). Additionally, cytokines produced by foal dendritic cells exposed to *Escherichia coli* and *Staphylococcus aureus* were lower than cytokine production by exposed dendritic cells from adult horses (Lopez et al., 2019). However, dendritic cells from foals infected with *R. equi* expressed IL-12 mRNA (a cytokine that stimulates T cell differentiation) comparably to dendritic cells from adult horses (Flaminio et al., 2009). Yet, the diminished MHC class II expression on dendritic cells from young foals limits their capacity to promote T cell maturation (Flaminio et al., 2009). Both cytokine signaling and antigen presentation by dendritic cells are critical initiators of the adaptive immune response. Delayed dendritic cell functional maturity in the foal may be a key factor in the age-window of susceptibility to *R. equi* pneumonia and warrants further research.

Adaptive Immunity

The cells of the adaptive arm of the immune system are called lymphocytes. Lymphocytes develop during gestation and are present in newborn mammals (Hodgins

& Shewen, 2012). However, these lymphocytes are naïve, and, for humans and many veterinary species including horses, it takes several months to respond to stimuli equivalently to responses seen in mature animals (Chase et al., 2008; Flaminio et al., 2000; Giguère & Polkes, 2005; Hodgins & Shewen, 2012).

The initial antigenic response of lymphocytes results in rapid cell proliferation (Murphy et al., 2012). Antigen-specific receptors are present on the surface of lymphocytes (Murphy et al., 2012). For proliferation to occur, lymphocytes need both surface receptor ligand binding and priming signals from cells of the innate immune system. Vast exposure to environmental antigens upon parturition induces mass expansion of lymphocyte populations within the first few months of life for most mammals, with total numbers even surpassing those of adults before expansion stabilizes (Flaminio et al., 2000; Hodgins & Shewen, 2012). Interestingly, expansion of lymphocyte populations in the microenvironment of the lungs of foals occurs at a slower rate than what is observed peripherally (Banks et al., 1999; Flaminio et al., 2000). Slower lymphocyte proliferation within the lungs may be an additional factor to consider in *R. equi* susceptibility, as local lymphocyte responses provide activation signals to alveolar macrophages that facilitate the clearance of intracellular pathogens (Flaminio et al., 2000).

T lymphocytes express an antigen-specific T-cell receptor (TCR) and are divided into subtypes based on accessory cell surface proteins. T-helper (Th) cells are characterized by the surface glycoprotein called cluster of differentiation 4 (CD4) while

cytotoxic-T cells (CTL) express CD8. The TCR and CD4 form a complex that binds to MHC class II on antigen-presenting cells, while the TCR and CD8 bind to MHC class I found on most nucleated cells. In addition to MHC binding, T cells also need to encounter costimulatory signals to become activated (Murphy et al., 2012). Costimulatory signals can be surface receptors, adhesion molecules, or cytokines (Hwang et al., 2020) and are not antigen-specific but are required for the T cell to proliferate, differentiate, and survive (Murphy et al., 2012). These signals enhance MHC-TCR binding and promote activation of downstream immune responses (Hwang et al., 2020), and also help T cell differentiation into T-helper- type 1 (Th1), type 2 (Th2), type 17 (Th17), and regulatory (Treg) subsets (Hwang et al., 2020). These T cell subsets are traditionally defined by the cytokines they secrete (see Figure 2.3).

The primary function of Th1 cells is rapid production of the cytokines IFN-γ and interleukin (IL)-2, and to activate and enhance responses of other immune cells. IFN-γ produced by Th1 cells is critical for macrophage activation to facilitate *R. equi* clearance (Hines et al., 2003). Th1 cell population numbers in foals are equivalent to adults by one week of age (Perkins & Wagner, 2015; Wagner et al., 2010). However, the quantity of cytokines produced by the foal's Th1 cells is greatly diminished for several months when compared to quantities produced by similar Th1 cells from adult horses (Perkins & Wagner, 2015). Insufficient cytokine production by the young foal's Th1 lymphocytes may thus play a crucial role in *R. equi* susceptibility.

The primary function of Th2 cells is activation of B lymphocytes, especially during parasite infections, and is characterized by production of the cytokines IL-4, IL-5, and IL-13 (Murphy et al., 2012). Most newborn mammals have a diminished capacity for Th1 responses with polarization to Th2 responses and production of IL-4 (Sedney & Harvill, 2023). This polarized response in the neonate is believed to balance the development of a robust immune response to harmful antigens while limiting tissue damage caused by inflammatory mediators (Perkins & Wagner, 2015). Interestingly, studies in the foal show a deficit in both IFN-γ and IL-4 production in response to mitogens, suggesting that the immune response of the newborn foal may differ from that of other species (Ryan & Giguère, 2010; Ryan et al., 2010; Wagner et al., 2010). The specific role of Th2 cells in *R. equi* pneumonia is unknown.

Th17 and Treg cells are among the newest T cell subtypes to be characterized, and their differentiation is concentration-dependent on the cytokine transforming growth factor-β (TGF-β). IL-17 cells are generated from Th precursors stimulated with inflammatory cytokines like IL-1β, IL-6, and IL-23, and are dependent on low concentrations of TGF-β and the presence of a retinoic acid receptor (RAR) orphan receptor gamma (RORγ2) (Lawrence et al., 2018; Tsai et al., 2013). Th17 cells are characterized by the production of the cytokine IL-17, which includes six known subtypes and is discussed in more detail below. IL-17 cells also produce IL-22, IL-26, TNF-α, and granulocyte macrophage colony stimulating factor (GM-CSF) (Lawrence et al., 2018). Th17 cells are primarily involved in the immune response to extracellular

pathogens but have also been implicated as being a contributing factor to autoimmunity and associated inflammation (Wagner et al., 2010).

In contrast, high concentrations of TGF-β drive the differentiation of Treg cells (Tsai et al., 2013). Treg cells are characterized by expression of the transcription factor forkhead box P3 (FOXP3) and by the production of IL-10 (Branchett et al., 2024). The general role of Treg cells is immune suppression and downregulation of activated T cells (Schmetterer et al., 2012). Specifically, Treg cells are critical for maintaining peripheral tolerance and preventing the development of autoimmune disease (Vignali et al., 2008). Treg cells are present in foals at birth, but IL-10 production is lower than production found in Treg cells from adult horses (Wagner et al., 2010). It is unclear when Treg cell function in foals reaches maturity. A specific role for Th17 cells and Treg cells during the immune response to *R. equi* has also not been established.

In simple terms, the function of $CD8^+$ CTLs is to recognize and kill damaged or infected cells through a mechanism of CD8 TCR binding to MHC class I that is found on all nucleated cells in mammals. *R. equi*-infected cells can be recognized by CTLs and killed through this mechanism (Harris et al., 2010; Hines et al., 2001; Hines et al., 2003). CTLs of adult horses can recognize *R. equ*i cell wall lipids expressed on the surface of infected macrophages (Harris et al., 2010). In murine infection models, CTL responses were shown to be critical for *R. equi* clearance (Nordmann et al., 1993). There are conflicting data in the literature regarding CTL responses in foals; some show that CTL responses in the foal are equivalent to adult responses by five days of age (Perkins &

Wagner, 2015), while other reports demonstrate delayed CTL responses in foals for several weeks after birth (Felippe, 2016; Harris et al., 2011). One report describes acceleration of *R. equi* specific CTL populations after administration of an *R. equi* oral vaccine (Harris et al., 2011). Further research in this area is also needed.

The other component of the adaptive immune response is the humoral immune response directed by B lymphocytes. B lymphocytes are also characterized by the presence of an antigen-specific cell receptor called the B-cell receptor (BCR). Unlike the TCR on T-lymphocytes, the BCR can also be released from the cell surface. Secreted BCRs are called antibodies [AKA immunoglobulins (Ig)], and antibody production is the primary function of the B lymphocyte. Antibodies are comprised of an antigen-binding component and a signaling component. B cell activation requires antigen recognition and binding of antigen to BCR and costimulatory signals from helper T cells, as shown in Figure 2.3 (Murphy et al., 2012). The type of antigen and source of costimulatory signals determines the B cell response and the class of antibody the B cell produces. B cells can differentiate into plasma cells that produce large quantities of antibody or memory B cells that circulate long-term, providing a fast and robust antibody response upon subsequent antigen encounter (Murphy et al., 2012).

There are five known Ig classes in mammals that are further divided into subclasses that vary by species (Bengtén et al., 2000; Perkins & Wagner, 2015). Immunoglobulins, regardless of class have a four-chain structure consisting of two light chains and two heavy chains held together by disulfide bonds, and a hinge portion

resulting in a structure that looks like a Y (Murphy et al., 2012). Class distinction is based on amino acid variations within the constant region of the heavy chain with smaller variations resulting in subclasses (Bengtén et al., 2000). Immunoglobulins have both an antigen-binding portion (the top of the Y) and a constant region (the trunk of the Y), called the Fc receptor that regulates immune cell function (Murphy et al., 2012). The number of antigen binding sites and effector function differs by class. Immunoglobulin subclass M (IgM) and IgD are present on the B cell surface and serve primarily as the BCR, although IgM is secreted in the early stages of an immune response (Murphy et al., 2012). Activated B cells can undergo isotype switching and produce IgG, IgA, or IgE antibodies (Bengtén et al., 2000). IgG is the most abundant in circulation and is highly effective at supporting phagocytosis of pathogens through opsonization and fixing complement, and can bind to most cells of the immune system through its Fc receptor (Murphy et al., 2012). Mucosal surfaces contain an abundance of IgA which serves to protect these areas from microbe invasion (Murphy et al., 2012). Lastly, IgE is essential for the immune response to parasites and binds to mast cells and basophils but also is involved in allergy (Murphy et al., 2012).

Newborn foals have a limited ability to produce most classes of antibodies; though, low concentrations of IgM and IgG can be found in foal serum pre-suckle (Hart & Wong, 2024). Endogenous antibody production increases in concert with the developing adaptive immune response through exposure to the large quantities of environmental organisms the foal encounters in the first days, weeks, and months of life

(Hart & Wong, 2024). As endogenous antibody production increases, maternal antibodies acquired through mare colostrum begin to decay, resulting in a total antibody nadir in foal serum at approximately 1 month of age (Hart & Wong, 2024). It has been theorized that this nadir represents an important deficiency in the young foal's immunity and contributes to susceptibility to infection in the young foal (Holznagel et al., 2003). Of particular importance is the endogenous production of IgA, the antibody predominantly found in the mucosal surfaces, which is not detected in the foal's airways until after 28 days of age (Sheoran et al., 2000). Additionally, some IgG subtypes are not produced in the foal for up to 4 months and IgE is not produced equivalently to adult horses until well after foals are weaned (Holznagel et al., 2003; Perkins & Wagner, 2015) As discussed in the section under maternal immunity, transfusing foals with *R. equi* hyperimmune plasma during the period where maternal antibodies are decaying has not consistently protected foals from bronchopneumonia; therefore, foal immunity remains an area where more research is warranted (Bordin et al., 2022).

Cytokine Signaling

Cytokines are signaling proteins produced by many cells throughout the body that regulate immune responses through autocrine, paracrine, or endocrine actions (Murphy et al., 2012). Cytokines are usually grouped into the following categories: pro-inflammatory, anti-inflammatory, or regulatory; however, some cytokines have multiple functions and can be categorized in more than one group (Murphy et al., 2012).

Colostrum from humans has been shown to contain cytokines form all three categories (Kim, 2021). Research on maternal cytokine transfer in equines is limited, but some studies have reported measurable quantities of tumor necrosis factor (TNF)-α, IL-6, and IFN-γ (pro-inflammatory cytokines), IL-4 and IL-13, (anti-inflammatory cytokines) and IL-8 (a chemokine important for neutrophil migration) in equine colostrum (Burton et al., 2009; Mariella et al., 2017; Vaske et al., 2024). However, cytokine concentrations in colostrum do not always correlate with quantities in foal serum post-suckle (Burton et al., 2009; Mariella et al., 2017). TNF-α, IL-4, and IL-6 have been shown to increase in foal serum 24 hours after colostrum intake, suggesting maternal passive transfer of these maternal cytokines likely occurs (Burton et al., 2009; Mariella et al., 2017). Thus far, only post-suckle IL-6 concentrations have been associated with decreased disease severity in septic foals (Burton et al., 2009; Mariella et al., 2017; Vaske et al., 2024). Serum IL-4 concentrations in foals post-suckle, however, were so low that they have not been considered relevant (Mariella et al., 2017). IFN-γ concentrations were the highest measured cytokine in mare colostrum but concentrations in foal serum pre- and post-suckling did not differ (Mariella et al., 2017). Therefore, it is unclear how much maternal IFN-γ is transferred via colostrum and, if transferred, what its exact immunological function is in the newborn (Mariella et al., 2017). Furthermore, it is unclear how long any maternal cytokines remain in the foal or what role they play in directing the foal's immune response post parturition. Importantly, circulating concentrations of cytokines in healthy foals from birth to weaning have not yet been reported.

As discussed above, cytokines produced by T-cells are particularly important in regulating the immune response to pathogens and directing the adaptive immune response (Perkins & Wagner, 2015). Several studies indicate that T-cell cytokine production and responsiveness to cytokine signaling is delayed in foals for several months (Breathnach et al., 2006; Wagner et al., 2010). The production of IFN-γ by Th1 cells is critical for the activation of macrophages and protection against intracellular pathogens (O'Garra, 1998). In fact, macrophage clearance of *R. equi* is dependent on cellular IFN-γ production (Hines et al., 2003; Kanaly et al., 1995). Controversy exists about the ability of T-cells from newborn foals to produce this important cytokine. A paper published in 2006 stated definitively that foals were IFN-γ deficient at birth and concluded that the newborn foal Th1-immune response was lacking (Breathnach et al., 2006). This conclusion was based on findings that both circulating and pulmonary leukocytes from foals at birth were unable to express the IFN-γ gene or produce IFN-γ protein when stimulated with mitogens specific for inducing intracellular signaling in T-cells (Breathnach et al., 2006). This work seemed to agree with findings of Th1 cell deficiencies (characterized by lack of IFN-γ production) in newborns of other species (Adkins et al., 2000; Lewis et al., 1986). Breathnach et al., however, did not differentiate functionality of T-cells by subgroups. When age-associated changes in intracellular cytokine production were examined in T-cell subsets from foals, evidence indicated that newborn foals have a functional Th1 cell response similar to that of adult horses by 5 days of age, including the ability to produce IFN-γ (Wagner et al., 2010). In fact, IFN-γ

was produced by both Th1 cells and cytotoxic T-cells from foals, with an apparent Th1-population bias, through 12 weeks of age (Wagner et al., 2010). Similar to the Breathnach study, however, mitogenic-stimulation [phorbol 12-myristate 13-acetate (PMA) and ionomycin] of T-cell cytokine production was quantitatively reduced in cells from foals up to 12 weeks of age regardless of T-cell subtype compared to T cells from adult horses (Wagner et al., 2010). Interestingly, bronchial lymph node cells from foals at approximately one week of age expressed higher IFN-γ mRNA in response to *R. equi* challenge than cells from adults challenged similarly (Jacks et al., 2007). Antigenic-specific T cell responses indicate that foals may be able to mount a protective response to specific pathogens, even if the overall T-cell response of the foal is diminished compared to adult horses (Jacks et al., 2007).

As mentioned before, Th2 responses are predominantly characterized by the production of IL-4 and regulate the production of antibody-mediated responses that are especially important for controlling parasitic infections (Mosmann & Coffman, 1989). Initially, it was assumed that foals were biased to a Th2 response, due to the lack of IFN-γ production in the presence of certain T-cell mitogens, as discussed above (Breathnach et al., 2006). Th2 bias initially seemed to be an important factor in *R. equi* susceptibility, as IL-4 was shown to induce granuloma formation in a mouse model of infection (Kanaly et al., 1996). However, several studies have now shown that Th2 responses in the foal are delayed for several months, even when these Th2 cells are stimulated with mitogens like PMA, ionomycin, or killed adjuvanted vaccines that have been shown to induce Th2

cytokine production in adult horse cells (Ryan & Giguère, 2010; Wagner et al., 2010). Moreover, in an *ex vivo* model of *R. equi* infection of equine monocyte-derived-macrophages, priming cells with IL-4 did not impact intracellular survival or replication of the bacteria (Berghaus et al., 2018).

The regulatory cytokine, IL-10, is produced primarily by monocytes and Treg cells, while its production by other cell types is poorly understood. Within its regulatory role, IL-10 suppresses the functions of macrophages and lymphocytes, dampens immune cell proliferation, and regulates B cell differentiation (Moore et al., 1993). Additionally, IL-10 can suppress the production of pro-inflammatory cytokines, like TNF-α, IL-6, and IFN-γ, by activated macrophages and inhibit production of ROS (de Waal Malefyt et al., 1991; Moore et al., 1993; Pusterla et al., 2006). Also, elevated IL-10 mRNA expression has been associated with non-survival in sick foals (Pusterla et al., 2006). Priming of monocyte-derived macrophages with IL-10 increased intracellular replication of *R. equi* in an *ex vivo* model of infection but was fully reversible in the presence of IFN-γ (Berghaus et al., 2018). If IL-10 is highly expressed by alveolar macrophages from newborn foals, this might contribute to the age-associated susceptibility to *R. equi* (Bordin et al., 2022).

Interleukin-17, and more specifically, the subtype IL-17A, is primarily produced by Th17 cells, but also by innate immune cells located in barrier tissues, such as the lung (Lawrence et al., 2018). In fact, the production of IL-17A is essential for granuloma formation during *Mtb* infection in humans (Okamoto Yoshida et al., 2010). While less

potent alone, in combination with other pro-inflammatory cytokines, IL-17A is a potent stimulator of neutrophil recruitment, phagocytosis, and production of antimicrobial peptides and is important for clearance of extracellular pathogens (Ishigame et al., 2009; Lawrence et al., 2018; Liang et al., 2006; Schwarzenberger et al., 1998). Production of IL-17A in human neonates is lower than adults and this is believed to be a contributing factor to bacterial susceptibility and increased risk of neonatal sepsis in preterm infants (Lawrence et al., 2018). However, excess IL-17A produced in response to bacterial infection can be detrimental and has been associated with tissue damage and death in an experimental sepsis model (Flierl et al., 2008).

The effects of IL-17 in the horse are comparatively unknown. Horses diagnosed with mild asthma have higher IL-17 mRNA expression, and IL-17 was also associated with inhibition of NET formation (Bond et al., 2020; Vargas et al., 2017). The role of IL-17 in equine sepsis has not yet been reported. However, in an *in vitro* monocyte-derived dendritic cell infection model, cells from adult horses produced significantly more IL-17 in response to bacterial stimulation compared to cells from one-day old foals (Lopez et al., 2019). Interestingly, when monocyte-derived dendritic cells from one-day old foals were cultured in the presence of plasma from adult horses, production of IL-17 was significantly higher than in similar cells cultured in serum-free medium (Lopez et al., 2020). These studies suggest that newborn foals may have similar IL-17 deficiencies as human neonates, and this may contribute to age-related susceptibility to bacterial infections.

The interaction of cytokines with cells represents a multifaceted system of both activation and suppression. Therefore, evaluating cytokines individually may not accurately represent the complete immune response picture. Understanding the balance between pro-, anti-, and regulatory cytokines may provide a clearer picture of what is happening within the entire immune system to regulate responses to pathogenic stressors. Cytokine measurements show foals have a significantly higher ratio of IFN-γ^+ lymphocyte/IL-4^+ lymphocyte populations at 6 and 12 weeks of age compared to adults, indicating the Th1 bias discussed earlier (Wagner et al., 2010). In fact, in foals infected with *R. equi*, the IFN-γ/IL-4 mRNA expression ratio was four times greater in bronchial lymph node cells than what was seen in infected adults (Jacks et al., 2007). However, ratios of lymphocytes producing IFN-γ to IL-10 was no different from adults in foals 5 days of age and older (Wagner et al., 2010). It has been suggested that measuring the ratio of pro-/anti-inflammatory cytokine concentrations may have value as a potential indicator of disease severity (Bordin et al., 2022). Additionally, other cytokine combinations have shown value as prognosticators for other infectious disease in horses and humans (Burton et al., 2009; Lam & Ng, 2008). While no single cytokine concentration has been found to be a perfect biomarker for a primary tuberculosis diagnosis in people, research suggests developing a biosignature of several cytokines shows promise as a diagnostic tool (Shaik et al., 2024).

As noted above, there are no established age-related reference values for circulating cytokine concentrations in healthy foals (or adult horses). In fact, data

related to serum cytokine protein concentrations in healthy foals greater than one week of age are lacking (Perkins & Wagner, 2015). Even reference values for cytokine concentrations in healthy human adults have not yet been fully established, and this is complicated by variability between subjects (Li et al., 2021; Monastero & Pentyala, 2017). While establishing cytokine concentration reference profiles in healthy equids would have similar challenges, their usefulness could be beneficial in diseases highly associated with specific cytokines, like IFN-γ and *R. equi* bronchopneumonia.

Recently, a group evaluated four cytokines (IFN-α, IFN-γ, IL-2, and IL-10) as potential biomarkers to predict resolution of *R. equi* infection in foals (Deniz et al., 2024). While the objective was to look for biomarkers to distinguish between foals that require or do not require antimicrobials to resolve *R. equi* infection, the authors only measured cytokines in foals with positive bacterial culture from transtracheal fluid and clinical signs of bronchopneumonia attributed to *R. equi.* Additionally, measurements of the four cytokines were made prior to and 14 days after antimicrobial treatment, and therefore, it is unclear if cytokine production changes were a result of responses to the bacteria or the antimicrobial. The authors concluded that baseline (Day 0 prior to treatment) measurements of cytokines in the serum of foals indicate activation of the immune response to *R. equi* infection, but a conclusion is impossible to corroborate in the absence of established normative protein values (Deniz et al., 2024). Evaluation of cytokine concentrations from birth to weaning in foals naturally exposed to *R. equi* and in relationship to disease development and severity have not yet been reported.

SECTION IV: STEROID HORMONES: CORTISOL AND VITAMIN D

Cortisol

Cortisol, a steroid hormone (structure shown in Figure 2.4), is also a key soluble factor that regulates the immune response. Cortisol is a glucocorticoid that is produced in the zona fasciculata of the adrenal cortex with the primary functions of regulating cellular metabolism, cardiovascular/hemodynamic responses, and immune responses (Chakraborty et al., 2021). The hypothalamic-pituitary-adrenal (HPA) axis regulates the production of cortisol and is activated by physiologic, pathophysiologic and environmental stressors in a negative feedback loop (Figure 2.5). Stress signals from the nervous system activate the hypothalamus to release both corticotrophin-releasing hormone (CRH) and arginine vasopressin into the hypothalamo-hypophyseal portal system connecting the hypothalamus to the anterior lobe of the pituitary gland. These two hormones, particularly CRH, stimulate the anterior lobe of the pituitary gland to release adrenocorticotrophic hormone (ACTH).

ACTH is released into the systemic circulation and stimulates the adrenal gland to synthesize and secrete cortisol (Stewart, 2008). Because it is lipophilic, secreted cortisol circulates in plasma primarily (approximately 90% in adults) bound to cortisol-binding globulin (CBG) or albumin (Lewis et al., 2005; Timmermans et al., 2019). Bound cortisol is unable to cross the cell membrane and is traditionally thought to be inactive, but recent reports describe potential immunoregulation by CBG-bound cortisol through a still-unidentified cell surface receptor on neutrophils (Fratto et al., 2017; Hammond et

al., 1990). In contrast, free unbound cortisol can easily cross the cell membrane. Cortisol then exerts its typical physiological actions through the glucocorticoid receptor (GR) located within the cytoplasm of steroid-target cells (Timmermans et al., 2019). In the cytoplasm of the cell, the GR is a monomer within a chaperone complex (Vandevyver et al., 2012). Upon ligand binding, GR dimerizes, translocates to the nucleus, and regulates transcription of thousands of steroid-responsive genes (Galon et al., 2002). In fact, it is estimated that glucocorticoids regulate up to 20% of the human genome (Galon et al., 2002). The primary result of cortisol-mediated gene regulation is to reduce the physiological stress signals that activated the HPA axis and return the body to homeostasis (Hart & Barton, 2011).

Immune Modulation by Cortisol

Cortisol is a powerful regulator of the inflammatory response from the time of initial danger signals released by infected cells to the resolution of inflammation and clearance of cellular debris (Cain & Cidlowski, 2017). Initially, pattern recognition receptor ligand binding on innate immune cells initiates a cascade of inflammatory mediators that initiate and direct the immune response and are regulated by cortisol. Downstream to initial immune signaling, cortisol attenuates the expression of many pro-inflammatory cytokines including IFN-γ and TNF-α, and even reduces the half-life of TNF-α mRNA (Bhattacharyya et al., 2011).

Leukocytes are recruited to sites of infection through chemotaxis, another important aspect of the initial inflammatory cascade. Cortisol downregulates the production of chemotaxis signals and modulates leukocyte adhesion molecules to reduce leukocyte infiltration into tissues (Cronstein et al., 1992; Heasman et al., 2003; Pitzalis et al., 2002). Cortisol stimulates macrophages to produce the anti-inflammatory cytokine, IL-10, and phagocytize apoptotic cells and debris during the resolution phase of inflammation (Cain & Cidlowski, 2017; Giles et al., 2001). Importantly, dose-dependent cortisol regulation of monocytes and macrophages has also been reported in human cells, with higher concentrations having suppressive effects and lower concentrations having stimulatory effects (Chakraborty et al., 2021).

Cortisol induces apoptosis in dendritic cells and suppresses their activation by reducing MHC II expression and inhibiting migration into the lymph nodes for antigen presentation (Kitajima et al., 1996; Moser et al., 1995; Piemonti et al., 1999). Cortisol also induces a tolerogenic phenotype in dendritic cells, suppressing dendritic cell maturation and effector functions (Chakraborty et al., 2021) (Rutella et al., 2006).

Cortisol regulates $CD4^+$ T-cell responses either indirectly by modulation of dendritic cells as described above, or directly by influencing the maturation and differentiation of T-cells in the thymus (Baschant & Tuckermann, 2010). Cortisol can also interfere with T-cell receptor signaling, proliferation, antigen-specific cytokine production, and promote lymphocyte apoptosis (Baumann et al., 2005; Cain & Cidlowski, 2017; Elenkov et al., 1996; Lowenberg et al., 2006). Cortisol preferentially

suppresses the response of Th1 cells and promotes the function of Th2 cells by modification of cytokine production by dendritic cells and attenuation of cytokine receptor expression on T-cells (Cain & Cidlowski, 2017).

As indicated above, dose-dependent effects of cortisol have been demonstrated. Overall, high concentrations of cortisol suppress the pro-inflammatory response, and lower concentrations enhance gene expression of surface receptors on immune cells (Chakraborty et al., 2021). One theory suggests that, in the absence of inflammation, normal cortisol rhythms enhance immune receptor expression facilitating quick cellular activation. However, during inflammation, cortisol acts to temper responses, reducing the risk of inflammatory mediated tissue damage (Cain & Cidlowski, 2017).

HPA axis dysregulation can occur in diseases that result in inflammation (Jones & Gwenin, 2021). During the immune response to *Mtb*, inflammatory cytokines function as a stress signal to stimulate the HPA axis and produce cortisol. Indeed, patients with active *Mtb* infections have increased plasma cortisol concentrations (Bozza et al., 2007). *In vitro* assays show cortisol inhibits T-cell proliferation and decreases IFN-γ production in response to *Mtb* stimulation (Mahuad et al., 2004). Direct associations between cortisol and diminished cytokine concentrations in patients with active *Mtb* infection have not been established (Bozza et al., 2007). However, the ratio of cortisol to dehydroepiandrosterone (DHEA) was negatively correlated with IFN-γ production in *Mtb* patients with lower cortisol/DHEA rations correlating with higher IFN-γ concentrations (Bozza et al., 2007). This suggests that steroid hormones may play a key role in

regulating the immune response to *Mtb*, as DHEA is a cortisol-related hormone primarily produced in the adrenal gland and in very simplistic terms, opposes the effects of cortisol (Kroboth et al., 1999).

Cortisol in the Horse

The production and secretion of cortisol in the horse is regulated by the HPA axis similarly to what is described in other species (Alexander et al., 1996; Silver et al., 1987). Basal concentrations of cortisol and concentrations produced in response to exogenous ACTH stimulation are also similar in healthy adult horses when compared to cortisol concentrations reported in other mammalian species (Bousquet-Melou et al., 2006; Martin et al., 2007; Stewart, 2008). In contrast to adult horses and newborns of other mammals, maturation of the HPA axis of the foal occurs much later, just before parturition and during for the first few weeks of life (Ousey, 2004; Silver & Fowden, 1994). The length of time it takes to reach HPA axis maturity in the foal is unknown. The immaturity of the HPA axis is evidenced by elevated ACTH concentrations and low basal cortisol concentrations in newborn foals (Hart et al., 2009a; Hedberg et al., 2007; Irvine & Alexander, 1987). In fact, exogenous ACTH administration to the foal does not result in the same cortisol secretion seen in adult horses (Bousquet-Melou et al., 2006; Hart et al., 2009a). This indicates that the HPA axis immaturity in the foal is at the level of the adrenal gland, with foals having either decreased sensitivity to ACTH or limited cortisol synthetic capacity or both (Hart & Barton, 2011; Hart et al., 2011). The immature HPA

axis of the foal may impact its ability to respond to physiologic or environmental stress in a manner comparable to that of adult horses, making them more susceptible to disease and immune dysregulation during the first few weeks of life (Hart et al., 2011).

Another unique feature of cortisol in the foal is higher concentrations of free (unbound) cortisol in circulation as compared to adult horses (Hart et al., 2011). In healthy adult horses, bound cortisol represents 80-90% of all cortisol in circulation. In the foal, bound cortisol represents less than 50% of total cortisol in the newborn and is still significantly less than adult horses at one week of age (Hart et al., 2011; Irvine & Alexander, 1987). This increase in unbound cortisol in the neonatal foal is most likely a result of decreased CBG and a reflection of decreased cortisol binding capacity (Hart et al., 2011). Free cortisol is preferentially metabolized in the liver and easily eliminated by the kidneys as compared to bound cortisol, resulting in rapid cortisol clearance and potentially leading to cortisol insufficiency during stress or illness (Bright & Darmaun, 1995; Hart et al., 2011). Additionally, cortisol immunomodulation is dose-dependent, and high concentrations of free cortisol may attenuate inflammatory signals, inhibiting the foal from effectively initiating the inflammatory cascade needed for bacterial clearance.

HPA axis immaturity and deficiencies in CBG binding capacity may impact the ability of the newborn foal to respond efficiently and effectively to bacterial pathogens, and are associated with disease severity in the septic neonatal foal (Dembek et al., 2017; Hart et al., 2009b). Since *R. equi* exposure and assumed infection occurs during

the first few days of life, these unique endocrine features of the newborn foal may also contribute to the development of *R. equi* disease. The role of cortisol, if any, in naturally occurring *R. equi* pneumonia has not yet been described.

Cortisol has been shown to modulate equine leukocyte function in an age-associated manner. When exposed to cortisol, monocyte-derived dendritic cells from adult horses expressed less MHC class II and produced less cytokines (IL-4, IL-17, IL-10, and IFN-γ) following bacterial antigen exposure (Lopez et al., 2024). Foal monocyte-derived dendritic cells were also impacted by cortisol resulting in decreased IL-4 and IL-10 production (Lopez et al., 2024).

Both free and CBG-bound cortisol decreases equine neutrophil function (Fratto et al., 2017). Specifically, CBG-bound cortisol reduced ROS production while increasing IL-8 production in neutrophils from foals and adults, but the increase of IL-8 was significantly higher in foals than in adults (Fratto et al., 2017). The effects of cortisol on other equine immune cells have not been determined.

Vitamin D

Vitamin D is also a steroid hormone that has immunoregulatory properties; its structure can be seen in Figure 2.4. Vitamin D deficiency or insufficiency is considered very common in people and has been associated with morbidity and mortality of many diseases, including infectious diseases like COVID-19, autoimmune-related diseases like inflammatory bowel disease and bacterial sepsis (Amrein et al., 2020; Johnson &

Thacher, 2023). Importantly, vitamin D deficiency is strongly associated with active *Mtb* infection in both adults and children (Buonsenso et al., 2018; Gois et al., 2017). Vitamin D deficiency has also been associated with disease severity in other respiratory infections and inflammatory pulmonary conditions like asthma (Amrein et al., 2020).

In people and most grazing animals, (but perhaps not the horse, as discussed later in this section) the majority of vitamin D requirements are met via ultraviolet light obtained through exposure to sunlight, with smaller amounts obtained through dietary sources, like fish and milk (Smith & Wright, 1984b; Uhl, 2018). Through UV and heat conversion of a cholesterol precursor or dietary sources, prohormones (generally referred to as vitamin D_2 and vitamin D_3) must go through a series of hydrolysis steps prior to becoming biologically active (see Figure 2.4) (Gil et al., 2018). Vitamin D metabolism and subsequent downstream effects (discussed below) are complex and involve multiple steps and body systems (Figure 2.6). The first hydrolysis step converts the prohormone D_2 or D_3 into 25,hydroxyvitamin D [25(OH)D] and is presumed to solely occur in the liver (Bikle, 2014). 25(OH)D is the inactive form of vitamin D and the predominant metabolite in circulation. The plasma half-life of 25(OH)D is thought to be around 3 weeks (Gil et al., 2018). Because of its stability and long half-life, 25(OH)D is the metabolite most commonly measured as a determinant of vitamin D heath status in people and animals (Casas et al., 2015; Dittmer & Thompson, 2011; Holick, 2008).

Vitamin D Endocrine Function

The second hydrolysis step occurs primarily in the kidney for the purpose of maintaining calcium and phosphorus homeostasis as its primary endocrine activity (Gil et al., 2018; Naveh-Many & Silver, 1990). This second hydrolysis step results in the biologically active form of vitamin D: 1, 25-dihydroxyvitamin D [$1,25(OH)_2D$]. Hydrolysis of $1,25(OH)_2D$ is catalyzed by the enzyme 1,α-hydroxylase (CYP27B1). Regulation of $1,25(OH)_2D$ hydrolysis is tightly controlled by three hormones - parathyroid hormone (PTH), fibroblast growth factor 23 (FGF23), and $1,25(OH)_2D$ itself - and by serum concentrations of calcium, phosphate, and $1,25(OH)_2D$ (Bikle, 2014). Low concentrations of circulating calcium, phosphate or vitamin D stimulate production of the PTH by the parathyroid glands (Serna & Bergwitz, 2020). PTH then initiates production of cytochrome P450 enzymes in the kidney to increase the synthesis of $1,25(OH)_2D$. Synthesis of $1,25(OH)_2D$ occurs in the mitochondria of the proximal convoluted tubule cells of the kidney (Gil et al., 2018). PTH also inhibits the production of 24-hydroxylase (CYP24A1), the enzyme responsible for degrading $1,25(OH)_2D$ (Gayan-Ramirez & Janssens, 2021). PTH, phosphorus, and $1,25(OH)_2D$ induce the production of fibroblast growth factor 23 (FGF23) by osteocytes and osteoblasts in bone (Serna & Bergwitz, 2020). Together these actions increase phosphate secretion and calcium reabsorption in the kidney and increase calcium absorption in the intestinal tract. (Gayan-Ramirez & Janssens, 2021). When calcium and phosphorus homeostasis has been reached,

CYP24A1 degrades excess 1,25$(OH)_2$D for biliary excretion (Gil et al., 2018). The half-life of 1,25$(OH)_2$D is only a few hours (Quraishi & Camargo, 2012).

The downstream cellular effects of 1,25$(OH)_2$D on target tissues are mediated by the VDR as discussed in Section I. The VDR is present in most cells of the body and, like other steroid hormone's receptors, are initially located in the cytoplasm of the cell (Deluca, 2014). Upon ligand (1,25$(OH)_2$D) binding, the VDR dimerizes with retinoid X receptor (RXR), translocates to the nucleus, and subsequently regulates the transcription of hundreds of genes (FeldmanD, 2013). Genes regulated by the vitamin D/VDR pathway are called vitamin D response elements (VDRE) and are important in many different tissues throughout the body (Deluca, 2014). VDRE are very tissue specific, and include osteocalcin produced by bone cells, cathelicidins antimicrobial peptide (CAMP), β-defensins, and CD14 (both critical for clearance of intracellular bacteria produced in macrophages), and fructose-1,6 diphosphatase (involved in cellular metabolism) (Carlberg, 2022).

Immune Modulation by Vitamin D

As stated above, the VDR is present in most cells of the body, including those of the immune system (Haussler et al., 1998). The discovery of the VDR in human leukocytes transformed vitamin D research, as it implicated a novel immunomodulatory role for this hormone (Provvedini et al., 1983). Thousands of reports have subsequently confirmed the importance of vitamin D actions within immune cells. In fact, many

immune cells, including human alveolar macrophages, even contain the hydrolyzing enzyme, CYP27B1, and directly synthesize 1,25$(OH)_2$D within the microenvironment of the cell (Adams et al., 1983; Bikle, 2009). 1,25$(OH)_2$D synthesis within immune cells is initiated by cytokine signaling and cell-surface receptor binding (Baeke et al., 2010). Local 1,25$(OH)_2$D synthesis within immune cells decreases the dependence on circulating concentrations of 1,25$(OH)_2$D; however, the availability of adequate 25(OH)D substrate remains crucial (Baeke et al., 2010).

Human immune cells, including macrophages, dendritic, and epithelial cells, but not neutrophils, express CYP27B1 for local 1,25$(OH)_2$D synthesis upon activation. T cell subsets ($CD4^+$ T cells and $CD8^+$ T cells) express both the VDR and CYP27B1, regardless of activation status (Bikle, 2022; Sirbe et al., 2022; Wang et al., 2012).

Vitamin D activity in immune cells regulates and modulates both innate and adaptive arms of the immune response in a time-dependent manner with an overall role of downregulation and inhibition of the pro-inflammatory response, as described in more detail below (Bikle, 2009; Bikle, 2022). TLR ligand binding in innate cells increases expression of both VDR and CYP27B1, resulting in local synthesis of active vitamin D (Bikle, 2022). Initially, during the response to bacteria, vitamin D immune regulation is more pro-inflammatory resulting in the upregulation of pattern recognition receptor expression on innate immune cells and production of critical antimicrobial peptides, including cathelicidins and defensins (Bikle, 2022; Martineau et al., 2007). Vitamin D also initially increases phagocytic and chemotactic capacities and promotes cellular

proliferation of innate cells (Bikle, 2022; Gauzzi et al., 2005; Lyakhovich et al., 2000; Pedersen et al., 2009). However, after this initial response, vitamin D blocks NF-kb nuclear translocation and the normal signaling cascade of proinflammatory cytokines, while upregulating the production of IL-10 and antioxidants (Gil et al., 2018; Gonzalez-Pardo et al., 2012). To prevent tissue damage that can occur as a result of chronic immune cell activation, vitamin D also decreases expression of TLR-2, -4 and -9 in the later stages of infection (Bikle, 2022; Khashim Alswailmi et al., 2021). Similar to cortisol, vitamin D-mediated regulation of the immune response is also concentration-dependent (Sadeghi et al., 2006).

As discussed in Section I, within human macrophages, vitamin D is essential for production of cathelicidins, a class of antimicrobial peptides critical for *Mtb* clearance (Figure 2.1) (Gombart et al., 2009; Wang et al., 2004). Additionally, within epithelial cells, an often overlooked but very important component of the innate immune system, vitamin D enhances production of cathelicidins resulting in increased barrier function (Wei & Christakos, 2015). This is especially important in the intestinal epithelium, as vitamin D depletion is associated with increased gut permeability, metabolic endotoxemia, and low-grade inflammation (Caricilli et al., 2011).

The adaptive immune response, including T-cell proliferation and differentiation, is also suppressed by the actions of vitamin D which inhibits the differentiation of Th1 and Th17 cells while promoting a shift to Th2 and Treg phenotypes (Bikle, 2022; Sadeghi et al., 2006). Vitamin D regulation of T cell differentiation is through dendritic cells.

Specifically, vitamin D reduces dendritic cell maturation, thus impacting their ability to effectively present antigen (Bikle, 2022). Vitamin D also influences cytokine signaling by inhibiting production of IL-12, IL-23, and IL-17, necessary for Th1 and Th17 development, and reduction of IL-2, which is important in T cell proliferation (Boonstra et al., 2001; Jeffery et al., 2009). Consequently, this shifts the T cell balance to a Th2 phenotype and an increase in Treg cells, resulting in more IL-10 and a downregulation of the adaptive immune response (Mahon et al., 2003; Tang & Meng, 2009). Within T cells, the expression and subsequent production of pro-inflammatory cytokines like TNF-α and IFN-γ are directly inhibited by vitamin D, as genes for both cytokines have a VDRE within their promoter region (Bikle, 2022). Vitamin D also directly inhibits B-cell proliferation, differentiation, and immunoglobulin class switching and induces B-cell apoptosis (Chen et al., 2007).

Vitamin D in the Horse

Relative to humans and other mammalian species, much less is known about vitamin D in the horse (Hymoller, 2015). However, several important species-associated differences in vitamin D metabolism and function have been identified (Azarpeykan et al., 2016a; Dosi et al., 2023; Harrington & Page, 1983; Hymoller, 2015; Smith & Wright, 1984a).

First, in other species, vitamin D is the most important regulator of calcium and phosphorous concentrations to maintain bone health, but vitamin D may not have the

equivalent role in the horse (Gil et al., 2018; Pozza et al., 2014). Horses have very low concentrations of serum vitamin D and phosphorus, coupled with very high concentrations of serum calcium and greater rates of calcium absorption in the small intestine compared to most other mammalian species (Breidenbach et al., 1998; Uhl, 2018; Wilkens et al., 2017). Horses also have increased urinary excretion of calcium and lower PTH sensitivity to changes in serum calcium concentrations (Toribio et al., 2003; Toribio et al., 2001). Administration of supraphysiological doses of vitamin D resulted in only small increases in serum calcium concentrations in horses, indicating vitamin D-independent calcium homeostasis likely occurs (Harmeyer & Schlumbohm, 2004). In contrast, calcium transport genes in the equine kidney are highly correlated with VDR expression and the enzymes that control vitamin D synthesis, indicating that vitamin D does still have some role in calcium homeostasis in the horse (Azarpeykan et al., 2016b; Rourke et al., 2010)

Secondly, dietary vitamin D_2 may be the primary source of prohormone vitamin D in horses. As already mentioned, vitamin D can either be obtained through UV exposure through the skin (vitamin D_3) or from diet (vitamin D_2 and D_3). In people, vitamin D_3 obtained through the skin has a longer half-life, and its hydrolysis results in a more physiologically potent metabolite compared to dietary vitamin D_2 (Armas et al., 2004; Jones et al., 2014; Romagnoli et al., 2008). It was assumed that vitamin D_3 would be the predominant metabolite in horses, as it is in other grazing animals. Smith and Wright first proposed in 1984 that vitamin D_3 might be limited in the horse due to the

lack of seasonal variability of serum vitamin D concentrations (as seen in sheep), and several recent studies have corroborated this finding (Breidenbach et al., 1998; Dosi et al., 2023; El Shorafa et al., 1979; Pozza et al., 2014; Smith & Wright, 1984a).

When the effect of horse blanketing on vitamin D metabolism was analyzed using ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) to carefully distinguish between the prohormone forms of vitamin D_2 (dietary) and vitamin D_3 (acquired through UV conversion in the skin) in equine samples, the concentration of vitamin D_3 was undetectable regardless of blanketing coverage (Azarpeykan et al., 2016a). In fact, Dosi *et al*, using similar technology, reported that vitamin D_3 was only detectable in horses given dietary vitamin D_3 supplementation (Dosi et al., 2023). Furthermore, skin samples from Thoroughbred horses exposed directly to UVB light were unable to convert 7-dehydrocholesterol (7-DHC) to vitamin D_3 as was seen in skin from other grazing animals (Azarpeykan et al., 2022). Interestingly, horses supplemented with extremely high doses of vitamin D_3 (10,000 IU per kg/daily for four days) maintained normal concentrations of serum $1,25(OH)_2D$, further indicating that vitamin D metabolism may be unique in the equid (Harmeyer & Schlumbohm, 2004).

Lastly, circulating concentrations of both active and inactive vitamin D metabolites are lower in healthy horses than found in other mammalian species (Harrington & Page, 1983; Maenpaa et al., 1988a). In fact, vitamin D metabolite concentrations in healthy horses are even lower than concentrations found in individuals from other mammalian species diagnosed with hypovitaminosis D-associated

diseases (Maenpaa et al., 1988b). Additionally, rickets, a known vitamin D deficiency-linked disease, is rarely found in horses and is even hard to induce in experimental models (Dittmer & Thompson, 2011; Wilkens et al., 2017). The reason for this species-associated peculiarity is unknown.

Age-associated changes in circulating vitamin D concentrations in human infants and babies have been described and indicate that vitamin D sufficiency differs during development (Mansbach et al., 2009; Zhang et al., 2024). This finding is not surprising, as vitamin D is critical in regulating serum calcium and phosphorus concentrations necessary to maintain the healthy bone growth and development that is especially important in young animals (Gil et al., 2018; Holick, 2007). Limited age-related changes have been described in serum concentrations of vitamin D in foals. The inactive vitamin D metabolite is lower in circulation in foals less than 2 months of age, as compared to yearlings or adult horses, but it is unclear when foals reach concentrations equivalent to those seen in adult horses (Pozza et al., 2014). Concentrations of $1,25(OH)_2D$ have only been reported in newborn foals with the specific aim of determining if hypovitaminosis D correlates with morbidity and mortality in sick neonatal foals. Sick foals (septic or non-septic) with the lowest concentrations of vitamin D were less likely to survive (Kamr et al., 2015). Low vitamin D concentrations may be a result of poor nursing in sick foals, but further research is warranted to determine if vitamin D insufficiency plays a role in dysregulation of the inflammatory response.

Currently, serum concentrations of 25(OH)D or 1,25$(OH)_2$D in healthy foals from birth to weaning, and in foals naturally or experimentally exposed to *R. equi*, have not been described. It is also unknown if vitamin D metabolite concentrations correlate with the development and severity of *R. equi* pneumonia in foals, as they do with similar *Mtb* infections in people (Chan, 2000; Iftikhar et al., 2013). Furthermore, the VDR has only been characterized in equine kidney and gastrointestinal tract cells, in relation to calcium regulation, and expression has not been examined in equine immune cells (Azarpeykan et al., 2016b; Rourke et al., 2010). It is also unknown if VDR expression is impacted by *R. equi* infection of equine macrophages, as is seen in human macrophages infected with *Mtb*.

The Vitamin D and Cortisol Connection

The relationship between vitamin D and cortisol is complex, highly intertwined, and ambiguous. During illness or infection, cortisol concentrations are often elevated, while vitmain D deficiency contributes to disease susceptibility and severity, indicating a possible negative association between these two steroid hormones (Holick, 2008; Yareshko & Kulish, 2022). The structural similarities of these hormones and their relationship to cholesterol can be seen in Figure 2.4. During acute stress, in order to restore homeostasis, cholesterol is preferentially utilized for the production of cortisol and aldosterone (a mineralcorticoid hormone produced in the adrenal cortex), at the expense of other adrenal hormones, however, the direct effect of acute stress on

vitamin D production is unknown (Gak et al., 2015). Additionally, during acute stress, the energy demands of many cells are increased, which involves activation of mitochondria, the organelle where hydrolysis of the active vitamin D metabolite occurs (Manoli et al., 2007). Thus, it is possible that during stress, the overall metabolism of vitamin D may be impacted. In a large population-based study of human patients receiveing glucocorticoid therapy for many diseases, there was a significant association with reduced serum 25(OH)D concentrations, further indicating that cortisol availability and vitamin D metabolism may be intertwined (Skversky et al., 2011)

Interestingly, both stress and the administration of exogenous corticosteroids are known contributors to the reactivation of *Mtb* infections (Baker et al., 2000; Haanaes & Bergmann, 1983). In fact, in patients with active tuberculosis the cortisol/cortisone ratio in bronchoalveolar lavage fluid (BAL), but not plasma, was significantly higher than in healthy controls. These data suggest that cortisol metabolism within the infected lung is preferentially driven to favor active cortisol metabolism (Baker et al., 2000). Concentrations of 25(OH)D in BAL fluid from patients with pulmonary tuberculosis are also lower than healthy controls, but concentrations of $1,25(OH)_2D$ have not been described (Ramirez-Ramos et al., 2021). It is possible that increased concentrations of cortisol within the microenvironment of the lung suppress vitamin D metabolism in alveolar macrophages during infection, and consequently reduce production of antimicrobial peptides, thus contributing to *Mtb* infectivity.

There also seems to be an interplay between the nuclear receptors of cortisol and vitamin D. VDR receptor signaling in the T cells of mice was dependent on the presence of a functional glucocorticoid receptor (Bagnoud et al., 2023). In neonatal rats, evidence shows that hydrocortisone injection induced intestinal VDR mRNA expression (Lee et al., 1991). However, cortisol has also been shown to suppress VDR gene expression in human adipocytes (Lahnalampi et al., 2010). Interestingly, vitamin D supplementation may also modulate the expression of glucocorticoid receptor isoforms by upregulating the expression of glucocorticoid receptor -α and enhancing the responsiveness to steroid treatment in asthmatic patients (Mahboub et al., 2021). Insufficient evidence currently exists to interpret the importance of this nuclear receptor interplay; however, based on their structural and functional similarities it is easy to theorize that they might influence the activation and activities of each other (Bagnoud et al., 2023). This warrants further investigation, especially when we consider that vitamin D and steroid hormones are common therapeutics recommended in human medicine.

Finally, there is also evidence that $1,25(OH)_2D$ regulates enzymes required for steroidogenesis and plays a role in adrenal disease. Using an adrenocortical cell line, $1,25(OH)_2D_3$ added to media significantly downregulated mRNA expression of 21-hydroxylase (an enzyme required for the production of cortisol and other hormones produced in the adrenal gland) (Lundqvist et al., 2010). In human medicine, there is evidence that the vitamin D/VDR pathway may play a role in the pathogenesis of several

adrenal-based human diseases such as Cushings syndrome, adrenal cortex tumors, Addison's disease, and hyperaldosteronism (Al Refaie et al., 2022).

The relationship between vitamin D and cortisol in the horse is even less clear. Vitamin D and cortisol concentrations have only been reported together in relation to their role in osteochondrosis, a disorder of mineral imbalance and endocrine dysfunction, but direct association between these hormones has not been described (Sloet van et al., 1999). Separate reports show evidence of increased cortisol concentrations and decreased vitamin D concentrations in septic newborn foals, suggesting that a negative correlation may exist between these two steroid hormones during illness in foals as well (Gold et al., 2007; Hart et al., 2009a; Kamr et al., 2015).

SECTION V: JUSTIFICATION FOR THE STUDIES HEREIN

As discussed above, *R. equi* pneumonia impacts the equine industry worldwide and currently an effecive vaccine or preventative is not available (Bordin et al., 2022). Antimicrobial-resistant *R. equi* isolates are increasing worldwide, and alternatives to our preferred antimicrobial therapies for foals with clinically apparent pneumonia are lacking (Bordin et al., 2022; Havemeyer Workshop on Rhodococcus equi Scientific Advisory, 2024). Additionally, we do not have a clear understanding if there are specific immune limitations within individual foals that impact their susceptibility to this disease.

Much of what we know about the pathogenicity of – and immune response to – *R. equi* was first garnered through the vast literature on *Mtb* (Goodfellow & Alderson,

1977; von Bargen & Haas, 2009). However, essential information regarding the equine immune response to *R.equi* is still lacking. Specifically of consequence to this body of work, a strong relationship exists between increased cortisol, vitamin D deficiency, and active *Mtb* infection in humans (Bottasso et al., 2007; Iftikhar et al., 2013; Mahuad et al., 2004; Rey et al., 2007). Importantly, *Mtb* clearance within human macrophages is contingent on immune regulation by the vitamin D/VDR pathway (Selvaraj, 2011; Wu & Sun, 2011). Immune responses mediated through the vitmain D/VDR pathway have not yet been explored in equine leukocytes. The similarities between *Mtb* and *R. equi* indicate that a relationship between vitamin D, cortisol, and bronchopneumonia caused by *R. equi* might also exist in the horse.

Age-associated differences in both vitamin D and cortisol concentrations have been described in neonatal foals infected with *Escherichia coli*, *Streptococcus species*, and other bacteria that can cause neonatal sepsis (Hart & Barton, 2011; Kamr et al., 2015). Given that foals encounter *R. equi* in the environment during the first few days of life, it is possible that these age-associated differences early in life play a role in directing the ultimate immune repsonse to this bacterium. Previous immunological studies have failed to identify a specific immune parameter that might predict whether an individual foal will remain healthy, resolve infection spontaneously, or become sick and need veterinary intervention after exposure to *R. equi*. However, the interplay between steroid hormones and immune regulation to *R. equi* within the foal has previously not been investigated. Therefore, the studies described in Chapters 3 through

5 were conducted to accomplish the following objectives: (1) assess the expression of the VDR in equine alveolar macrophages; (2) investigate age-associated differences in VDR expression in equine alveolar macrophages; (3) determine if the VDR within alveolar macrohages is altered by *R. equi* infection in an age-dependent fashion; (4) investigate the association between circulating steroid hormone (cortisol and vitamin D) concentrations, foal age, and disease during naturally occuring *R. equi* exposure; (5) evaluate circulating concentrations of cortisol, vitamin D, cytokines, and combinations of cytokines from birth to weaning as potential predictors of *R. equi* pneumonia susceptibility and severity in foals on a farm endemic for the disease.

SECTION VI: REFERENCES

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FIGURES

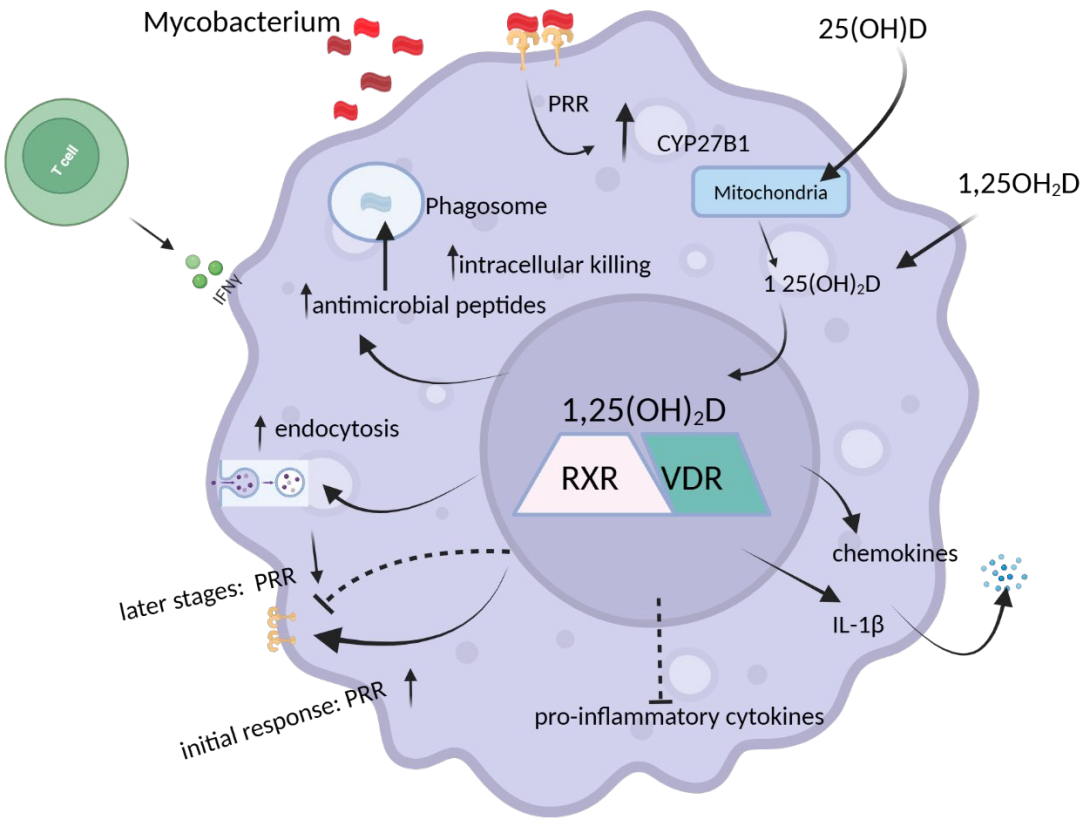

Figure 2.1. Vitamin D/vitamin D Receptor (VDR) pathway in human macrophages during the immune response to *Mycobacterium tuberculosis* (*Mtb*). Binding of *Mtb* to pattern recognition receptors (PRR) on interferon gamma (IFN-γ)-activated macrophages results in upregulation of the vitamin D receptor (VDR) and 1,α hydroxylase (CYP27B1), the enzyme required for hydrolysis of the active vitamin D (1,25$(OH)_2$D) metabolite within the mitochondria of the cell. The ligand-bound VDR dimerizes with the retinoid X receptor (RXR) and moves to the nucleus to up- or downregulate transcription of genes involved in the production of chemokines, cytokines, PRR, and antimicrobial peptides to enhance bactericidal activities within the macrophage. Stimulatory interactions are illustrated with solid arrows and inhibitory interactions are illustrated with dashed lines with bars. Illustration created with BioRender.com and adapted from Ismailova et al. 2022 (Ismailova & White, 2022).

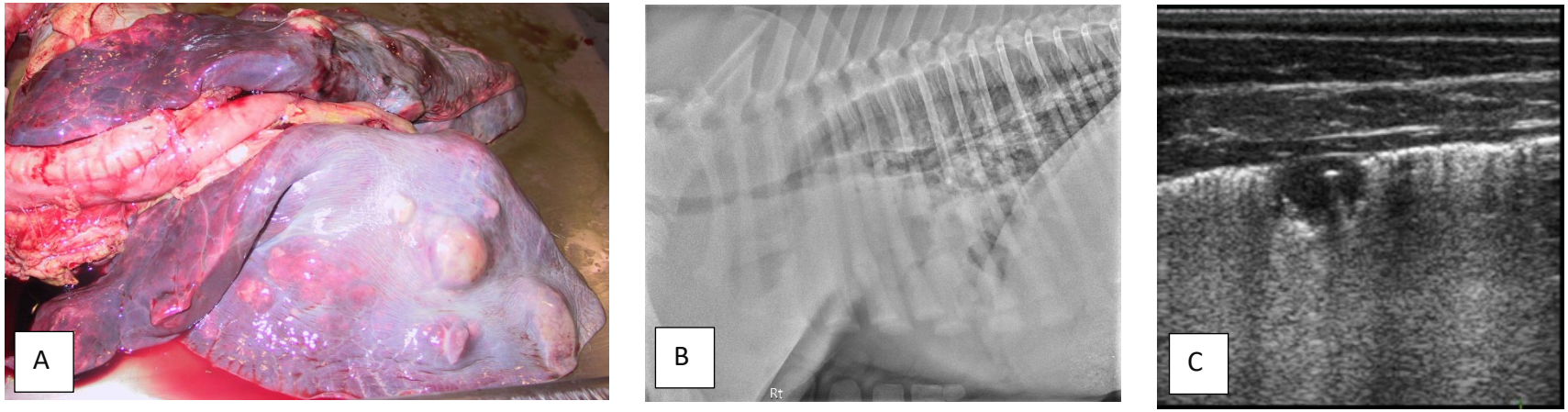

FIGURE 2.2. Images of foal lungs with pyogranulomatous pneumonia caused by *Rhodococcus equi* (*R. equi*). (A) Lungs from a foal that died of *R. equi* bronchopneumonia with areas of consolidation and abscessation. (B and C) Radiographic (B) and ultrasonographic (C) images of lesions caused by *R. equi* in a live foal. Photos courtesy of Drs. Steeve Giguère and Erin Beasley.

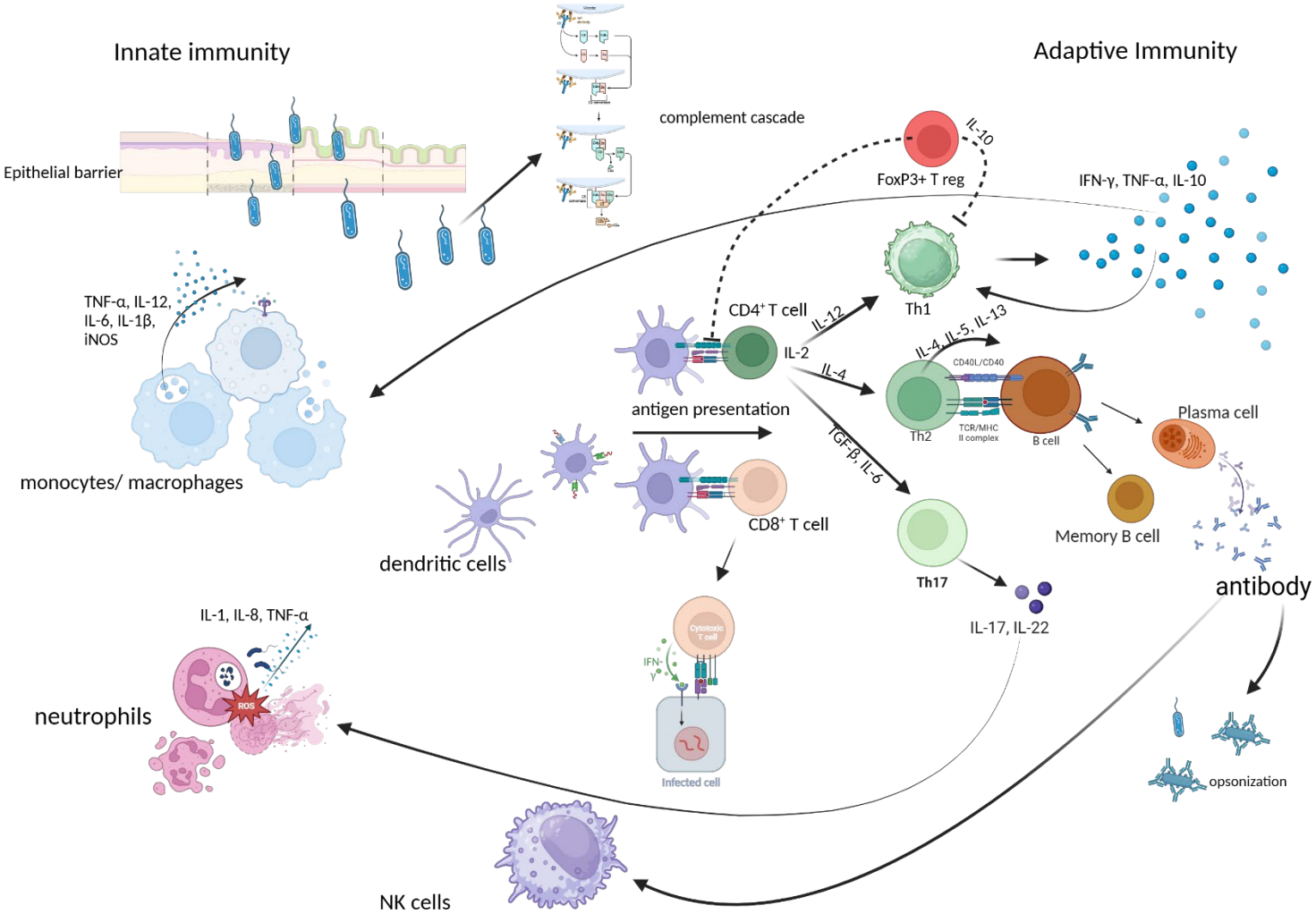
Innate immunity
Adaptive Immunity
Epithelial barrier
complement cascade
FoxP3+ T reg
IL-10
IFN-γ, TNF-α, IL-10
TNF-α, IL-12,
IL-6, IL-1β,
iNOS
CD4⁺ T cell
IL-2
IL-12
Th1
IL-4, IL-5, IL-13
IL-4
CD40L/CD40
Th2
TCR/MHC
II complex
B cell
Plasma cell
Memory B cell
antigen presentation
TGF-β, IL-6
monocytes/ macrophages
dendritic cells
CD8⁺ T cell
Th17
IL-17, IL-22
antibody
IL-1, IL-8, TNF-α
ROS
neutrophils
Cytotoxic
T cell
IFN-
γ
Infected cell
opsonization
NK cells

FIGURE 2.3. Basic components of a mature immune response. The mature immune response consists of both innate and adaptive arms. The skin, mucosa, and chemical barriers provide the first line of defense against invading pathogens. Once this barrier has been crossed, the cells of the innate immune response, monocytes/macrophages, neutrophils, and dendritic cells respond by phagocytosing the pathogen and releasing a myriad of defense mechanisms (including production of cytokines, reactive oxygen intermediates, digesting enzymes, and extracellular traps) for pathogen clearance. Antigen presentation results in rapid proliferation of adaptive immune cells and subsequent production of antibodies and cytokines that direct the appropriate immune response to neutralize the invading pathogen. Stimulatory interactions are illustrated with solid arrows and inhibitory interactions are illustrated with dashed lines with bars. Created with BioRender.com. C = complement. CD = cluster of differentiation. $FoxP3^{+}$ = Forkhead box P3 IFN-γ = interferon gamma. Ig = immunoglobulin. IL = interleukin. iNos = nitric oxide synthase. NK = natural killer. MHC = major histocompatibility complex. TCR = T cell receptor. Th = T helper. TNF-α = tumor necrosis factor alpha.

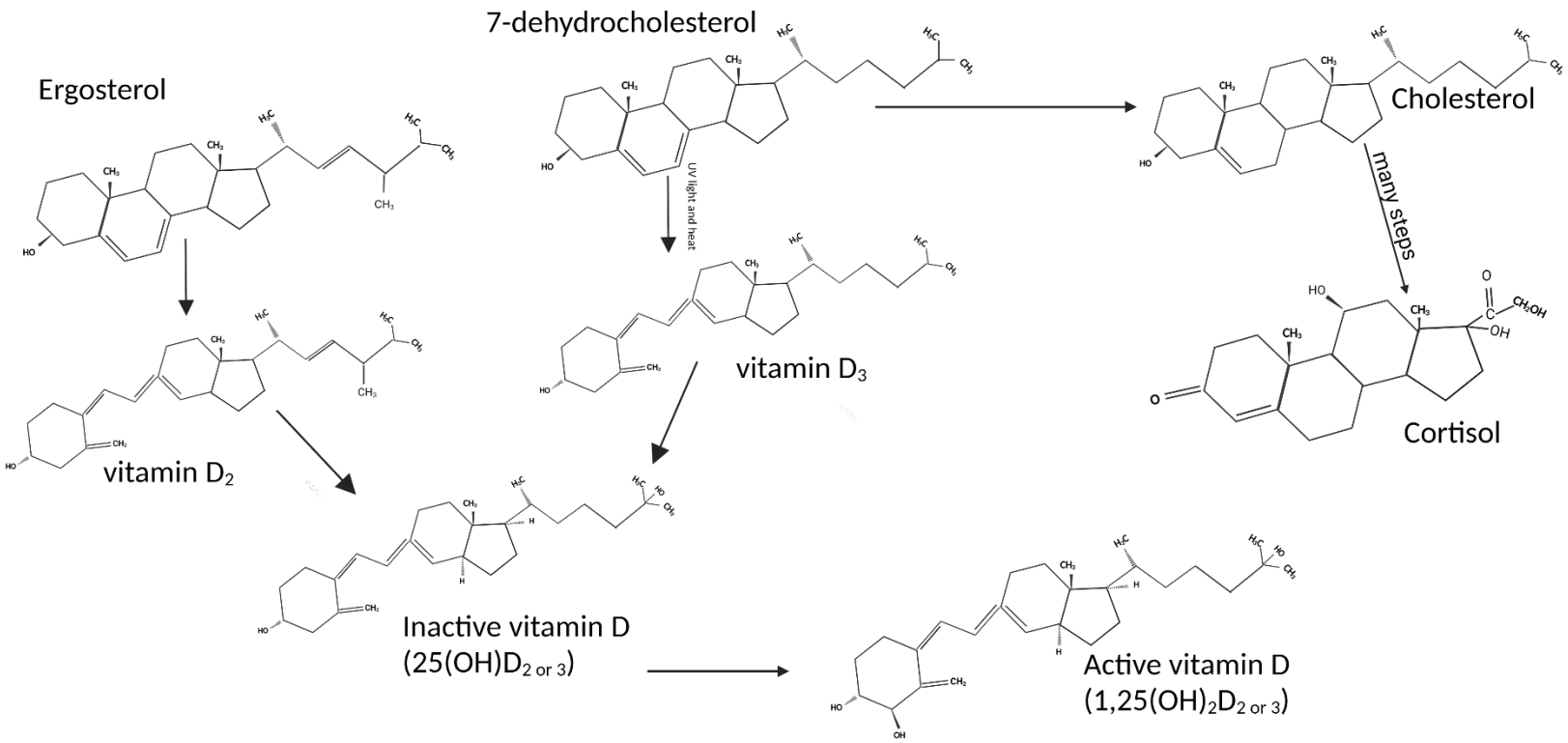

Figure 2.4. Cholesterol, cortisol, and vitamin D structural relationship. 7-dehydrocholesterol is a precursor to cholesterol and is also converted to vitamin D3 in the skin via ultraviolet (UV) light and heat. Cholesterol is converted to cortisol through a series of enzymatic steps in the adrenal gland. Vitamin D3 and vitamin D2 (produced from the plant derived sterol: Ergosterol) undergo initial hydrolysis to become the inactive vitamin D metabolite [25(OH)D]. A second hydrolysis step results in active vitamin D metabolite ([1,25$(OH)_2$D]. Created with BioRender.com.

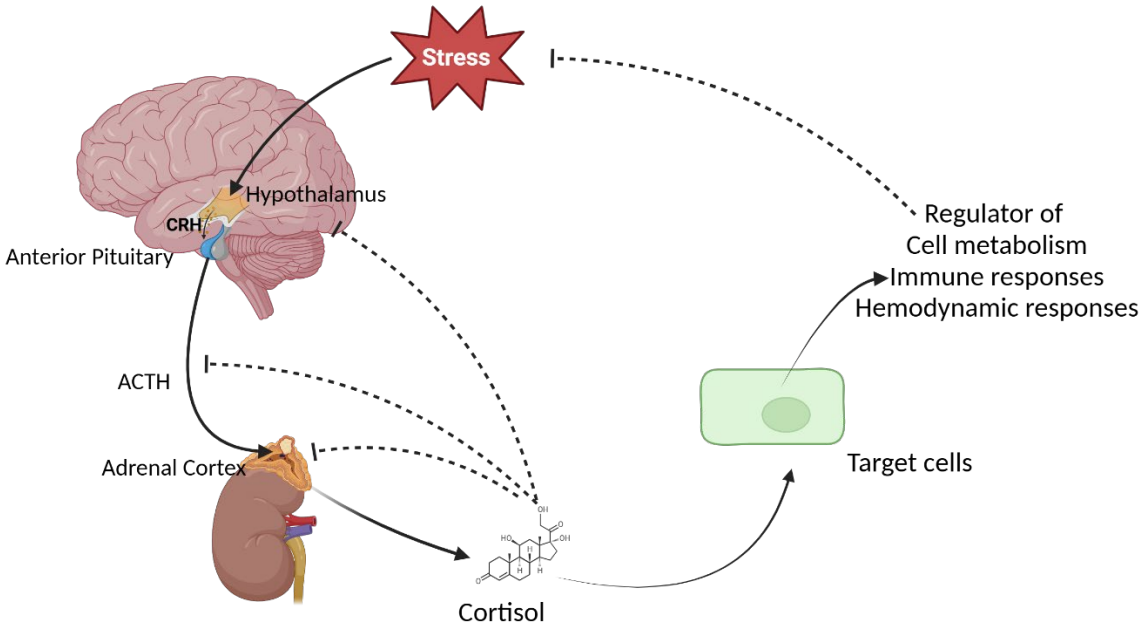

FIGURE 2.5. The hypothalamic-pituitary-adrenal (HPA) axis. Stress signals activate the hypothalamus to produce corticotrophin-releasing hormone (CRH) and arginine vasopressin to stimulate the anterior lobe of the pituitary gland to release adrenocorticotrophic hormone (ACTH) into the circulation, which stimulates the adrenal gland to synthesize and secrete cortisol. Cortisol regulation occurs in target cells throughout the body to reduce the stress signals that initiated the HPA axis and return the body to homeostasis. Stimulatory interactions are illustrated with solid arrows and inhibitory interactions are illustrated with dashed lines with bars. Created with BioRender.com.

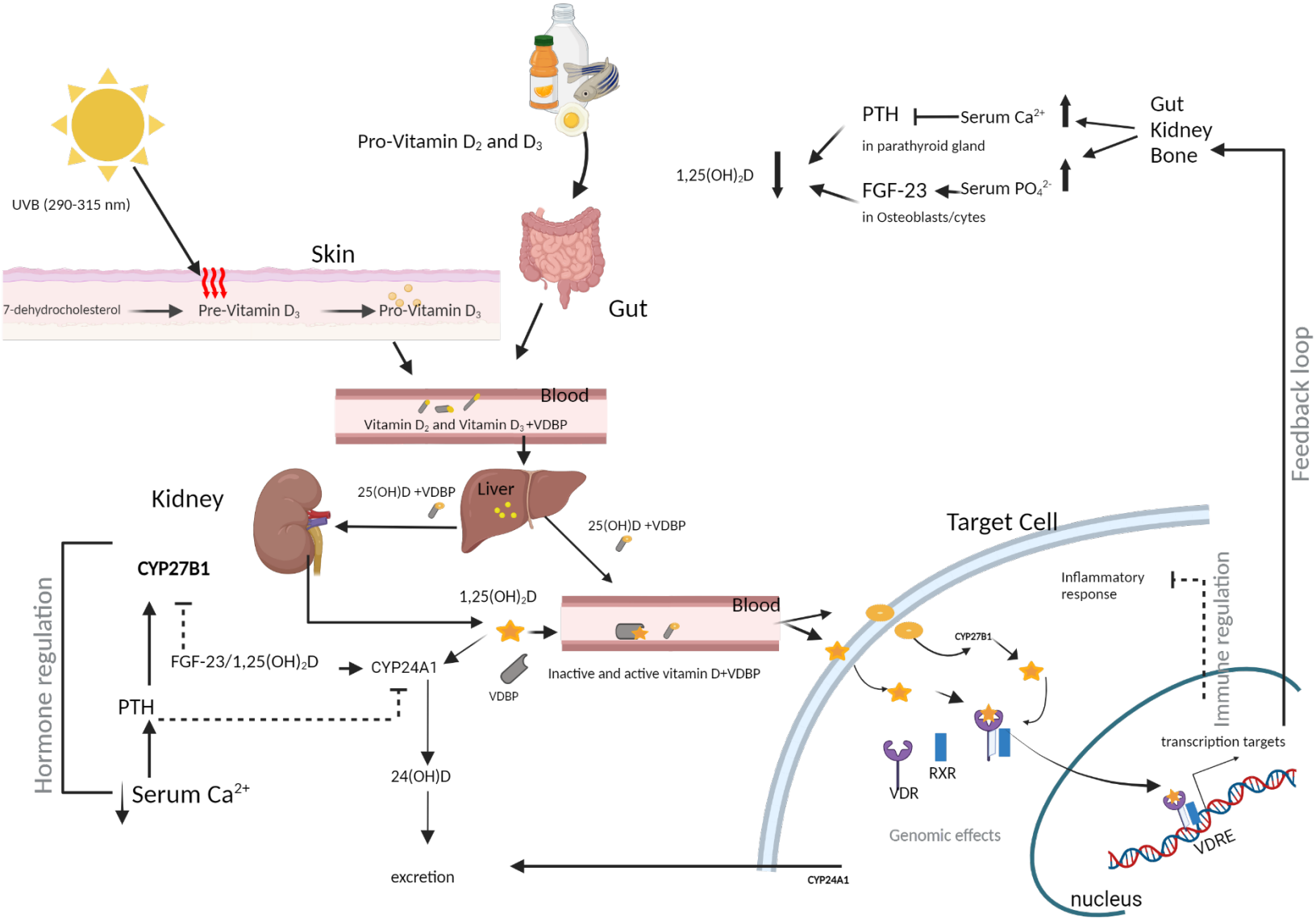
UVB (290-315 nm)
Skin
7-dehydrocholesterol
Pre-Vitamin D3
Pro-Vitamin D3
Pro-Vitamin D2 and D3
Gut
Blood
Vitamin D2 and Vitamin D3+VDBP
Kidney
25(OH)D +VDBP
Liver
25(OH)D +VDBP
CYP27B1
1,25(OH)2D
Blood
FGF-23/1,25(OH)2D
CYP24A1
VDBP
Inactive and active vitamin D+VDBP
PTH
Serum Ca2+
24(OH)D
excretion
Hormone regulation
1,25(OH)2D
PTH
Serum Ca2+
in parathyroid gland
FGF-23
Serum PO4 2-
in Osteoblasts/cytes
Gut
Kidney
Bone
Feedback loop
Target Cell
Inflammatory response
Immune regulation
CYP27B1
transcription targets
VDR
RXR
Genomic effects
VDRE
nucleus
CYP24A1

FIGURE 2.6. Vitamin D metabolism with classical and non-classical actions. Prohormones obtained by UV radiation conversion of 7-dehydrocholesterol and heat in the skin (vitamin D_3) or through diet (vitamin D_2 or D_3), and thought to be most important in the horse, undergo initial hydrolysis in the liver to produce the inactive vitamin D metabolite [25(OH)D]. 25(OH)D bound to vitamin D binding protein (VDBD) is the primary metabolite in circulation but requires a second hydrolysis step, which occurs in the kidney or locally in immune cells. The second hydrolysis step is catalyzed by the enzyme 1, α-hydroxylase (CYP27B1) and results in the active vitamin D metabolite [$1,25(OH)_2D$]. Regulation of $1,25(OH)_2D$ hydrolysis is controlled by parathyroid hormone (PTH), fibroblast growth factor 23 (FGF-23), and $1,25(OH)_2D$, and serum concentrations of calcium, (CA^{2+}) phosphate (PO_4), and $1,25(OH)_2D$ in a feedback loop. Degradation of $1,25(OH)_2D$, facilitated by 24-hydroxylase (CYP24A1), to 24(OH)D for excretion occurs when homeostasis has been reached. Local $1,25(OH)_2D$ synthesis within immune cells increases expression of the vitamin D receptor (VDR), which dimerizes with the retinoid D receptor (RXR) prior to translocating to nucleus to regulate transcription of target genes called vitamin D response elements (VDRE) that regulate the immune response. Stimulatory interactions are illustrated with solid arrows and inhibitory interactions are illustrated with dashed lines with bars. Created with BioRender.com.

CHAPTER 3

AGE-RELATED CHANGES IN VITAMIN D METABOLISM AND VITAMIN D RECEPTOR EXPRESSION IN EQUINE ALVEOLAR MACROPHAGES: A PRELIMINARY STUDY[1]

[1]*1* Berghaus, LJ, Cathcart, J, Berghaus, RD, Hart, KA 2023. Age-related changes in vitamin D metabolism and vitamin D receptor expression in equine alveolar macrophages: A preliminary study. Veterinary Immunology and Immunopathology. 259 (2023) 110593 Reprinted here with permission from the publisher.

Abstract

The vitamin D receptor (VDR)-vitamin D axis modulates pulmonary immunity in people but its role in equine immunity is unknown. Bacterial pneumonia causes high morbidity/mortality in foals, and alveolar macrophages (AMϕ) are important for pulmonary defenses. Age-related variations in vitamin D-mediated function of AMϕ might contribute to the foal's susceptibility to pneumonia. Our aim was to assess the impact of age on equine vitamin D metabolism and VDR expression in AMϕ. AMϕ and plasma were collected from healthy foals (2, 4, and 8 weeks old) and adult horses (once). AMϕ VDR expression was determined via RT-qPCR and plasma vitamin D metabolites quantified via immunoassays. Data were analyzed with linear mixed models. Inactive-vitamin D metabolite concentrations were lowest in foals at 2 weeks and lower at 2 and 4 weeks compared to adults ($P < 0.001$). Active-vitamin D metabolite concentrations were higher in foals than adults ($P < 0.05$). VDR expression was detected in AMϕ in all animals and was highest in 2-week-old foals. Vitamin D metabolism and AMϕ VDR expression are impacted by age in horses. This may have immunological consequences in foals given the key role that the VDR-vitamin D axis has in pulmonary immunity in other species.

Keywords

foal, neonatal immunity, calcitriol, calcidiol, pulmonary

Introduction

Foals are uniquely susceptible to primary bacterial pneumonia when compared with adult horses, but reasons for this age-related susceptibility are poorly understood (Perkins & Wagner, 2015). Pulmonary alveolar macrophages (AMϕ) are key cells that initiate and direct the immune response to respiratory pathogens, and are involved in uptake and, ideally, clearance of bacteria that cause pneumonia (Giguère et al., 1999). Decreased phagocytosis and bacterial killing have been described in AMϕ from neonatal foals and in neonates of other species when compared to adults (Bakker et al., 1998; Dickie et al., 2009; Fogarty & Leadon, 1987). *Rhodococcus equi* is an important cause of bacterial pneumonia in the foal, but almost never causes pneumonia in immunocompetent adult horses. *Rhodococcus equi* has greater survival and replication rates within foal AMϕ when compared to foal macrophages of other lineages (Berghaus et al., 2014). These functional changes in AMϕ are likely instrumental in the age-related susceptibility to primary bacterial pneumonia seen in the foal, but mechanisms impacting this altered function of foal AMϕ are poorly characterized.

The vitamin D receptor (VDR) is a nuclear receptor that mediates the function of its ligand, the active metabolite of the steroid hormone vitamin D (Haussler, 1986). Vitamin D is classically understood to be important in calcium and phosphorus homeostasis, and bone growth and development, and is primarily regulated by the kidney (D. Bikle, 2009; Haussler, 1986). However, the discovery of the VDR within human leukocytes uncovered an additional role for vitamin D as a modulator of the

immune response (Manolagas et al., 1994; Provvedini et al., 1983). Extrarenal synthesis of the active vitamin D metabolite $1,25(OH)_2D_3$ (actVitD) was found to also occur in these immune cells that express the VDR (D. D. Bikle, 2009). More specifically, the VDR is present in human pulmonary leukocytes, including AMϕ (Hu et al., 2019; Nguyen et al., 1987; Nguyen et al., 2004). *In vitro*, human AMϕ stimulated with microbial ligands produce actVitD, which works through the VDR to then modulate the immune response in respiratory disease (Gayan-Ramirez & Janssens, 2021; Haussler et al., 2008; Reichel et al., 1987). The VDR-vitamin D axis regulates hundreds of genes via vitamin D response elements (VDRE) and is well described in many mammalian species (Adams & Hewison, 2008; Haussler et al., 1998; Jaffey et al., 2018; Nagpal et al., 2005; Nelson et al., 2012). In human macrophages, VDRE are involved in pathways that synthesize antimicrobial peptides, alter redox homeostasis, and regulate cytokine and acute phase proteins production (Baeke et al., 2010; Lemire, 2000; Nelson et al., 2012; Pfeffer et al., 2018; Prietl et al., 2013).

Functions of vitamin D in the horse are less well understood (Hymoller, 2015). In horses, circulating vitamin D metabolite concentrations are 5 to 10 times lower than concentrations reported in humans and other veterinary species (Breidenbach et al., 1998; Hurst et al., 2020). This has led to the conclusion that calcium and phosphorous homeostasis and bone growth and development are not solely regulated by vitamin D in the horse as is seen in other mammalian species (Breidenbach et al., 1998; Rourke et al.,

2010). However, the immunomodulatory role of vitamin D in horses remains unexplored.

Thus, the objective of this study was to determine if the VDR is expressed in equine AMϕ and if there are age-related differences in both VDR expression and vitamin D metabolism in horses. We hypothesized that equine AMϕ would express the VDR, and that both VDR expression and vitamin D metabolism would be impacted by age in horses.

Materials and Methods

Animals

Ten healthy Quarter Horse foals and nine healthy adult horses of various breeds (five Quarter horses, one each of Dutch Warmblood, Thoroughbred, Irish sport horse, and Oldenburg breeds) from the University of Georgia College of Veterinary Medicine's equine research herd were used for this study. Foals (seven males and three females) were considered healthy based on physical examination and IgG concentration (> 800 mg/dL) at 24 h of age. Adults (five geldings, four mares) were considered healthy on the basis of physical examination and ranged in age from 8-25 years. Foals were housed with their dams and adult horses were housed in groups on mixed-grass pastures on the same farm during the course of the study with *ad libitum* access to grass, hay, and water. All animals were fed varying amounts (based on body condition and size) of a commercial complete pelleted feed (Senior Formula, Seminole Feed, Ocala, FL) twice

daily during the study period. During the same season (spring), adult horses were sampled once, and foals were sampled at 2, 4, and 8 weeks of age. Sampling and animal care protocols were approved by the University of Georgia's Institutional Animal Care and Use Committee. *A priori* sample size calculations using previously described vitamin D metabolite concentrations in foals determined that eight foals and eight adults would be needed to detect significant age-related differences (Kamr et al., 2015; Pozza et al., 2014). Extra animals were included in each group in the event that an animal needed to be removed due to injury or unforeseen circumstances.

Plasma collection and alveolar macrophage collection

Blood for measurement of vitamin D metabolites from foals at each age (2, 4, and 8 weeks) and from adult horses (once) was obtained via direct jugular venipuncture into lithium heparin tubes. Plasma was separated by centrifugation and stored at -80^{o}C until batched vitamin D metabolite analysis. At each sample time point, AMϕ were obtained by bronchoalveolar lavage. Animals were sedated intravenously with xylazine (0.5 mg/kg) and butorphanol (0.04 mg/kg) prior to lavage. 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. Aspirated aliquots were combined into sterile 50 mL conical tubes, and cells were pelleted by centrifugation (400 x g for 10 minutes) and combined pellets were washed three times with phosphate buffered

saline (PBS). A total nucleated cell count was determined by use of a cell counter (Cellometer Auto T4, Nexelom Bioscience, Lawrence, MA). Cells were resuspended at 1 x 10^6 cells/mL, and triplicate aliquots were transferred to microcentrifuge tubes. Cells were pelleted (400 x g for 10 minutes) and supernatant carefully removed. Three hundred microliters of RNAcell protect reagent (Qiagen, Valencia, CA) was added to the cell pellets and tubes were briefly vortexed. Samples were stored at -80^{o}C until batch RNA extraction.

mRNA expression of VDR

Total RNA was isolated from AMϕ and from archived frozen equine renal tissue (positive control) with the RNeasy kit (QIAGEN Inc., Valencia, CA) according to the manufacturer's instructions including treatment with amplification-grade DNase I (Life Technologies, Grand Island, NY). The RNA concentration was measured by optical density at 260 nm (Nanodrop, Thermofisher, and Waltham, MA). cDNA synthesis was performed using a High-Capacity cDNA Reverse Transcription Kit, (Applied Biosystems, Foster City, Ca) with 100 ng of total RNA as a template. Expression of mRNA of equine VDR and the housekeeping genes zeta polypeptide (YWHAZ), and hypoxanthine phosphoribosyl transferase (HRT1) was performed as previously described in equine renal tissue (Table 3.1) (Azarpeykan et al., 2016). RT-qPCR was performed using a CFX96 Detection System (Bio-Rad, Hercules, CA). Each reaction mixture contained 2x Fast SYBR Green master mix (Applied Biosystems, Foster City, Ca), 10 ng template cDNA, primers

at 500 nM concentration, and RNAase-DNAase-free water. Amplification and detection were performed with the initial incubation steps at 95^{o}C for 20 seconds and then 40 cycles of 95^{o}C for 3 seconds and 60^{o}C for 30 seconds, followed by melt curve analysis of 60^{o}C for 15 seconds and 94.5^{o}C for 30 seconds. cDNA from equine renal tissue 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 as negative controls. Relative gene expression was calculated using the average C_t of VDR and the geometric mean of the reference genes for each sample via the method described by Pfaffl (2001) using the equine renal sample as the calibrator and the lowest expression of VDR to normalize relative expression in AMϕ samples (Pfaffl, 2001).

Vitamin D metabolite analysis

Vitamin D metabolite analysis [inactive vitamin D metabolite, 25-(OH)D (inactVitD) and actVitD] was performed utilizing previously validated and published enzyme immunoassays (IDS, Tyne & Wear, UK)(Kamr et al., 2015; Pozza et al., 2014). Briefly, for the inactVitD analysis, 25 µl of equine plasma, assay calibrators, and controls were diluted in biotin-labeled inactVitD analysis. The diluted samples were incubated in a plate precoated with an inactive vitamin D metabolite 25-(OH)D antibody at room temperature for 2 hours. Samples were aspirated and plates washed using PBS with Tween-20. Horseradish peroxidase-labeled avidin was added to bind to complexed

biotin within samples. Color was developed using a 3,3',5,5'-Tetramethylbenzidine (TMB) substrate for 30 minutes prior to addition of stop solution. Absorbance was measured at 450 nm using a microplate reader (BioTek Synergy H1, Santa Clara, Ca). For actVitD analysis, 500 µl of each sample was delipidated and immunoextracted following manufacturer's guidelines. Immunopurified samples were incubated overnight at 4^{o}C with sheep anti-actVitD primary antibody. One hundred and fifty microliters of sample, plate controls and plate calibrators were added to pre-coated anti-sheep microtiter plates and incubated for 90 minutes at room temperature. ActVitD biotin solution was added to all wells and incubated for an additional 60 minutes. Samples were aspirated and plates washed using PBS containing Tween-20. Horseradish peroxidase labeled avidin was added to bind to complexed biotin within samples. Colors were developed using a TMB substrate for 30 minutes prior to stop solution being added. Absorbance was measured at 450 nm using the same microplate reader. Sample concentration calculations for both metabolites were performed using a 4-parameter logistic curve using GraphPad Prism version 9.3.1 (Graphpad Software, LLC San Diego, Ca), and control samples fell within the manufacturer's suggested acceptable range.

Statistical analysis

Normality of the data and equality of variances were assessed using the Shapiro–Wilk and Levene's tests, respectively. Variables that did not meet the assumptions for parametric testing were log-transformed prior to analysis. VDR expression and vitamin D

metabolites were compared between foal ages using linear mixed models. Age was modeled as a fixed nominal effect, and foal was included as a random effect to account for repeated measurements within the same foals. Estimation was performed using the method of restricted maximum likelihood, and denominator degrees of freedom for F-tests were approximated using the Kenward-Roger procedure. Pairwise comparisons were performed using the Bonferroni procedure to limit the type I error probability to 5% over all comparisons. The responses of adult horses were compared to those of foals at 2, 4, and 8 weeks of age using independent Bonferroni-adjusted two-sample t-tests. All tests assumed a two-sided alternative hypothesis, and values of $P < 0.05$ were considered statistically significant. Analyses were performed using commercially available statistical software (Stata version 17.0, StataCorp LLC; College Station, TX).

Results and Discussion

VDR expression in equine pulmonary alveolar macrophages

Quantifiable VDR expression in AMϕ from foals at all ages and from adult horses was achieved (Figure 3.1). Relative expression of VDR in AMϕ of foals was significantly higher at 2 weeks of age compared to 4 weeks ($P = 0.024$). VDR expression in foals at 8 weeks of age was not significantly different from that at either 2 weeks ($P = 0.051$) or 4 weeks ($P = 1.00$). Relative expression of VDR in adult horses was significantly lower than that of foals at 2 weeks ($P = 0.023$) but was not different from that of foals at 4 and 8 weeks ($P = 1.00$).

The results of our study support our hypotheses that the VDR would be expressed in equine AMϕ, and that VDR expression would be impacted by age in AMϕ from healthy foals compared to adults. We also found expected age-related differences in plasma concentrations of active and inactive vitamin D metabolites. This is the first study to measure VDR expression in equine leukocytes, and to serially characterize age-related changes in vitamin D metabolite concentrations in young, growing foals. In other species, VDR activation and ligand (actVitD) binding within leukocytes directly govern downstream immune activities (Haussler, 1986; Nelson et al., 2012). Documentation of VDR expression in equine pulmonary leukocytes in the study herein suggests that the VDR-vitamin D axis may have a similar immunomodulatory role in the horse.

There are few studies evaluating age-related changes in VDR expression in leukocytes from any species. Hirsh et al. (2011) reported a significant decrease in VDR expression in neutrophils from human infants compared to neutrophils from adults, and suggested that this decreased VDR expression may contribute to infants' susceptibility to inflammatory disease (Hirsch et al., 2011). Foals are uniquely susceptible to primary bronchopneumonia caused by *R. equi* and other pathogens. Equine AMϕ are key cells in the pathogenesis of this disease, and age-related changes in *R. equi* replication within equine AMϕ have been reported (Chaffin et al., 2003; Giguère et al., 1999). In contrast to the study in infants mentioned above, AMϕ from 2-week-old foals in our healthy population had significantly higher VDR expression than AMϕ from older foals or adult horses. This might reflect species differences between horses and humans, or may be

related to key functional differences between the circulating neutrophils evaluated in the human study and the pulmonary tissue macrophages evaluated in our foals (McTaggart et al., 2001). VDR expression in foal neutrophils warrants further investigation, as important age-related variation in equine neutrophil function has also been reported (McTaggart et al., 2001).

Foals are exposed to many airborne pathogens immediately at and after birth, so the upregulation of the VDR in AMϕ of 2-week-old foals might be an indication of an active and developing pulmonary immune system. The expression decreased between 2 and 4 weeks of age for 7 out of 10 foals and remained low, while expression increased in three foals at the 8-week time-point. It has been suggested that the balance between the pro-inflammatory cytokine IFN-γ, produced by T helper-1 (Th1) cells, and the anti-inflammatory cytokine IL-10, mainly produced by monocytes, may be critical for clearance of intracellular bacteria like *R. equi* (Bordin et al., 2022). Inadequate Th1 responses, as characterized by IFN-γ deficiencies, have been implicated as contributing in some way to the foals susceptibility to *R. equi* infection (Flaminio et al., 1998). The vitamin D/VDR complex in human macrophages has been shown to both attenuate the expression of IFN-γ and enhance the expression of IL-10. The individual differences in VDR expression that we observed may contribute to a particular foal's ability to clear intracellular pathogens. Furthermore, in human macrophages, the VDR has been demonstrated to modulate expression of important antimicrobial peptides and pattern recognition receptors involved in bacterial clearance (Ismailova & White, 2022). Further

investigation in adult horses and foals will be necessary to know if similar regulation occurs within equine macrophages and might contribute in some way to the foal's susceptibility to bronchopneumonia caused by *R. equi*.

Age-related changes in vitamin D metabolism

Concentrations of inactVitD and actVitD in plasma are shown in Figure 3.2. InactVitD concentrations increased as the foal aged. Plasma concentrations did not differ significantly between 2 and 4 weeks of age ($P = 0.091$), but concentrations at 8 weeks were significantly higher than those at both 2 weeks ($P < 0.001$) and 4 weeks ($P = 0.017$). Foals at 2 and 4 weeks of age also had lower concentrations of inactVitD compared to adult horses ($P < 0.001$), while the concentrations in 8-week-old foals did not differ from those of adults ($P = 0.072$; Figure 3.2A). Concentrations of actVitD did not differ significantly between foals at 2, 4, or 8 weeks of age ($P = 0.43$), but concentrations measured in foals at all ages were higher than those measured in healthy adult horses ($P < 0.05$; Figure 3.2B).

Vitamin D metabolite concentrations in healthy adult horses described in our study are consistent with previous reports documenting lower circulating concentrations of vitamin D in the horse as compared to most other mammalian species (Smith & Wright, 1984). The inactVitD is most commonly used to assess vitamin D status. However, there are no established reference ranges for vitamin D sufficiency/insufficiency in most veterinary species (Hurst et al., 2020). Previously, Pozza

et al. (2014) reported circulating inactVitD concentrations at a single timepoint in foals at either < 1 week of age or at 2 months of age, and found concentrations in foals at both ages were lower than those reported in healthy adult horses (Pozza et al., 2014). Our findings in foals at 2 weeks of age corroborate these findings; however, with repeated measurements in this group of healthy growing foals, we found concentrations of the inactVitD metabolite were not different than adults by 8 weeks of age. In humans, maternal vitamin D insufficiency/deficiency is often transferred to the neonate and as a result vitamin D concentration in human infants are often lower than in older children and adults (Holick et al., 2011; Kimball et al., 2008). In the current study, vitamin D metabolite concentrations in the dams were not measured and there are currently no established guidelines on whether vitamin D supplementation in mares would be necessary or beneficial for foals (Hymoller, 2015).

Plasma concentrations of the actVitD in hospitalized neonatal foals (septic and sick-non-septic, < 1 week of age) were significantly lower when compared to age-matched healthy foals (Kamr et al., 2015). In our study, circulating concentrations of the actVitD were higher in healthy foals at all ages compared to healthy adult horses. In humans, circulating actVitD concentrations are under renal control and are tightly regulated by plasma calcium, phosphorus, and parathyroid hormone concentrations, with sufficient quantities of the active metabolite required for proper bone growth and mineralization (Kimball et al., 2008). The increased circulating actVitD concentrations in the healthy foals in our study might reflect the needs of a growing foal related to bone

growth and development (Dittmer & Thompson, 2011). However, it has been suggested by Briedenbach (1998) and others that, in the horse, calcium and phosphorus homeostasis is untethered from vitamin D regulation, and, therefore, circulating vitamin D concentrations are unrelated to bone health (Breidenbach et al., 1998; Hymoller, 2015; Rourke et al., 2010). In fact, because of the critical role that vitamin D plays in immunomodulation, it has been suggested that measures of immunocompetence might be the best determinant of vitamin D sufficiency in horses (Hymoller, 2015). It is possible that the higher concentrations of actVitD metabolite we observed in healthy foals may also indicate an active and robust developing immune system in the foal.

Important limitations of the current study include lack of concurrent assessment of vitamin D-related clinicopathologic parameters such as calcium, phosphorus, and parathyroid hormone concentrations in our study population. These measurements might have allowed us to better characterize the role of the active vitamin D metabolite in calcium regulation and bone growth in healthy growing foals.

Another key limitation is the omission of differential cell counts on the bronchoalveolar lavage samples. From published reports and a long history of equine bronchoalveolar lavage in our lab and others, AMϕ should be the primary cell type collected in healthy animals (McGorum, 1994). However, it is possible that the VDR expression reported in our study could be from other leukocytes present in the lavage fluid. The foals in our study remained healthy throughout all sampling time points (2-8 weeks of age), were monitored until weaning, and all foals and horses remained free of

clinically detectable respiratory disease. Bronchopneumonia caused by *R. equi* usually presents in foals at 3-5 months of age, so it is possible that if we had sampled foals out to 20 weeks of age, other changes in VDR expression relevant to *R.equi*-specific immunity might have been detected. However, this report is the first describing the expression of VDR in equine leukocytes of any type, which is still an important finding related to equine pulmonary immunity.

Conclusion

The results of this study support our hypotheses and indicate that there are age-related differences in both vitamin D metabolism and pulmonary leukocyte VDR expression in the horse. Plasma concentrations of both the inactive and active metabolites of vitamin D were different in healthy foals compared to adult horses. VDR expression could be measured in AMϕ from foals and horses at all time points, and VDR expression was also impacted by age. In conclusion, these data suggest that vitamin D and the VDR may play an important role in the equine pulmonary immune response and the immunomodulatory functions of vitamin D warrant further investigation in the horse.

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Table 3.1. Vitamin D and housekeeping primers. Previously validated primers used to quantify expression of equine vitamin D receptor (VDR) gene and housekeeping genes Zeta polypeptide (YWHAZ) and hypoxanthine phosphoribosyltransferase 1 (HPRT1) in alveolar macrophages from horses (n = 9) and foals (n = 10) (Azarpeykan et al., 2016).

Vitamin D receptor (VDR)	Forward: 5'-ACAGCATCCAAAAGGTGGTC-3'
	Reverse: 5'-TGACTTCAGCAGCACGATCT-3'
Zeta polypeptide (YWHAZ)	Forward: 5'-TGTTGTAGGAGCCCCGTAGGT-3'
	Reverse: 5'-ATTCTCCGAGCCTCTGCTGT-3'
Hypoxanthine phosphoribosyltransferase 1 (HPRT1)	Forward: 5'-TTGCTGACCTGCTGGATTAT-3'
	Reverse: 5'-TTATGTCCCCTGTTGACTGGT-3'

Figures

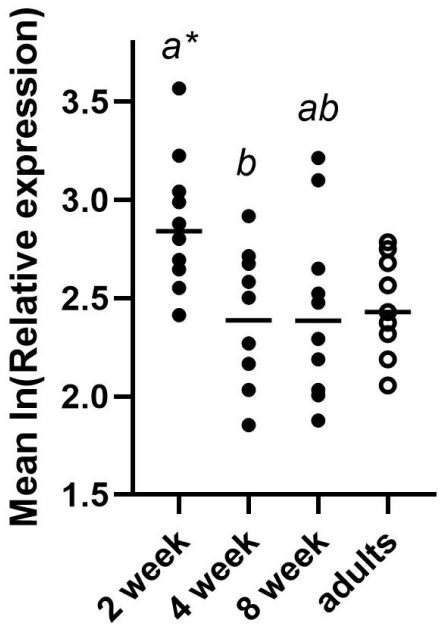

Figure 3.1. Vitamin D receptor (VDR) expression. The mean natural log of the relative expression of the VDR in pulmonary alveolar macrophages (AMϕ) in foals at 2, 4 and 8 weeks of age (n = 10) and adult horses (n = 9) as determined by RT-qPCR using VDR specific primers and two housekeeping genes (YWHAZ and HRT1), analyzed according to the method described by Pfaffl (Pfaffl, 2001). Each closed circle represents one foal, and each open circle represents one adult horse, with the horizontal line depicting the median for each group. Foal ages with a superscript letter in common do not differ with a level of significance of 5%. Significant differences between foal ages and adults are indicated with *. Significance was set at $P < 0.05$.

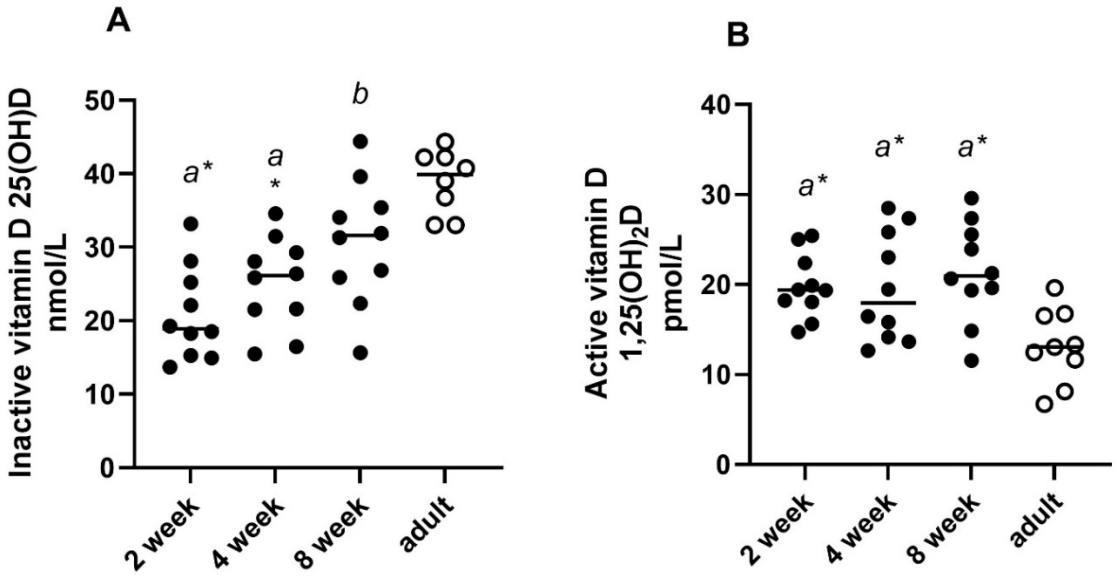

Figure 3.2. Vitamin D metabolite concentration. Vitamin D metabolite concentrations in plasma as measured by enzyme immunoassay (Immunodiagnostic Systems, Tyne and Wear, UK) in foals at 2, 4, and 8 weeks of age (n = 10) and adult horses (n = 9). (A): inactive vitamin D, 25(OH) D measurements in nmol/L and (B): active vitamin D metabolite $1,25(OH)_2D_3$ concentrations in pmol/L. Each closed circle represents one foal and each open circle represents one adult. The horizontal bar represents the median for each group. Foal ages with a superscript letter in common do not differ with a level of significance of 5%. Significant differences between foal ages and adults are indicated with *. Significance was set at $P < 0.05$.

CHAPTER 4

THE IMPACT OF AGE ON VITAMIN D RECEPTOR EXPRESSION, VITAMIN D METABOLISM, AND ASSOCIATED CYTOKINES IN *EX VIVO RHODOCOCCUS EQUI* INFECTION OF EQUINE ALVEOLAR MACROPHAGES [1]

[1]Berghaus, LJ, Cathcart, J, Berghaus, RD, Ryan, C, Toribio, RE, Hart, KA 2024. The impact of age on vitamin D receptor expression, vitamin D metabolism and cytokine production in *ex vivo Rhodococcus equi* infection of equine alveolar macrophages. Veterinary Immunology and Immunopathology 268 (2024) 110707 Reprinted here with permission from the publisher.

Abstract

Rhodococcus equi (*R. equi*), a pneumonia-causing intracellular bacterium, results in significant morbidity and mortality in young foals, while healthy adult horses rarely develop disease. Survival and replication within alveolar macrophages (AMϕ) are the hallmarks of *R. equi*'s pathogenicity. The vitamin D receptor (VDR) and its ligand, the active vitamin D metabolite 1,25$(OH)_2$D, are important in immune responses to intracellular bacteria. The vitamin D/VDR pathway regulates the downstream production of cytokines in infected human AMϕ. The immunomodulatory role of the vitamin D/VDR pathway in equine leukocytes is unknown. The objective of the current study was to determine the impact of age on synthesis of 1,25$(OH)_2$D, VDR expression, and regulation of vitamin D-associated cytokines in an *ex vivo* model of *R. equi* infection in equine AMϕ. AMϕ were collected from ten healthy foals at 2, 4, and 8 weeks old and from nine healthy adult horses once via bronchoalveolar lavage. AMϕ were mock infected (CONTROL) or infected with a virulent laboratory strain of *R. equi* for 7 days (INFECTED). VDR expression was determined via RT-qPCR from cell lysates. 1,25$(OH)_2$D and cytokines were measured in cell supernatants by immunoassays. VDR expression was impacted by age ($P = 0.001$) with higher expression in AMϕ from 8-week-old foals than from 2-week-old foals and adults. There was no significant effect of infection in foal AMϕ, but in adults, relative VDR expression was significantly lower in INFECTED AMϕ compared to CONTROL AMϕ ($P = 0.002$). There was no effect of age or infection on 1,25$(OH)_2$D concentration ($P > 0.37$). Mean TNF-α production was significantly higher from INFECTED compared to CONTROL AMϕ from 4- and 8-week-old foals and adults (*P*

< 0.005). Mean IFN-γ production was significantly higher from AMϕ from foals at 8 weeks old compared to 2 weeks old (P = 0.013) and higher from INFECTED AMϕ than from CONTROL AMϕ in foals at 4 weeks old and in adults (P < 0.027). The proportion of samples producing IL-1β and IL-10 was also significantly higher from INFECTED compared to CONTROL AMϕ isolated from 4-week-old foals (P < 0.008). Similarly, in adult samples, IL-17 was produced from a greater proportion of INFECTED compared to CONTROL samples (P = 0.031). These data document age-associated changes in VDR expression and related cytokine production in equine AMϕ in response to R. *equi* infection. Further research is needed to fully elucidate the immunomodulatory role of the vitamin D pathway in the horse.

Keywords

foal, neonatal immunity, calcitriol, vitamin D receptor, pulmonary, pneumonia

Introduction

Rhodococcus equi (*R. equi*) is a ubiquitous soil saprophyte and a leading cause of pneumonia in young horses (foals). The organism's ability to replicate within alveolar macrophages (AMϕ) is key to the development of granulomatous lesions in the lungs of affected animals (Giguère *et al.*, 2011; Zink *et al.*, 1985). Newborn foals are exposed to the bacteria shortly after birth by inhalation of environmental organisms present in both

soil and feces (Cohen *et al.*, 2013; Prescott, 1987; Willingham-Lane *et al*., 2019). *R. equi* pneumonia is endemic on some farms, but susceptibility and disease severity in individual foals can vary greatly on these farms, and healthy adult horses are rarely clinically affected (Chaffin *et al.*, 2003; Giguère *et al*., 2015; Venner *et al.*, 2012). To date, there is no clear consensus on immunological factors that explain differences in age-related susceptibility or allow practitioners to predict disease severity in individual foals (Felippe, 2016).

R. equi has key similarities to *Mycobacterium tuberculosis* (*Mtb*), the facultative intercellular bacterium the causes tuberculosis in people (Meijer & Prescott, 2004). Both organisms are from the order Actinomycetes and can arrest normal phagosome maturation, facilitating their survival and replication within pulmonary macrophages (Meijer & Prescott, 2004). Tuberculosis has been extensively studied in humans, and findings from *Mtb* research may be valuable to understanding *R. equi* pneumonia in the foal (von Bargen & Haas, 2009).

Vitamin D and the vitamin D receptor (VDR) are immune modulators in response to *Mtb* infection in people and in experimental models of *Mtb* infection (Lemire, 1992; Selvaraj, 2011). Human AMϕ synthesize the active metabolite of vitamin D, $1,25(OH)_2D$, and upregulate the expression of the VDR in response to bacterial stimuli (Liu *et al*., 2006). Vitamin D, bound to the VDR, translocates to the cell nucleus and regulates many downstream response elements including the production of cytokines and antimicrobial peptides (Prietl *et al*., 2013). The role of the vitamin D/VDR pathway in equine pulmonary immunity in general, and in response to *R. equi* infection in particular, is

unknown. Recently, our group demonstrated VDR expression in equine AMϕ, the first report of VDR expression in any equine leukocyte type (Berghaus *et al.*, 2023). In that preliminary study, AMϕ from 2-week-old foals had the highest expression of the VDR when measured directly after collection by bronchoalveolar lavage when compared to older foals (4 and 8 weeks old) and adult horses (Berghaus *et al.*, 2023). These findings indicate that the vitamin D/VDR pathway and the age-related variation in its activity might play an immunomodulatory role in the horse.

Therefore, the objective of the current study was to assess age-related differences in VDR expression, 1,25$(OH)_2$D (active vitamin D) synthesis, and vitamin D-related cytokine production in an *ex vivo* model of *R. equi* infection in equine AMϕ. We hypothesized that infection with *R. equi* would impact VDR expression, active vitamin D synthesis, and associated cytokine production in equine AMϕ isolated from healthy foals and adult horses in an age-dependent manner.

Materials and Methods

Animals

Ten healthy Quarter Horse foals and nine,healthy adult horses of various breeds (five Quarter Horses, one each of Dutch Warmblood, Thoroughbred, Irish sport horse, and Oldenburg breeds) from the University of Georgia College of Veterinary Medicine's equine research herd were used for this study. Horses used in this study were also used in the previous study examining baseline VDR expression in equine AMϕ (Berghaus et al., 2023). Foals (seven males and three females) were considered healthy based on

physical examination and IgG concentration (> 800 mg/dL) at 24 hours of age. All foals were considered free of active pulmonary disease based on an ultrasonographic pulmonary abscess score < 1 cm prior to each sample collection (Venner *et al*., 2012). Adult horses (five geldings, four mares) were considered healthy based on lack of historical evidence of respiratory disease and normal physical examination at the time of sample and ranged in age from 8-25 years.

Horses were housed in groups (foals with their dams) on mixed-grass pastures at the same farm for the entire study period with *ad libitum* access to grass, hay, and water. All animals were fed varying amounts (based on body condition and size) of a commercial complete pelleted feed (Senior Formula, Seminole Feed, Ocala, FL) twice daily during the study period. During the same season (spring), adult horses were sampled once, and foals were sampled at 2, 4, and 8 weeks of age. The University of Georgia's Institutional Animal Care and Use Committee approved sampling and animal care protocols. *A priori* sample size calculations using previously described vitamin D metabolite concentrations in foals determined that eight foals and eight adult horses would be needed to detect significant age-related differences (Kamr *et al*., 2015; Pozza *et al*., 2014). Extra animals were included in each group in case an animal needed to be removed due to injury or unforeseen circumstances.

Alveolar macrophage collection and culture

AMϕ were obtained by bronchoalveolar lavage at 2, 4, and 8 weeks of age in foals and once in adult horses. Animals were sedated intravenously with xylazine (0.5

mg/kg) and butorphanol (0.04 mg/kg) prior to lavage. 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 four aliquots of 50 mL of sterile physiologic saline (0.9% NaCl) solution infused and aspirated immediately. Aspirated aliquots were combined into sterile 50 mL conical tubes, and cells were pelleted by centrifugation (400 x g for 10 minutes) before combined pellets were washed three times with phosphate-buffered saline (PBS). Total nucleated cell count was determined by use of a cell counter (Cellometer Auto T4, Nexelom Bioscience, Lawrence, MA). Cells were resuspended at 1 x 10^6 cells/mL in complete cell culture medium (α-MEM with L-glutamine [Life Technologies, Grand Island, NY] and 10% heat-inactivated donor horse serum [Hyclone, GE Healthcare, Logan, UT). Donor horse serum was confirmed to have inactive vitamin D metabolite (25(OH)D concentrations (18.02 +/-1.89 nmol/L) comparable to concentrations previously reported in healthy adult horses (Kamr *et al.*, 2015), thus ensuring adequate substrate availability for active vitamin D synthesis. One milliliter of the suspended cells was added to wells of two 24-well cell culture plates and allowed to adhere overnight at 37^{o}C with 6% CO_2. Plate one was used to lyse cells for determination of colony-forming units (CFU) at baseline and 7 days post infection (dpi), and plate two was used for cell supernatant cytokine and 1,25$(OH)_2$D metabolite analysis quantification and for cell monolayer VDR expression analysis. Following overnight incubation, non-adherent cells were removed with the supernatant, and the monolayer of cells was washed with warm PBS. Cells were then infected with a virulent laboratory strain of *R. equi* (ATCC #33701, Rockville, MD) or mock infected (media only)

as previously described (Berghaus *et al*., 2014). Briefly, bacterial cultures were diluted in α-MEM containing 10% donor horse serum (not heat inactivated to aid in phagocytosis) at a multiplicity of infection (MOI) of 0.1. One milliliter of bacteria in media or media only was added to a minimum of triplicate wells for the following three conditions: 1) BASELINE: infected cells harvested 40 minutes post infection for confirmation of bacterial phagocytosis and to determine initial intracellular bacterial load; 2) CONTROL: mock-infected cells (media only) cultured for seven dpi; and 3) INFECTED: cells infected with virulent *R. equi* cultured for seven dpi. Following 40 minutes of incubation with bacteria or mock infection to permit infection of cells, the supernatant was removed from all wells. For BASELINE wells, 1 mL of sterile water was added to lyse the monolayer. To ensure complete cell lysis, monolayers treated with water were incubated for 30 minutes before transfer to collection tubes for quantification. Serial dilutions of lysed monolayers were plated on tryptic soy agar to determine the concentration of phagocytosed bacteria by manually counting colonies and calculating CFU/mL. For CONTROL and INFECTED wells, 2 mL of complete media [supplemented with 8 μg/mL of amikacin (USP, Rockville MD)] was added and returned to the incubator at 37^{o}C in 6% CO_2. Monolayers were visually inspected daily for evidence of fungal infection or significant cell death, and only triplicate wells clear of fungal contamination and with intact monolayers were used for day seven analysis. An additional 1 mL of complete media was added to wells at three dpi to help provide adequate nutrition to maintain cell health. Seven dpi monolayers from INFECTED wells of plate one was lysed for assessment of intracellular replication of *R. equi* as described above for BASELINE

conditions. Cell lysate data was not collected for two adult horses at seven dpi as two horses had fungal contamination in all wells of plate one. BAL samples from two other adult horses had insufficient cells to seed both plates; since intracellular replication within AMϕ from adult horses has previously been reported, plate two was prioritized in those cases (Berghaus *et al.*, 2014; Bordin *et al.*, 2021). Intracellular replication of *R. equi* was calculated by determining the change in CFU/mL at seven dpi compared to BASELINE CFU/mL.

From plate two, cell supernatants were collected and centrifuged at 400 x g for 10 minutes to pellet any non-adherent cells. Supernatant was transferred to collection tubes and stored at -80^{o}C for later batch analysis of vitamin D-associated-cytokines and 1,25$(OH)_2$D. After removal of the supernatant, the cell monolayer from plate two was gently washed with PBS prior to the addition of 300 µL of RNAprotect cell reagent (Qiagen, Valencia, CA). Protected cells were then transferred to 1.5 mL Eppendorf tubes and stored at -80^{o}C until future RNA extraction.

Determination of vitamin D receptor mRNA expression

Total RNA was isolated from cultured AMϕ and from archived frozen equine renal tissue (positive control) with a RNeasy kit (QIAGEN Inc., Valencia, CA) according to the manufacturer's instructions, including treatment with amplification-grade DNase I (Life Technologies, Grand Island, NY). The RNA concentration was measured by optical density at 260 nm (Nanodrop, Thermofisher, Waltham, MA). cDNA synthesis was performed using a High-Capacity cDNA Reverse Transcription kit, (Applied Biosystems,

Foster City, CA) with 100 ng of total RNA as a template. Expression of the equine VDR mRNA, and the housekeeping genes zeta polypeptide (YWHAZ) and hypoxanthine phosphoribosyl transferase (HRT1), was determined using validated primers (Table 4.1) as previously described in equine renal tissue (Azarpeykan *et al.*, 2016; Berghaus *et al.,* 2023). RT-qPCR was performed using a CFX96 Detection System (Bio-Rad, Hercules, CA). Each reaction mixture contained 2x Fast SYBR Green master mix (Applied Biosystems, Foster City, CA), 10 ng template cDNA, primers at 500 nM concentration, and RNAase-DNAase-free water. Amplification and detection were performed with the initial incubation steps at 95^{o}C for 20 seconds, then 40 cycles of 95^{o}C for 3 seconds and 60^{o}C for 30 seconds, followed by melt curve analysis of 60^{o}C for 15 seconds and 94.5^{o}C for 30 seconds. cDNA from equine renal tissue was used as positive control and run on each plate as a calibrator sample. Samples without cDNA were included in the amplification reactions as negative controls. Each sample was assayed in triplicate, and the mean value was used for comparisons. Relative gene expression was calculated using the average C_t of VDR and the geometric mean of the reference genes for each sample via the method described by Pfaffl (2001) using the equine renal sample as the calibrator and the lowest expression of VDR to normalize relative expression in AMϕ samples (Pfaffl, 2001).

Analysis of the active vitamin D metabolite 1,25(OH)$_2$D

Supernatant 1,25(OH)$_2$D was analyzed utilizing a previously validated and published enzyme immunoassay (IDS, Tyne & Wear, UK) (Kamr *et al*., 2015; Pozza *et al.*,

2014). Briefly, 500 µl of each sample was delipidated and immuno-extracted following manufacturer guidelines. Immunopurified samples were incubated overnight at 4^{o}C with sheep anti-vitamin D primary antibody. One hundred and fifty microliters of sample, plate controls, and plate calibrators were added to pre-coated, anti-sheep, microtiter plates and incubated for 90 minutes at room temperature. Vitamin D-biotin solution was added to all wells and incubated for an additional 60 minutes. Samples were aspirated and plates washed using PBS containing Tween. Horseradish peroxidase-labeled avidin was added to bind to complexed biotin in samples. Colors were developed using a 3,3',5,5'-tetramethylbenzidine (TMB) substrate 30 minutes prior to stop solution being added. Absorbance was measured at 450 nm using a microplate reader (BioTek Synergy H1, Santa Clara, CA). Sample 1,25$(OH)_2$D concentration calculations were performed using a 4-parameter logistic regression model using GraphPad Prism version 9.5.1 (Graphpad Software, LLC San Diego, CA), and control samples fell within the manufacturer's suggested acceptable range.

Cytokine analysis

Supernatant from INFECTED or CONTROL cells was analyzed for the presence of the following cytokines: interleukin (IL)-4, IL-6, IL-10, IL-17, IL-1β, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α. These cytokines were selected for their importance in either foal immunity or association with vitamin D regulation. IL-4, IL-10, IL-17, and IFN-γ concentrations were measured with a previously validated bead-based immunoassay (5-plex, Animal Health Diagnostic Center, Cornell University, Ithaca, NY) as previously

described (Wagner & Freer, 2009). Cytokine concentrations were calculated based on a standard curve for each cytokine, with the following lower and upper limits of detection: IL-4: 40-80,000 pg/mL, IL-10: 15-35,000 pg/mL, IFN-α: 12-3000 pg/mL, IFN-γ: 10-5000 Units/mL, and IL-17: 10-10,000 Units/mL. IL-1β, TNF-α, and IL-6 concentrations were measured using commercial enzyme-linked immunosorbent assays (ELISA) kits (R&D Duoset, Minneapolis, MN) according to manufacturer directions, as previously described (Burton *et al.*, 2009; Lopez *et al*., 2019; Naskou *et al.*, 2018). Briefly, 100 µL of capture antibody diluted in the provided coating buffer was added to each well of a 96-well strip plate and incubated overnight at room temperature. Unbound capture antibody was then aspirated and 300 µL of provided wash buffer used to wash wells three times using a commercial plate washer (Bio-Tek H1, Santa Clara, CA). Reagent diluent (300 µL) was added to each well and incubated for one hour at room temperature as a blocking step, followed by another washing step as described above. Serial dilutions of the provided recombinant standard were performed to prepare a 7-point standard curve. Standard or sample (100 µL) was added to duplicate wells, with reagent diluent alone added to duplicate wells to serve as a blank sample, and the plate was incubated for 2 hours at room temperature. After another wash step, biotin-labeled detection antibody (100 µL) was added to each well, incubated for 2 hours at room temperature, and washed again. Streptavidin-horseradish peroxidase (200 µL) was added to each well, and the plate was incubated in the dark for 20 minutes prior to the final wash step. The 3, 3’,5,5’-tetramethylbenzidine (TMB) substrate (200 µL) was added and color developed for approximately 20 minutes prior to the addition of a stop solution (50 µL). Optical density

was read at 450 nm with correction set at 570 nm. Reported lower and upper limits of detection are as follows: IL-1β: 125-8000 pg/mL, TNF-α: 31.3-2000 pg/mL, and IL-6: 125-8000 pg/mL. Samples above the standard curve were diluted as necessary to fall within the linear portion of the standard curve. A four-parameter logistic regression analysis was created using GraphPad Prism version 9.5.1 (Graphpad Software, LLC San Diego, CA) to interpolate the concentration of each sample against the standard curve.

Statistical analysis

Normality of the data and equality of variances were assessed using the Shapiro–Wilk and Levene's tests, respectively. Variables that did not meet the assumptions for parametric testing were log-transformed prior to analysis. VDR expression, vitamin-D-associated cytokines (IFN-γ, TNF-α, IL-6), and $1,25(OH)_2D_3$ concentrations were compared between foal ages using linear mixed models. Age and infection were modeled as fixed nominal effects, and foal was included as a random effect to account for repeated measurements within the same foals. Estimation was performed using the method of restricted maximum likelihood, and denominator degrees of freedom for F-tests were approximated using the Kenward-Roger procedure. Pairwise comparisons were performed using the Bonferroni procedure to limit the type I error probability to 5% over all comparisons. For cytokines IL-17, IL-10, and IL-1β, the proportion of samples with a concentration greater than the lower limit of detection of the assay was compared between INFECTED and CONTROL samples of each age using an exact McNemar's test. All tests assumed a two-sided alternative hypothesis, and values of $P <$

0.05 were considered statistically significant. Analyses were performed using commercially available statistical software (Stata version 17.0, StataCorp LLC; College Station, TX).

Results

VDR expression in equine pulmonary macrophages

Relative expression of the VDR was quantifiable in cultured AMϕ from horses of all ages and in both INFECTED and CONTROL AMϕ. (Figure 4.1). In a factorial analysis, relative VDR expression was significantly impacted by age ($P < 0.001$), with higher mean expression in 8-week-old foals compared to both 2-week-old foals and adults. There was no significant overall effect of infection ($P = 0.47$), and no significant interaction between the effects of age and infection ($P = 0.21$). When the effect of infection was evaluated separately for each age of foals, there was no significant difference in VDR expression between INFECTED and CONTROL AMϕ of foals at 2 weeks ($P = 0.96$), 4 weeks ($P = 0.81$), or 8 weeks ($P = 0.67$) of age. In contrast, relative VDR expression in AMϕ isolated from adult horses was significantly lower for INFECTED compared to CONTROL ($P = 0.002$).

Vitamin D metabolite concentrations in cultured equine pulmonary macrophages

Concentrations of $1,25(OH)_2D$ in the supernatant of cultured AMϕ under CONTROL or INFECTED conditions are shown in Figure 4.2. In a factorial analysis, there

was no significant effect of age (P = 0.37) or infection (P = 0.64), and no significant interaction between the effects of age and infection (P = 0.74).

Intracellular replication of *R. equi* in cultured equine pulmonary macrophages

Intracellular survival and replication of *R. equi* within cultured equine AMϕ measured by plating and counting CFU/mL in cell lysates are shown in Figure 4.3. There was no impact of age on intracellular survival of *R. equi* in equine pulmonary macrophages (P = 0.17).

Cytokine production in cultured equine pulmonary macrophage supernatant

The median and interquartile range for IFN-γ and TNF-α production in the culture supernatant of INFECTED and CONTROL AMϕ from all ages are shown in Figure 4.4. For TNF-α concentrations, there was a significant interaction between the effects of age and infection (P = 0.024). There was no significant effect of INFECTED and CONTROL conditions in AMϕ isolated from 2-week-old foals (P = 0.097), but mean TNF-α concentrations were significantly higher in supernatants from INFECTED AMϕ compared to CONTROL AMϕ from 4-week-old foals (P < 0.001), 8-week-old foals (P < 0.001), and adult horses (P = 0.005). Likewise, there was a significant effect of age for INFECTED (P = 0.001) cells, but not for CONTROL AMϕ (P = 0.13). The mean TNF-α concentration was significantly higher in supernatants from INFECTED AMϕ from 8-week-old foals than INFECTED AMϕ from both 2-week-old foals and adult horses.

Regardless of infection status, there was an overall significant effect of age on mean IFN-γ concentrations ($P = 0.032$), with significantly higher production by AMϕ from 8-week-old foals than cells from 2-week-old foals. Mean IFN-γ concentrations from AMϕ isolated from 4-week-old foals and adult horses were intermediate and did not differ significantly from those of other ages ($P > 0.07$, $P > 0.16$, respectively). There was no significant overall effect of infection ($P = 0.13$) and no significant interaction between the effects of age and infection ($P = 0.37$) on mean IFN-γ concentrations. Separate comparisons of INFECTED and CONTROL conditions were performed for each age. For these comparisons, there was no significant effect of infection at 2 weeks ($P = 0.88$) or 8 weeks ($P = 0.93$) of age, but the mean IFN-γ production from INFECTED AMϕ was higher than that of CONTROL AMϕ at 4 weeks of age ($P = 0.015$) and in adult horses ($P = 0.027$).

Many samples had concentrations of IL-1β, IL-10, and IL-17 that were below the limit of detection of the assay, impacting the ability to accurately evaluate for differences in mean cytokine production. Therefore, the probability that detectable cytokine concentrations were generated for different ages and infection states was evaluated and is shown in Figure 4.5. The proportion of samples producing IL-1β and IL-10 concentrations above the limit of detection was significantly higher in INFECTED AMϕ compared to CONTROL AMϕ isolated from 4-week-old foals ($P = 0.004$ and $P = 0.008$, respectively). For IL-17, the proportion of samples producing concentrations above the limit of detection was significantly higher in INFECTED AMϕ compared to CONTROL AMϕ only for adult horses ($P = 0.031$).

The concentrations of IL-4 and IFN-α in cultured AMϕ supernatant were below the limit of detection for all samples for all ages and culture conditions. In a factorial analysis of IL-6 concentrations in AMϕ supernatant, there was no significant effect of age (P = 0.76) or infection (P = 0.65), and no significant interaction between the effects of age and infection (P = 0.39; data not shown).

Discussion

VDR expression in AMϕ is impacted by age and *R. equi* infection

Results of this study support our hypothesis that VDR expression in equine AMϕ is impacted by age. VDR expression was significantly higher in infected AMϕ from 8-week-old foals compared to adults, indicating age-related differences. Additionally, our findings demonstrate downregulated VDR expression by AMϕ from adult horses after *R. equi* infection, representing the first report of changes in VDR expression after bacterial exposure in equine leukocytes.

Differences in VDR expression between AMϕ from 8-week-old foals and adult horses in our study may be associated with age-related differences in susceptibility to *R. equi* infection. Clinical signs of *R. equi* pneumonia are often not seen until foals are at least 8-12 weeks old (Cohen *et al*., 2013; Liu *et al*., 2011). The increased VDR expression in AMϕ from 8-week-old foals seen in our study, therefore, mirrors the age at which clinical disease becomes apparent in many foals. Previous studies indicate that pro-inflammatory cytokine production required for clearance of *R. equi* is altered in young

foals (Breathnach *et al.*, 2006; Jacks *et al*., 2007). In humans, the vitamin D/VDR pathway attenuates pro-inflammatory cytokine production while augmenting release of anti-inflammatory and regulatory cytokines (Bikle, 2022). It is possible that increased VDR expression by AMϕ from 8-week-old foals facilitates activation of the vitamin D/VDR pathway, thereby reducing production of protective pro-inflammatory cytokines and allowing replication of *R. equi* that culminates in clinically apparent pneumonia in the foal.

Previously, we reported significantly higher VDR expression in AMϕ isolated from healthy foals at 2 weeks of age compared to AMϕ from older foals and adult horses, when quantified directly after bronchoalveolar lavage (Berghaus *et al.*, 2023). This differs from the current study, where VDR expression from cultured AMϕ at 7 dpi was significantly higher at 8 versus 2 weeks of life. The *ex vivo* infection and cell culture methodology utilized in this study may have affected VDR expression, since the cell culture environment cannot provide the full dynamic regulation of hormonal pathways. Other microbiologic, immunologic, and physiologic factors that direct cellular function *in vivo* but are absent in the cell culture environment may also account for differences between studies. Regardless, age-related increases in VDR expression by AMϕ were documented in both investigations. The timing of these increases in VDR expression, coupled with other previously documented concurrent increases in immune parameters, such as B-cell populations, antibody responses and T-cell responses (Ryan & Giguère, 2010; Stoneham, 2008), may support an immunomodulatory role for the vitamin D/VDR pathway in the horse. In neonates of several species, including foals,

inflammatory responses are reduced during the earliest days of extrauterine life, and it is possible that higher VDR expression in young foals contributes to these specialized immunological responses (Dickie *et al.*, 2009; Hirsch *et al*., 2011; Ryan & Giguère, 2010). Additional studies are required to further investigate the proposed relationship between components of the vitamin D/VDR pathway, cytokine profiles, and responses to *R. equi* infection in foals.

R. equi infection was not associated with changes in VDR expression in cells from foals in the current study. The *R. equi*-mediated downregulation of VDR observed here in AMϕ from adult horses is interesting and surprising, as *up*regulation of VDR expression in activated pulmonary macrophages from humans with *Mtb* is widely reported (Bikle, 2022; Liu & Modlin, 2008; Liu *et al.*, 2006; Xu *et al*., 2014). It is possible that in *R. equi*-infected adult equine AMϕ, downregulation of the VDR allows for the production of IFN-γ, an inflammatory cytokine required for bacterial clearance (Hines *et al.*, 2003; Kanaly *et al*., 1995). This could explain why adult horses are almost universally protected from *R. equi* pneumonia, yet adult humans are susceptible to *Mtb*. Additional studies characterizing downstream impacts of these changes in VDR regulation and to determine if a *R. equi*-mediated decrease in VDR expression is detectable in older foals, once they are past the age of *R. equi* pneumonia susceptibility, are needed.

Vitamin D metabolite synthesis in cultured AMϕ was detected but did not vary with age or infection

Neither age nor *R. equi* infection impacted active vitamin D synthesis in our cultured AMϕ from healthy foals or horses, in contrast to what has been shown in similar intracellular infections in other species (Reichel *et al.*, 1991; Reichel, Koeffler, *et al.*, 1987). As adequate substrate for active vitamin D synthesis was provided by donor horse serum in our complete media, we believe that low cell numbers in our samples and timing of supernatant collection may have limited our ability to detect differences. Reichel *et al*. demonstrated the ability to measure $1,25(OH)_2D$ from cultured human and porcine alveolar macrophages, either constitutively (porcine) or in response to activation with lipopolysaccharide or IFN-γ (humans) (Reichel *et al*., 1991; Reichel, Koeffler, & Norman, 1987). In human macrophages, $1,25(OH)_2D$ was measured after 6 days in culture from large flasks containing 30 times more cells than in our study. The AMϕ from pigs were seeded at a similar density as in our study, but $1,25(OH)_2D$ was measured after just 24 hours in culture compared to 7 days in our study. Additionally, $1,25(OH)_2D$ concentrations were quantified using HPLC, a technique with a four-fold lower limit of detection than the immunoassay used herein (Farrell & Herrmann, 2013). It is possible that subtle changes in $1,25(OH)_2D$ synthesis by activated cells in our study may have occurred but were undetectable with the current experimental design. Our data suggest that equine AMϕ constitutively produce $1,25(OH)_2D$, which might allow activated cells to make the most efficient necessary genomic responses. Future studies evaluating infection-related kinetics of $1,25(OH)_2D$ synthesis in larger quantities of

cultured equine AMϕ, rates of steroid hormone degradation, and use of HPLC analysis, or *in vivo* infection models, would be necessary to definitively address these questions.

Intracellular survival and replication of *R. equi* within cultured AMϕ was not impacted by age

Intracellular replication of *R. equi* within cultured equine AMϕ has been demonstrated previously in cells isolated from both foals and adults, with intracellular survival being highest in foals at 8-weeks-old compared to younger foals or adults (Berghaus *et al*., 2014; Bordin *et al*., 2021). In the current study, however, significant age-related differences in intracellular replication were not detected. Small sample size is likely responsible for our inability to detect differences. Intracellular replication data could only be collected in five of the nine adult horses due to fungal contamination in cells or low cell recovery in our BAL samples, likely impacting our ability to detect significant differences in intracellular replication in adults compared to foals, representing an important limitation of this study. Several samples from adult horses resulted in a negative-fold change in intracellular bacteria, and there is a clear trend toward a significant decrease in intracellular replication in adult horse samples compared to foals at all ages, when the data are visually inspected. A similar reduction was not apparent in foal cells at any age.

An additional factor that may have affected our ability to detect differences in bacterial growth is the MOI used in this study. In a previous *ex vivo* model of *R. equi*

infection in AMϕ, an MOI of one or ten virulent *R. equi* per cell was utilized, and intracellular survival and replication was measured at just 2 days post infection (Berghaus *et al*., 2014; Bordin *et al*., 2021). In the study herein, MOI of 0.1 with a longer duration of culture time (7 days) was chosen to more closely mimic the low but persistent environmental exposure to virulent *R. equi* that most foals experience in natural infection states (Cohen *et al.*, 2013). Importantly, the chosen MOI for our study was still sufficient to maintain measurable evidence of intracellular survival and replication with minimal disruption to the AMϕ monolayer in culture and represents a viable technique for use in future studies.

Cytokine production was impacted by *R. equi* infection in an age-dependent fashion

Cytokine responses to infection are thought to play a pivotal role in the unique age-related susceptibility of foals to *R. equi* pneumonia. To the authors' knowledge, this is the first report of IL-17 or IL-1β protein production in response to *R. equi* infection in AMϕ from either foals or adult horses. IL-17 is a pro-inflammatory cytokine that regulates inflammatory responses and is important in the clearance of intracellular bacteria (Korn *et al.*, 2007; Kuwabara *et al*., 2017). Previously, stimulation of pulmonary leukocytes from foals older than 1 week of age with *R. equi* was shown to significantly induce IL-17 mRNA expression, but samples from adults were not evaluated (Liu *et al.*, 2011). In the current study, despite wide variation in concentrations and many samples below the limit of detection, production of IL-17 by cultured AMϕ was detected in at

least some foals at all ages and in the majority (66%) of adult horses. In adult horses, only infected cells produced IL-17, indicating this cytokine may be more tightly regulated in the mature immune cells in the uninfected state. An adequate IL-17 response has been shown to be important for bacterial clearance of *Mtb* infections (Torrado & Cooper, 2010). However, the role of IL-17 in disease progression and severity of naturally occurring *R. equi* is unclear, and T-helper 17 immune responses in *R. equi* infection in foals warrant further investigation.

The balance between both pro-inflammatory and anti-inflammatory cytokines, rather than individual cytokine concentrations, likely plays a role in effective killing of intracellular bacteria (Bordin *et al*., 2022). Findings from our study support this, as the proportion of infected cells that produced both IL-1β (an important pro-inflammatory cytokine) and IL-10 (an important anti-inflammatory cytokine) were significantly higher in cells from 4-week-old foals than all other ages. Four weeks of age is often when initial evidence of lung lesions can be seen by ultrasonographic screening for foal pneumonia but is often prior to clinical signs of disease, if and when they manifest in foals (McCracken *et al*., 2009; Slovis, 2005). Individual disease severity or organism clearance during this age window may hinge upon maintenance of pro-and anti-inflammatory cytokine balance.

Findings from our study are consistent with previous work indicating that IFN-γ production, in response to *R. equi* stimulation, is limited in neonatal foals (Breathnach *et al.*, 2006; Hooper-McGrevy et al., 2003). In our study, IFN-γ production by AMϕ from 8-week-old foals was higher than production by cells from younger foals regardless of

infection status. In contrast to the lack of response to infection seen in AMϕ from foals, increased IFN-γ production in response to *R. equi* infection was noted in AMϕ from adult horses. This age-related post-infection change in IFN-γ production likely reflects maturation of the immune system. TNF-α has similarly been implicated as an important pro-inflammatory cytokine in *R. equi* disease, and several studies have shown an increase in TNF-α gene expression in cells infected with *R. equi ex vivo* (Giguère *et al*., 1999; Kasuga-Aoki *et al*., 1999; Liu *et al*., 2009; Nordmann *et al*., 1993). In our study, *R. equi* infection resulted in TNF-α production by AMϕ from adult horses and from foals at 4 and 8 weeks of age, but not in cells isolated from these foals at 2 weeks old. Previously, we showed that priming with TNF-α significantly reduced intracellular replication of *R. equ*i in AMϕ in an *ex vivo* infection model (Berghaus *et al.*, 2018). It is possible that the limited TNF-α production after *R. equi* infection, seen in AMϕ from the youngest foals, contributes to establishment of initial infection and the insidious progression of the disease (Liu *et al.*, 2009).

The vitamin D/vitamin D receptor pathway may be activated in *R. equi* infection

Lastly, as described above, we demonstrated modulation of VDR expression and vitamin D-regulated cytokines in response to *ex vivo R. equi* infection in AMϕ from adult horses and in some circumstances, older foals. These findings support the hypothesis that the vitamin D/VDR pathway may play a role in the immune response to *R. equi* infection in the horse. However, our finding of down-regulated VDR expression in

infected cells from adult horses is surprising, considering the role that the vitamin D/VDR pathway plays in human *Mtb* infection. Upon activation with *Mtb*, human AMϕ demonstrate upregulated VDR expression and 1,25$(OH)_2$D synthesis, and regulation of important down-stream response elements, including specific cathelicidins (LL_37) and antimicrobial peptides (Liu *et al*., 2007; Selvaraj, 2011). It is unknown if the vitamin D/VDR pathway regulates production of any antimicrobial peptides in the horse in any infections. Additionally, in activated human and rodent macrophages, upregulation of the vitamin D/VDR pathway is associated with a reduction in pro-inflammatory cytokine production and increases the production of the anti-inflammatory cytokine, IL-10. In cells from our adult horses, the down-regulation of VDR expression coincided with an increase in pro-inflammatory cytokines (TNF-α and IFN-γ) and limited production of the anti-inflammatory cytokine, IL-10, suggesting that the vitamin D/VDR pathway may regulate similar genomic responses as seen in human and rodent macrophages. Further study is needed to determine if the vitamin D/VDR pathway directly regulates cytokine responses and production of antimicrobial peptides in the horse, and to examine the role of this pathway in age-related susceptibility to *R.equi*.

There were several limitations to our study. The small sample size and unobtained intracellular replication data from several adult horse samples limited our ability to corroborate previous findings of age-related differences in *R. equi* clearance between horses and foals (Berghaus *et al.*, 2014). Additionally, samples were not collected from these foals after 2 months of age; inclusion of samples from older ages may have elucidated more changes in the vitamin D/VDR axis that might impact

pulmonary immunity and overall age-related susceptibility to *R. equi* pneumonia. Finally, direct comparisons of our study results to other study results cannot be made because the lower MOI and longer duration in culture in our study, chosen to mimic chronic low-grade exposure in naturally occurring disease, differs from methodology used in earlier studies.

Conclusion

The findings from this study support our hypothesis that age-associated differences in VDR expression and vitamin D-associated cytokines would be detected in this *ex vivo* model of *R. equi* infection in equine AMϕ. Age- or infection-related differences in $1,25(OH)_2D$ synthesis in equine AMϕ were not detected, potentially due to species differences in the horse or limitations of the culture system and assays used. This report is the first to describe modulation of the expression of the VDR in equine leukocytes of any type in response to bacterial infection. These findings demonstrate a potential role for the VDR pathway in equine pulmonary immunity and suggest that further study is warranted.

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Table 4.1. Previously validated primers used to quantify gene expression of equine vitamin D receptor (VDR), and housekeeping genes Zeta polypeptide (YWHAZ) and hypoxanthine phosphoribosyltransferase 1 (HPRT1).

Vitamin D receptor (VDR)	Forward: 5'-ACAGCATCCAAAAGGTGGTC-3'
	Reverse: 5'-TGACTTCAGCAGCACGATCT
Zeta polypeptide (YWHAZ)	Forward: TGTTGTAGGAGCCCCGTAGGT
	Reverse: ATTCTCCGAGCCTCTGCTGT
Hypoxanthine phosphoribosyltransferase 1 (HPRT1)	Forward: TTGCTGACCTGCTGGATTAT
	Reverse: TTATGTCCCCTGTTGACTGGT

Figures

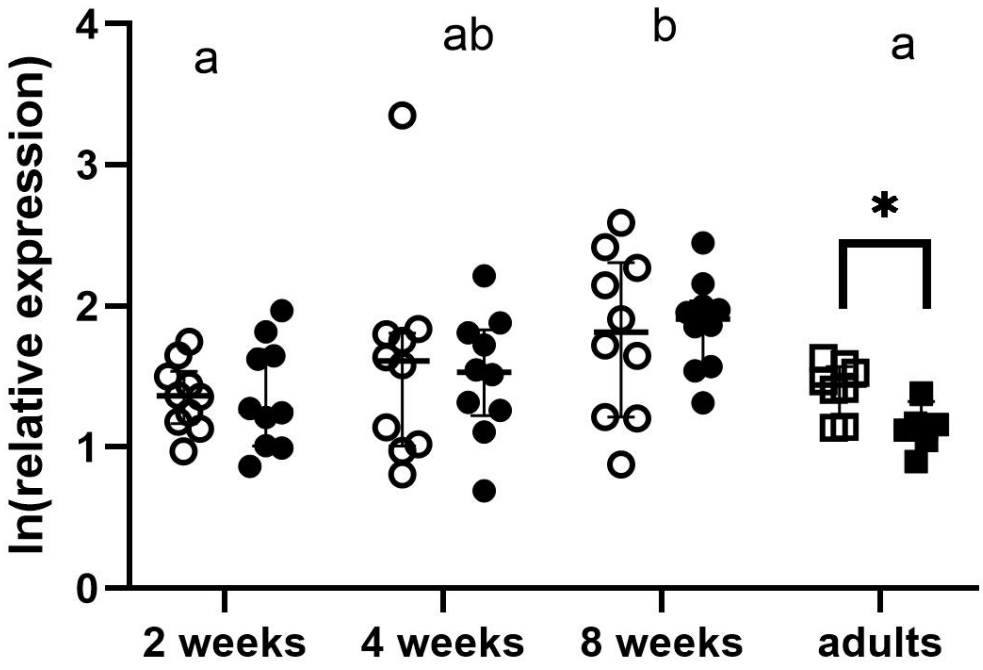

Figure 4.1. Vitamin D receptor (VDR) expression. The mean natural log of the relative expression of VDR in cultured pulmonary alveolar macrophages (AMϕ), 7 days post-mock infection (CONTROL) or *R. equi* infection (INFECTED). AMϕ were obtained from foals at 2, 4, and 8 weeks of age (n = 10) and from adult horses (n = 9), and VDR expression was determined by RT-qPCR using VDR-specific primers and two housekeeping genes (YWHAZ and HRT1), analyzed according to the method described by Pfaffl (Pfaffl, 2001). The median with interquartile range for each age of foal is shown by vertical and horizontal lines on each figure, and measurements from individual foals are represented by open circles (CONTROL) and closed circles (INFECTED), and individual adult horses by open squares (CONTROL) or closed squares (INFECTED). Ages with a superscript letter in common do not differ with a level of significance of 5%. Significant differences between ages are indicated by different superscript letters. Significant effect of infection within a specific age is indicated by *. Significance was set at $P < 0.05$.

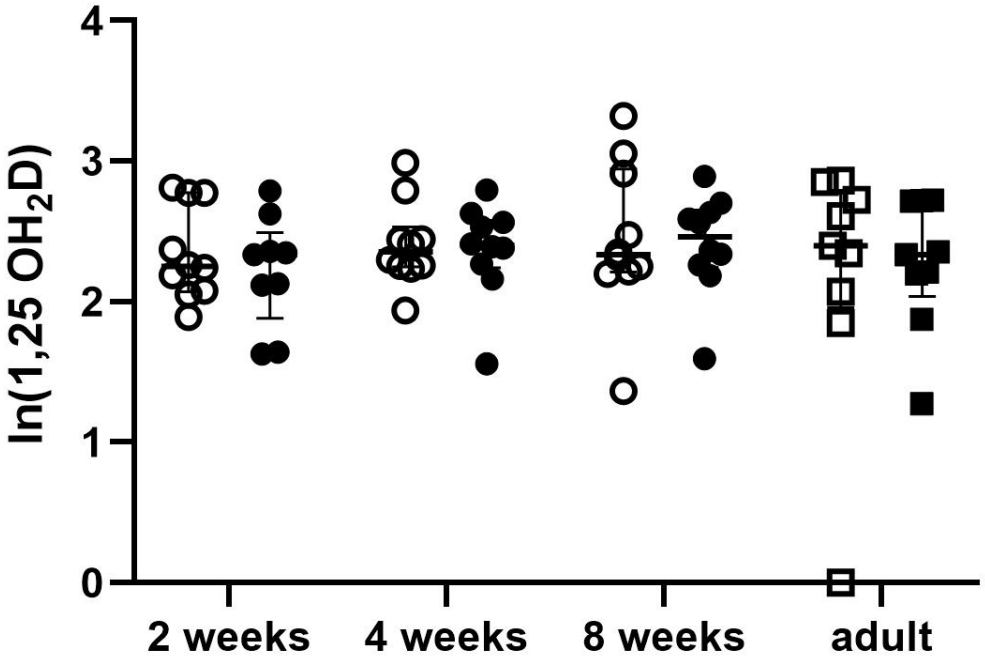

Figure 4.2. Vitamin D synthesis measured in culture supernatant. The mean natural log of 1,25-dihydroxyvitamin D OH_2D (pmol/L) in the supernatant of cultured pulmonary alveolar macrophages (AMϕ), 7 days post-mock infection (CONTROL) or *R. equi* infection (INFECTED) in foals at 2, 4, and 8 weeks of age (n = 10) and adult horses (n = 9) as determined by enzyme immunoassays (IDS, Tyne & Wear, UK) (Kamr *et al.*, 2015; Pozza *et al*., 2014). The median and interquartile range for each age of foal are represented by vertical and horizontal lines, and measurements from individual foals are represented by an open circle (CONTROL) and closed circle (INFECTED), and for individual adults by open square (CONTROL) or closed square (INFECTED). No significant differences were detected between age comparisons or infection status. Significance was set at $P < 0.05$.

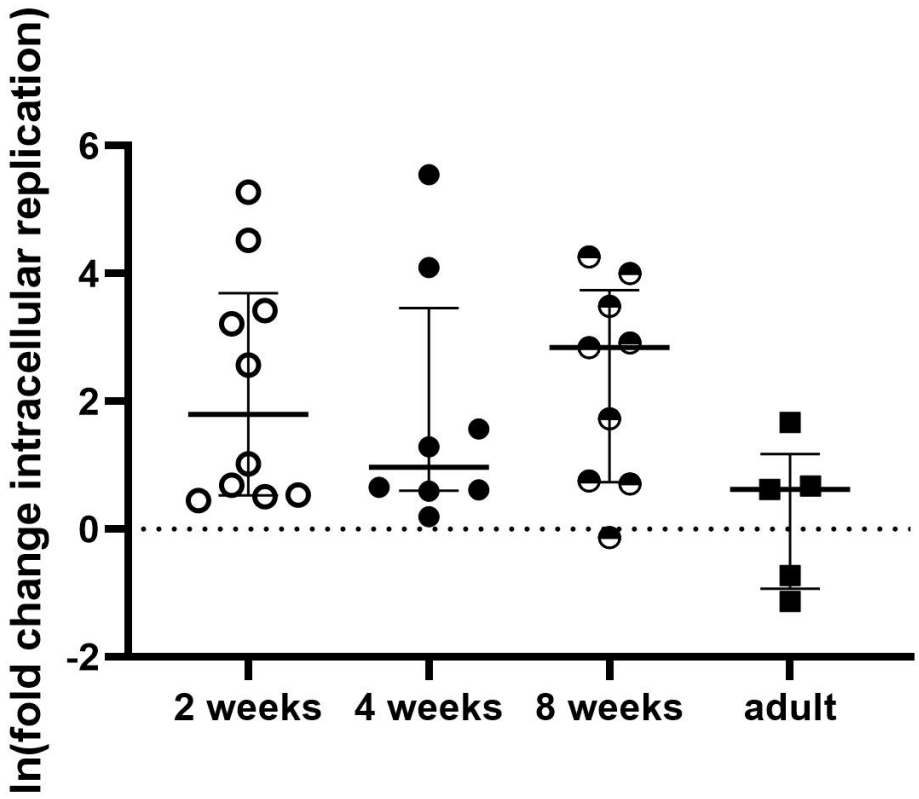

Figure 4.3. Intracellular replication of *Rhodococcus equi* (*R. equi*). The natural log of fold change of intracellular replication of *R. equi* in cultured pulmonary alveolar macrophages (AMϕ) obtained via bronchoalveolar lavage, at 7 days post-*R. equi* infection at a multiplicity of infection of 0.1 bacteria per cell, as measured by counting colony forming units/mL (CFU/mL) in cells from foals collected at 2, 4, and 8 weeks of age (n = 10) and from adult horses once (n = 9). The median and interquartile range for each age of foal is represented by vertical and horizontal lines, and measurements from individual animals are represented by an open circle (2-week-old foals), closed circle, (4-week-old foals), half-closed circle (8-week-old foals), and closed square (adult horses). No significant differences were detected between age comparisons or infection status. Significance was set at $P < 0.05$.

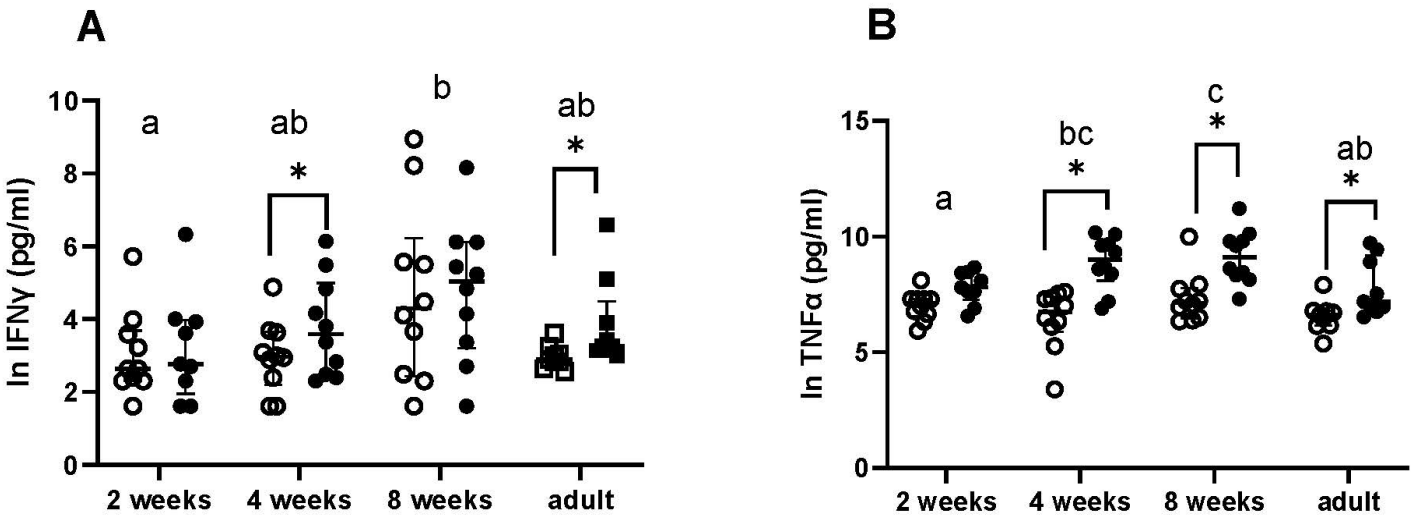

Figure 4.4. Interferon (IFN)-γ and tumor necrosis factor (TNF)-α concentrations. The natural log of the mean concentrations of cytokines IFN-γ (A) and TNF-α (B), measured in the supernatant of cultured pulmonary alveolar macrophages (AMϕ) obtained via bronchoalveolar lavage, 7 days post-mock infection (CONTROL) or *R. equi* infection (INFECTED) in foals at 2, 4, and 8 weeks of age (n = 10) and adult horses (n = 9) as determined via validated immunoassays (Naskou *et al*., 2018; Wagner & Freer, 2009). The median and interquartile range for each age of foal is represented vertical and horizontal lines, and measurements of individual foals are represented by an open circle (CONTROL) or closed circle (INFECTED), and for individual adult horses by open square (CONTROL) or closed square (INFECTED). Ages with a superscript letter in common do not differ with a level of significance of 5%. Significant ($P < 0.05$) effect of infection within each age is indicated by *.

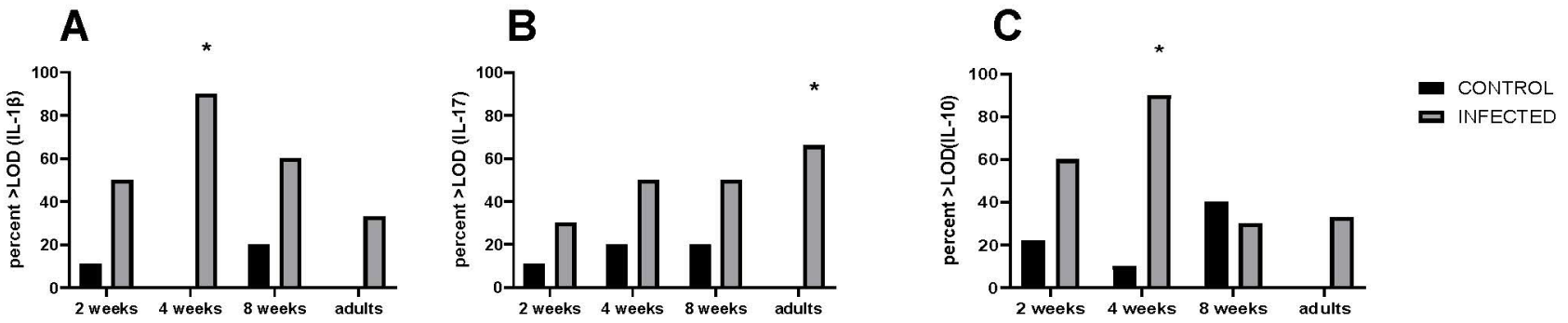

Figure 4.5. Samples of interleukin (IL)-1β, IL-17, and IL-10 above the limit of detection. The percentage of samples with IL-1β (A), IL-17 (B), and IL-10 (C) concentrations above the respective assay limit of detection in the supernatant of cultured pulmonary alveolar macrophages (AMϕ) obtained via bronchoalveolar lavage at 7 days post-mock infection (CONTROL) or *R. equi* infection (INFECTED) in cells isolated from foals at 2, 4, and 8 weeks of age (n = 10) and adult horses (n = 9). Cytokine concentrations were determined via previously validated immunoassays (Lopez *et al.*, 2019; Naskou *et al*., 2018; Wagner & Freer, 2009). Mean concentrations were not directly comparable due to the number of samples below the assay limit of detection (LOD). The percentage of samples above the assay limit of detection for each cytokine is represented by a black bar (CONTROL cells) or a gray bar (INFECTED cells) at each age. Significant ($P < 0.05$) effect of infection is indicated by *.

CHAPTER 5

CYTOKINE, CORTISOL, AND VITAMIN D PROFILES IN FOALS FROM BIRTH TO WEANING ON A FARM ENDEMIC FOR *RHODOCOCCUS EQUI* PNEUMONIA[1]

[1]*1* Berghaus, LJ, Venner, M, Helbig, H, Hildebrandt, D Hart, KA 2024. Cytokine, cortisol, and vitamin D profiles in foals from birth to weaning on a farm endemic for Rhodococcus equi pneumonia. To be Submitted American Journal of Veterinary Research 2024

Abstract

Objectives: *Rhodococcus equi* (*R. equi*) causes pneumonia in young foals, but disease susceptibility and severity vary. Cortisol and vitamin D modulate immune responses and cytokine production during bacterial infection, and altered concentrations are associated with sepsis in neonatal foals. We hypothesized an age- and disease-effect on circulating steroid hormone concentrations in foals, and that differences in cytokines and steroid hormone concentrations would predict disease severity in pneumonic foals.

Methods: Blood samples were collected from two hundred initially healthy foals, on a pneumonia-endemic breeding farm after birth and at 1, 2, 4, 8, 12, and 20 weeks of age. Health status was tracked weekly, and at weaning (20 weeks), foals were divided into three health groups: 1) foals that remained healthy, 2) foals that developed subclinical, self-resolving pneumonia, and 3) foals that developed clinically apparent pneumonia necessitating antimicrobial treatment. Foals were randomly selected (n = 30/group) for cortisol, vitamin D and cytokine analysis using validated assays. Data were analyzed with mixed linear regression with Bonferroni post-hoc analysis ($P < 0.05$).

Results: We observed disease-associated differences for IFN-γ at 4 weeks ($P < 0.007$) and 20 weeks ($P < 0.008$) and age-associated differences for cortisol, vitamin D and other cytokines ($P < 0.001$).

Conclusions: Periodic measurement of circulating steroid hormones and cytokines from birth to weaning was not predictive of pneumonia susceptibility and severity in foals on a farm with endemic pneumonia. Disease-associated IFN-γ differences warrant further investigation.

Key words

equine, pneumonia, vitamin D, cortisol, cytokines, *Rhodococcus equi*

Introduction

*Rhodococcus equ*i (*R. equi*) is a ubiquitous, soil saprophyte that can cause endemic pneumonia on some horse breeding farms. Characterized by pyogranulomatous bronchopneumonia, *R. equi* pneumonia is most often found in foals from two to five months of age and is rarely seen in healthy older foals or adult horses (Giguère et al., 2011a). On endemic farms, appreciable variability in individual responses to the bacteria occurs for unknown reasons. Most foals will clear the bacteria without the need for veterinary intervention, while other foals require extensive antimicrobial treatment that may or may not be successful (Venner et al., 2012). Treatment of pneumonic foals is expensive in terms of both cost of medication and farm labor, and affected foals are less successful athletes as adult horses (Ainsworth et al., 1998; Giguère et al., 2011b). Currently it is not possible to differentiate between individual foals that will clear the bacteria and those that will require antimicrobial treatment. Discovering specific factors that could predict disease severity in individual foals would benefit the equine industry, potentially reducing both costs of treatment for breeders and overall antimicrobial usage on horse-breeding farms.

In the host, bacterial infections lead to immune responses that include dynamic changes in cytokine profiles (Adkins et al., 2004). For intracellular bacteria like *R. equi*, the cytokine interferon-gamma (IFN-γ), has been considered a vital mediator for

clearance (Kanaly et al., 1995; Murphy et al., 2012; Nordmann et al., 1993). At birth foal leukocytes stimulated with mitogens display a limited mRNA expression and ability to produce IFN-γ protein, and it is assumed that this immunological deficiency may play a role in the foal's susceptibility to *R. equi* pneumonia (Bordin et al., 2022; Boyd et al., 2003; Breathnach et al., 2006). The availability of tumor necrosis factor (TNF)-α and interleukin (IL)-10 (and the balance between these) has also been shown to impact the immune response to *R. equi* (Berghaus et al., 2018; Bordin et al., 2021; Harris et al., 2011). However, most of what we understand about *R. equi*-associated cytokine dynamics are from challenge models of infection (murine and equine), evaluation of cytokine gene expression, or in vitro infections of equine leukocytes (Berghaus et al., 2018; Berghaus et al., 2014; Bordin et al., 2021; Giguère & Prescott, 1998; Giguère et al., 1999). A thorough investigation of *in vivo* cytokine profiles of foals during natural exposure to *R. equi* and subsequent clearance of bacteria or development of pneumonia is an important gap in our knowledge.

The steroid hormones cortisol and vitamin D are mediators of the immune response and regulate inflammatory cytokine production and are important in supporting bacterial clearance during infection (Ikuta et al., 2022). Age-associated differences in circulating concentrations of these hormones between newborn foals and adults have been reported (Hart et al., 2011a; Hart et al., 2009a; Pozza et al., 2014). Furthermore, disease-associated differences for both cortisol and vitamin D concentrations have been demonstrated in septic newborn foals compared to healthy newborns (Hart et al., 2009b; Kamr et al., 2015). Foals are initially exposed to *R. equi*

shortly after birth, so these age-associated differences in cortisol and vitamin D in neonatal foals may play a role during this age-window of susceptibility to disease caused by *R. equi*. Concentrations of cortisol and vitamin D have not been investigated in foals that develop pneumonia after natural exposure to *R. equi* on a farm endemic for disease.

The objective herein was to investigate circulating concentrations of cytokines previously shown to be associated with experimental *R. equi* infection and the immunomodulatory steroid hormones cortisol and vitamin D, as potential predictors of individual disease severity in foals naturally exposed to *R. equi* on a farm endemic for disease. Specifically, we measured concentrations of circulating cytokines [IFN-γ, TNF-α and interleukins (IL-) 4, 10, and 17], cortisol [total and percent free cortisol (%FC)] and vitamin D metabolites [25(OH)D and 1,25(OH)D] to determine their association with respiratory disease incidence and severity. We hypothesized that there would be a significant effect of age and respiratory disease on steroid hormone concentrations and that differences in cytokine and steroid hormone concentrations, would predict disease severity in foals with clinical pneumonia.

Materials and Methods

Ethics statement

The University of Georgia (UGA), College of Veterinary Medicine's Clinical Research Committee approved all sample and medical record data collection protocols. Informed client consent was obtained prior to farm sampling.

Study population

Two hundred healthy warmblood foals were enrolled in this prospective cohort study during the 2021 (n = 100) and 2022 (n = 100) foaling seasons on a large European breeding farm that has a history of pneumonia attributed to *R. equi*. All enrolled foals were born between the months of March and May each year. Foals were enrolled if they were born via an uncomplicated vaginal delivery after full-term (≥ 330 days) gestation, had a normal, initial, physical examination after birth, and had adequate transfer of passive immunity (plasma IgG concentration > 800 mg/dL at 12-24 hours of age). All enrolled foals were subject to similar management, housing, and routine veterinary care practices during the entire sampling period per farm standard practices.

Foal monitoring

Weekly monitoring of each foal, from birth through weaning, included physical exam, peripheral white blood cell count, and trans-thoracic ultrasonography performed using a portable unit and a 7.5-MHz linear transducer to evaluate for pulmonary consolidation consistent with pulmonary abscessation (Arnold-Lehna et al., 2019). A focal hypoechoic area of consolidation, with a diameter of > 1.0 cm was considered an abscess. Both the number of abscesses and the total diameter of each abscess were recorded at each examination. A clinical scoring system (body temperature, respiratory rate, presence and type of nasal discharge, palpitation of mandibular lymph nodes, presence of dyspnea, and auscultation of the lungs and trachea) combined with lung abscess score was utilized to determine a foal's health status as previously described

(Venner et al., 2007; Venner et al., 2009). Foals with clinically apparent pneumonia and abscess scores > 15 cm were placed on a 6-week antimicrobial therapy regimen according to standard farm practices (Arnold-Lehna et al., 2019; Venner et al., 2013). Foals with mild to moderate pneumonia were treated with the combination of rifampin (10 mg/kg once daily orally) and tulathromycin (2.5 mg/kg once weekly IM), and foals with more severe clinical signs of pneumonia were treated with rifampin (10 mg/kg once daily orally) and azithromycin (10 mg/kg once daily orally). Foals on antimicrobial treatment were monitored daily for adverse reactions, such as diarrhea, lameness, colic, or injection site swelling (foals treated with tulathromycin), and therapy was adjusted as needed. Additionally, if abscess scores were not reduced following 1 week of therapy, then the macrolide option was changed. Antimicrobial therapy was extended by 2 or more weeks for foals with persistent sonographic evidence of pulmonary lesions at the end of the initial 6 weeks of treatment.

Sample collection

Approximately 30 mL of blood was collected via direct venipuncture from all 200 enrolled foals during their weekly exam beginning shortly after birth and again at 1, 2, 4, 8, 12, and 20 weeks of life. Blood samples were divided between serum, lithium heparin, and ethylenediaminetetraacetic acid (EDTA) tubes, centrifuged, and serum or plasma separated, aliquoted into 1.5 mL Eppendorf tubes, and stored at -80^{o}C until completion of foal sample collection each season. Frozen samples were shipped on dry ice to the University of Georgia (UGA) (Athens, Georgia, USA) for processing. A commercial

shipping service accustomed to managing biological sample transport (World Courier, New Hyde Park, NY) was employed to transport and maintain temperature of samples throughout shipment process. Samples were then stored at -80^{o}C at UGA until batch analysis.

Foal randomization

After weaning, medical records data were reviewed for all enrolled foals, and foals were categorized into one of three health categories. For the purposes of our investigation, a foal was considered HEALTHY if it had no abnormal physical exam findings or evidence of pulmonary lesions via trans-thoracic ultrasonography on weekly exams from birth to 20 weeks of life. A foal was considered SUBCLINICAL if, on at least one weekly exam sonographic pulmonary lesions were detected, but the foal showed no clinical signs of pneumonia and had a lung abscess score of < 15cm and was never placed on antimicrobial therapy. Lastly, foals that showed clinical signs of respiratory disease and/or had a lung abscess score of > 15 cm that prompted antimicrobial therapy for treatment of pneumonia at any point from birth to weaning were categorized as CLINICAL. Then, 90 foals (n = 30 in each of the HEALTHY, SUBCLINCIAL, and CLINICAL groups) were randomly chosen for sample analysis (Research Randomizer randomizer.org). More specifically, to eliminate sample year bias, 15 foals from each health category were randomly selected from each foaling season (2021 and 2022). These group sizes based on *a priori* sample size calculations were based on published vitamin D data in healthy and sick, non-septic, neonatal foals that revealed to be able to

detect a 20% difference in vitamin D concentrations between groups with α set at 0.5 and statistical power set at 80%, 25 foals in each group would be needed (PASS Version 16.0.9; NCSS, LLC; Kaysville, Utah, USA) (Kamr et al., 2015). We chose to include thirty foals per group in case other measured parameters had more variation.

Sample analysis

Evaluation of cytokine concentrations in plasma

Plasma concentrations of the following cytokines were measured with validated immunoassays: IL-4, IL-10, IL-17, IFN-γ, and TNF-α. These cytokines were selected for their importance in *R. equi* immune function or association with steroid hormone regulation (Berghaus et al., 2018; Giguère et al., 1999; Hines et al., 2003). IL-4, IL-10, IL-17 and IFN-γ concentrations were measured with a previously validated, bead-based immunoassay (5-plex, Animal Health Diagnostic Center, Cornell University, Ithaca, NY) as previously described (Wagner and Freer 2009). Cytokine concentrations were calculated based on a standard curve for each cytokine, with the following lower and upper limits of detection: IL-4: 40-80,000 pg/mL, IL-10: 15-35,000 pg/mL, IFN-γ: 10-5000 Units/mL, and IL-17: 10-10,000 Units/mL. TNF-α concentrations were measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit (R&D Duoset, Minneapolis, MN) according to manufacturer directions as previously described ((Berghaus et al., 2024; Burton et al., 2009; Lopez et al., 2019; Naskou et al., 2018). Reported lower and upper limits of detection for TNF-α are 31.3-2000 pg/mL. Samples above the standard curve were diluted as necessary to fall within the linear portion of the standard curve. A

four-parameter logistic regression analysis was created (GraphPad Prism version 10.5.1, GraphPad Software, LLC San Diego, CA) to interpolate the concentration of each sample against the standard curve. Ratios of cytokine concentrations were made for IFN-γ/IL-10, and TNF-α/IL-10 by dividing the concentration of the proinflammatory cytokine (IFN-γ or TNF-α) by the concentration of the anti-inflammatory cytokine (IL-10).

Cortisol

Circulating total cortisol concentrations in serum (hereafter referred to as total cortisol) were measured using a previously validated chemiluminescent immunoassay with a lower limit of detection of 0.2 ug/dL (Reimers et al., 1996; Singh et al., 1997). Serum-free cortisol fraction was determined by a modification of an ultrafiltration assay previously described for use in horses (Hart et al., 2011a; Lewis et al., 2005). Briefly, frozen aliquots of serum were allowed to thaw at room temperature, before equilibrating for 1 hour at 37^{o}C. Micron-filters (size 10 kiloDalton) (placed in 1.5 mL tubes) were preconditioned by the addition of 400 µL of a 0.1% gelatin solution and centrifuging at 14,000 x g for 30 minutes. Following the initial centrifugation, the preconditioned filters were transferred to new 1.5 mL tubes. Four hundred microliters of equilibrated serum were added to the preconditioned filters and centrifuged at 14000 x g for one hour at room temperature. Cortisol concentrations of both equilibrated serum, before and after filtration were determined using a commercially available ELISA (Salimetrics, State College, PA) previously validated for use on equine serum and optimized for use of filtrate in our laboratory as calculated by four-parameter logistic

regression model using commercial software (GraphPad Prism version 10.5.1, GraphPad software, LLC San Diego, CA) (Hart et al., 2016). Validation of this modified free cortisol fraction assay resulted in an inter-assay coefficient of variance (CV) of 14.71% and intraassay CV of 14.16%. The Salimetrics assay has a range of 0.012-3.00 µg/dL, and samples were diluted as necessary to fall within the linear portion of the standard curve. Free cortisol fraction is expressed as % free cortisol (%FC), and estimated by dividing calculated concentration of filtrate (representing unbound cortisol) by the calculated concentration of equilibrated serum before filtration (representing total cortisol) and multiplied by 100 as previously described (Hart et al., 2011a).

Vitamin D metabolites

Serum vitamin D metabolite [25(OH)D and 1,25(OH)D] were measured utilizing previously validated enzyme immunoassays (Immunodiagnostic Systems, Tyne & Wear, Boldon, UK) according to manufacturer's guidelines and as previously described (Berghaus et al., 2023; Pozza et al., 2014). For 25(OH)D, briefly, 25 µl of equine plasma, assay calibrators, and controls were diluted in biotin-labeled-25(OH)D. The diluted samples were incubated in microtiter wells of a plate precoated with a 25(OH)D antibody at room temperature for two hours. Samples were aspirated and plates washed using phosphate-buffered saline (PBS) containing Tween-20. Horseradish peroxidase labeled avidin was added to bind to complexed biotin within samples. Color was developed using a TMB substrate for 30 minutes. Stop solution was added and absorbance was measured at 450 nm using a microplate reader. Concentration of

samples was determined using GraphPad Prism version 10.5.1 (GraphPad Software, LLC San Diego, CA, USA), and all control samples fell within acceptable range. 25(OH)D concentrations for a subset of these foals born in 2021 have been previously reported but are included here because they were used to compute the ratio of 1,25(OH)D to 25(OH)D (Hart et al., 2023).

For serum 1,25(OH)D concentrations, 500 µL of each sample was delipidated and immunoextracted following manufacturer guidelines. Immunopurified samples were incubated overnight at 4^{o}C with sheep anti-vitamin D primary antibody. One hundred and fifty microliters of sample, plate controls, and plate calibrators were added to pre-coated anti-sheep microtiter plates and incubated for 90 minutes at room temperature. Vitamin D-biotin solution was added to all wells and incubated for an additional 60 minutes. Samples were aspirated and plates washed using PBS containing Tween-20. Horseradish peroxidase-labeled avidin was added to bind to complexed biotin in samples. Color was developed using a 3,3',5,5'-tetramethylbenzidine (TMB) substrate 30 minutes prior to stop solution being added. Absorbance was measured at 450 nm using a microplate reader (Bio Tek Synergy H1, Santa Clara, CA). Sample 1,25(OH)D concentration calculations were performed using a 4-parameter logistic regression model using GraphPad Prism version 10.5.1 (GraphPad Software, LLC San Diego, CA) and control samples fell within the manufacturer's suggested acceptable range. The ratio of 1,25(OH)D to 25(OH)D was calculated by dividing the 1,25(OH)D calculated concentration by the 25(OH)D calculated concentration.

Statistical analysis

Normality and equality of variance of the data was assessed by the Shapiro-Wilk test. Variables that did not meet the assumptions for parametric testing were log-transformed prior to analysis. Total cortisol, %FC, 25(OH)D, 1,25(OH)D, TNF-α, and IL-10 concentrations were compared using linear mixed models. Age and health status were modeled as fixed nominal effects, and foal was included as a random effect to account for repeated measurements within the same foals. Estimation was performed using the method of restricted maximum likelihood, and denominator degrees of freedom for F-tests were approximated using the Kenward-Roger procedure. Model fit was assessed using Akaike´s information criterion. Residuals were modeled using a first-order autoregressive (i.e., AR1) correlation structure. Pairwise comparisons were performed using the Bonferroni procedure to limit the type I error probability to 5% over all comparisons.

For cytokines (IL-17 and IFN-γ), the proportion of samples with a concentration above the lower limit of detection (> LOD) of the assay was compared between groups at each age using a generalized estimating equation logistic model to account for repeated measurements in the same foal with robust standard errors and an exchangeable working correlation structure. Mixed tobit censored regression models with fixed effects for health status and age, and a random effect for foal, were also used to compare IL-17 and IFN-γ concentrations (and age of diagnosis within CLINICAL foals), with values below the lower limit of detection being censored.

Comparisons of the age at subclinical diagnosis between SUBCLINICAL and CLINICAL foals were analyzed by student's *t* test. All tests assumed a two-sided alternative hypothesis, and values of $P < 0.05$ were considered statistically significant. Analyses were performed using commercially available statistical software (Stata version 18.0, StataCorp LLC; College Station, TX).

Results

Foals

During the 2021 and 2022 foaling seasons, 218 foals were initially enrolled into the study between the months of April and May. Six foals in 2021 and eight foals in 2022 were excluded for failure of transfer of passive immunity. One foal in 2021 and four foals in 2022 died before 20 weeks of age (due to injuries unrelated to respiratory disease) and were excluded from the study, leaving 199 foals with samples from birth to 20 weeks for comparison. Approximately 82% (163/199) of the enrolled foals had evidence of pulmonary lesions on ultrasonography, at some point from birth to weaning, with 42.9% (70/163) of those foals resolving these lesions without antimicrobial treatment. As described above, fifteen foals from each group were randomly selected from each year, with the exception of HEALTHY foals from 2021, as there were only 15 enrolled foals that remained healthy through 20 weeks that year; therefore, all 15 HEALTHY 2021 foals were used. The number of foals in each health category and mean age in weeks at the time of respiratory disease diagnosis for SUBCLINCAL and CLINCIAL foals is shown in Table 5.1. Seventy-six percent of foals randomly chosen for downstream sample analysis

(23/30) in the CLINICAL group had a subclinical diagnosis of ultrasonographic pulmonary lesions prior to diagnosis of clinically apparent respiratory disease. The age at which the CLINICAL foals had subclinically detectable ultrasonographic pulmonary lesions was significantly earlier (median of 9.6 weeks) than the age of detectable pulmonary lesions in foals in the SUBCLINICAL group (median of 13.6 weeks) ($P < 0.009$). Of the foals randomly selected in our CLINICAL group, three required additional antimicrobial treatment after the initial 6-week treatment regimen, as per standard farm protocol.

Effect of foal age on circulating cytokine and steroid hormone concentrations

Comparisons of cytokine concentrations at sampled foal ages are shown in Figures 5.1- 5.3. Concentrations of IFN-γ greater than the limit of detection (LOD) were found in only 58% of our samples (371/630), so both the percentage of foals from each group that produced IFN-γ > LOD and the concentrations > LOD were compared among foal ages and health statuses. Age-associated changes in IFN-γ are shown in Figure 5.1A and 5.1B. There was an overall significant effect of age for both the proportion of foals producing IFN-γ and the concentration of IFN-γ produced > LOD ($P < 0.001$). Both the proportion of foals with measurable concentrations of IFN-γ and the concentration of IFN-γ was significantly higher at 4 and 8 weeks of age compared to all other sample ages ($P < 0.050$).

Most foals (> 90%) had measurable concentrations of IL-10 and TNF-α at all timepoints (613/630 and 570/630 samples respectively), but only 45% (284/630) of samples had detectible concentrations of IL-17. Both the percentage of foals from each

group that produced IL-17 > LOD and the concentrations > LOD were compared for IL-17. Overall age-associated changes for IL-10 (Figure 5.2A), TNF-α (Figure 5.2B), and IL-17 (Figure 5.2C) were significant ($P < 0.001$). Concentrations of IL-10 dropped dramatically from birth concentrations to a nadir at 1 and 2 weeks of age before increasing to above birth concentrations at 8 weeks of age and then decreasing back to concentrations comparable to birth at 12 weeks of age ($P < 0.005$). The concentration of TNF-α was highest at birth and 1 week, before significantly decreasing incrementally through 20 weeks ($P < 0.001$). Both the proportion of foals with measurable concentrations of IL-17 (data not shown) and the concentration of IL-17 produced displayed a similar pattern with both increasing to a peak at 4 weeks of age before decreasing to observations equivalent to birth by 20 weeks ($P < 0.050$).

The ratios of the pro-inflammatory cytokines (IFN-γ and TNF-α) to the anti-inflammatory cytokine, IL-10, were also compared (Figure 5.3A and 5.3B). For IFN-γ/IL-10 ratios, a concentration of half the lowest interpolated concentration was assigned to samples that were < LOD. An overall significant effect of age for ratios of both IFN-γ/IL-10 and TNF-α/IL-10 was found ($P < 0.001$). The ratio of IFN-γ/IL-10 increased significantly by 1 week of age and remained high before decreasing to back to birth ratio at 12 weeks of age ($P < 0.020$). TNF-α/IL-10 ratios increased at 1 week of age from birth ratios and then incrementally decreased and were below birth ratios by 4 weeks of age ($P < 0.022$).

Only 17 foals (5 HEALTHY, 6 SUBCLINICAL AND 6 CLINICAL) produced concentrations of IL-4 > LOD, with only one HEALTHY foal producing IL-4 at multiple

timepoints. Thus, meaningful IL-4 comparisons across foal ages were not achievable (data not shown).

Total cortisol and %FC across foal ages is shown in Figures 5.4A and 5.4B. Significant age-associated differences in steroid hormone concentrations were found for both total cortisol and %FC ($P < 0.001$). Total cortisol concentrations (Figure 5.4A) decreased from birth to 1 week of age ($P = 0.008$) but were comparable to birth concentrations by 2 weeks and continued to rise through 12 weeks of age. Percent FC (Figure 5.4B) was highest at birth and dropped significantly ($P < 0.001$) by 1 week and was lower at 20 weeks compared to birth or 1 week ($P < 0.044$).

Concentrations of vitamin D metabolites, 25(OH)D and 1,25(OH)D, and the ratio of 1,25(OH)D to 25(OH)D in foals from birth to 20 weeks of age are shown in Figure 5.5. There was a significant effect of age for both vitamin D metabolites and the ratio of 1,25(OH)D to 25(OH)D ($P < 0.001$). The nadir of both 25(OH)D and 1,25(OH)D was found in birth samples. Concentrations of 25(OH)D gradually increased through 20 weeks with significant increases occurring between 1 and 2 weeks, and between 4 and 8 weeks of age ($P < 0.033$). Concentrations of 1,25(OH)D significantly increased from birth to 1 week of age ($P < 0.001$) and then remained stable before decreasing at 20 weeks of age ($P < 0.001$).

Effect of respiratory disease on circulating cytokine and steroid hormone concentrations

The effect of respiratory disease on circulating concentrations of cytokines is also shown in Figures 5.1-5.3. There was a significant interaction between age and health for the proportion of foals producing IFN-γ ($P < 0.001$, Figure 5.1A) and the interaction between age and health approached significance for the concentration of IFN-γ > LOD ($P < 0.07$, Figure 5.1B). Therefore, separate comparisons were made among health status groups for each timepoint. The proportion of foals producing IFN-γ was higher in CLINICAL foals at 4w compared to the proportion of SUBCLINICAL or HEALTHY foals ($P < 0.033$), but the proportion of CLINICAL foals producing IFN-γ was lower at 20 weeks compared to the proportion of HEALTHY foals producing IFN-γ at 20 weeks ($P < 0.007$). Additionally, the concentration of IFN-γ produced by foals in the CLINICAL group was lower at 20 weeks ($P = 0.008$) compared to concentration produced by foals in the HEALTHY group at that age. There were no differences in either the proportion of foals producing IFN-γ or IFN-γ concentrations between HEALTHY and SUBCLICAL foals at any age ($P > 0.088$).

There was no effect of health status on concentrations of IL-10 (Figure 5.2A), TNF-α, (Figure 5.2B), and IL-17 (Figure 5.2C) ($P > 0.438$) with little variability between groups at any timepoint. Moreover, there were no significant differences between health groups for the ratios of IFN-γ/IL-10 or TNF-α/IL-10 ($P > 0.225$) (Figure 5.3). Meaningful comparisons of disease associated differences for IL-4 concentrations were not achievable because of the substantial number of samples < LOD.

The effect of health status on circulating concentrations of steroid hormones is shown in Figures 5.4 and 5.5. There were no differences between foal health groups for

total cortisol or %FC ($P > 0.310$), and no significant interactions between time and health ($P > 0.899$) (Figure 5.4A and 5.4B). Likewise, there was no effect of respiratory disease for concentrations of 25(OH)D, 1,25(OH)D and ratio of 1,25(OH)D to 25(OH)D ($P > 0.386$) and no significant interactions between time and health status ($P > 0.213$) (Figure 5.5A, 5.5B and 5.5C).

Cytokines and steroid hormones as early predictors of future respiratory disease

To determine if plasma concentrations of cytokines or steroid hormones in early life might predict eventual clinically important respiratory disease in individual foals, we conducted additional analysis to compare parameters only from foals in the HEALTHY and CLINCIAL groups from birth to 4 weeks of age. Because we were specifically looking at these parameters as potential predictors of future disease, we eliminated foals from the CLINICAL group that were diagnosed before 8 weeks of age, because we theorized that circulating concentrations could have been impacted by the initial stages of developing pulmonary disease (n = 5). Thus, we used data from 30 HEALTHY and 25 CLINICAL foals for this analysis.

Concentrations of IL-10 ($P = 0.712$), TNF-α ($P = 0.9180$), IL-17 ($P = 0.501$) or the ratios of TNF-α/IL-10 (P = 0.977) or IFN-γ/IL-10 ($P = 0.64$) did not differ between groups. (Figure 5.2A-C 5.3B). Concentrations of IFN-γ were not different between groups ($P = 0.862$), but there was a significantly higher proportion of CLINCAL foals that produced IFN-γ at 4 weeks ($P = 0.012$), compared to HEALTHY and SUBCLINICAL foals at that age. (Figure 5.6A-D).

There were no between-group differences at any timepoint for total cortisol (P = 0.220) or %FC (P = 0.816) (Figure 5.4). There were also no differences between groups at any timepoint for 25(OH)D (P = 0.842), 1,25(OH)D (P = 0.435) or the ratio of 1,25(OH)D to 25(OH)D (P = 0.436) (Figure 5.5A-C).

Effect of age at respiratory disease diagnosis on IFN-γ concentrations in CLINICAL foals

Because IFN-γ is critical for the clearance of intracellular bacteria and heath-associated differences were found for both the number of foals producing IFN-γ by 4 weeks of age and the concentration of IFN-γ produced at 20 weeks of age, we sought to determine if age at respiratory disease diagnosis was associated with plasma IFN-γ concentration. The median age of CLINICAL diagnosis was 15.4 weeks (Table 5.1). CLINICAL foals were subdivided into two groups: foals diagnosed with clinical pneumonia at less than the median age or at greater than the median age (n = 15/group). Age of diagnosis was not significantly associated with IFN-γ concentration at any age (P > 0.589, Figure 5.7).

Discussion

The aim of this investigation was to look for immunological and steroid hormone dynamics that predict individual differences in foal response to bacterial respiratory pathogens exposure during early life. These data illustrate important previously unknown respiratory disease-associated changes in circulating cytokine concentrations in growing foals during natural exposure to *R. equi* and other respiratory pathogens, and

in the number of foals producing IFN-γ. Additionally, we found age-associated changes in circulating steroid hormones and cytokines that could impact immune system development and function during the period at which foals are at risk of pneumonia associated with *R. equi* and other bacteria. The results from our study did not, however, support our hypothesis that alterations in circulating cytokine, cortisol, or vitamin D concentrations would be associated with disease or be useful predictors of disease severity in foals with naturally occurring pneumonia after *R. equi* exposure. This is, however, to the authors' knowledge the most comprehensive report of age-associated changes in cytokines and steroid hormones in foals from birth to weaning, and the only report to date to examine the role of steroid hormones and cytokine concentrations during naturally occurring *R. equi* exposure on a farm endemic for disease. Furthermore, the results herein expand our understanding of the foal's maturing immune system during natural exposure to respiratory pathogens and provides a valuable foundation of future studies.

Newborn foals have been shown, under some circumstances, to be deficient in T-helper type-1 (Th1) responses, including the production of cytokines like IFN-γ (Breathnach et al., 2006); Hooper-McGrevy et al. (2003); (Lopez et al., 2019; Ryan & Giguère, 2010). IFN-γ has been shown to be critical for clearance of virulent *R. equi* from pulmonary macrophages during *ex vivo* infection (Giguère et al., 1999; Kasuga-Aoki et al., 1999). Thus, it has been hypothesized and generally accepted that newborn foals have a limited capacity to produce IFN-γ, and this deficiency is a key contributor to *R. equi* susceptibility in foals (Bordin et al., 2022). Measurable concentrations of IFN-γ in

mare colostrum and pre-suckle foal serum have been described, but are not associated with an increase in post-suckle IFN-γ concentrations in foal serum (Mariella et al., 2017). Therefore, the source of serum IFN-γ in the newborn foal appears to be endogenous. Approximately 44% of foals sampled had measurable concentrations of IFN-γ in the blood sample collected at birth (post-suckle), with both the number of foals producing IFN-γ and the concentration of IFN-γ produced then increasing during the first month of life. Our findings corroborate work reported by Jacks *et al.* in 2007, which showed that foals at 4 weeks of age were able to mount a Th1-type response to *in vivo* experimental *R. equi* challenge, as demonstrated by upregulation of leukocyte gene expression of IFN-γ (Jacks et al., 2007). Furthermore, our group recently demonstrated that alveolar macrophages obtained from 4-week-old foals were able to produce IFN-γ in response to low dose *ex vivo* exposure, with *R. equi* comparable to adult responses (Berghaus et al., 2024). Significantly more foals with clinically apparent respiratory disease were producing IFN-γ by 4 weeks of age compared to either healthy foals or foals with subclinical respiratory diseases. Furthermore, increased production of IFN-γ compared to healthy foals was found in clinical foals prior to any clinical or ultrasonographic evidence of pulmonary disease. These results indicate that while there may be a degree of limited IFN-γ capacity in newborn foals, this deficiency resolves quickly and may not be directly linked to the age-window of susceptibility to *R. equi* pneumonia.

A critical balance between pro- and anti-inflammatory cytokines has been proposed as an important factor for clearance of intracellular pathogens like *R. equi* from foal lungs (Berghaus et al., 2018; Bordin et al., 2021; Bordin et al., 2022). Our results

showed only minor differences in the ratio of TNF-α/IL-10 regardless of health status and while the ratio of IFN-γ/IL-10 for the HEALTHY group appears visibly higher at most time points, differences never approached significance. Therefore, based on our results, measuring ratios of IFN-γ or TNF-α to IL-10 may not be as useful for predicting naturally occurring respiratory disease.

It is important to note that serum cytokine concentrations as measured in our study might not reflect pulmonary immune responses. In foals, lymphocyte populations are diminished in lung tissue for the first three months of life. Furthermore, dendritic cells (professional antigen-presenting cells that help direct lymphocyte responses) are immature in both phenotype and function for at least the first month of life (Flaminio et al., 2000; Lopez et al., 2019; Lopez et al., 2020). Thus, within the microenvironment of the lung, some foals may struggle to maintain the sustained robust immune response necessary for clearance of a bacterium like *R. equi* that is encountered continuously in the environment and replicates in such an insidious manner. Furthermore, *Mycobacterium tuberculosis*, a related intracellular bacterium that causes respiratory infection in people, has evolved survival mechanisms that decrease T-cell effector responses within pulmonary granulomas (Gern et al., 2021; Wik & Skalhegg, 2022). It is possible that *R. equi* has developed similar survival strategies. The foals in our CLINICAL group had ultrasonographic evidence of infection significantly earlier than foals in the SUBCLINICAL group (Table 1), suggesting that these foals likely needed a longer, more sustained immune response, just when adaptive immunity within the lung is most immature. Though CLINICAL foals were capable of increased IFN-γ production early on (4

weeks of age), by 20 weeks of age, fewer foals in this group produced IFN-γ and the quantity of IFN-γ, they produced was lower than foals in the HEALTHY group.

In order to determine if this diminishing IFN-γ production in CLINICAL foals at 20 weeks might be attributed to bacterial clearance and resolving infection, we stratified IFN-γ concentrations by the age at which foals were diagnosed with clinical pneumonia. Concentrations of IFN-γ were not different between subgroups in this small group of foals, but concentrations of IFN-γ from foals diagnosed at older ages showed a continual decrease through 20 weeks of age, which approached significance (P = 0.069). Therefore, while we cannot definitely know from these data that exhaustion of IFN-γ production played a role in the development of clinical pneumonia in the foals we studied, these data strongly suggest that further investigation into the mechanisms and kinetics of IFN-γ production by leukocytes during naturally occurring foal pneumonia after *R.equi* exposure is warranted.

Cortisol has been shown to modulate the immune function, and cytokine production, of both neutrophils and dendritic cells during in vitro studies using equine cells stimulated with bacterial antigen (Hart et al., 2011b; Lopez et al., 2024). In fact, responses to cortisol by foal immune cells were altered compared to responses from immune cells of adult horses, and it has been proposed that age-associated differences in cortisol immunomodulation may play a role in disease susceptibility in the foal (Fratto et al., 2017; Lopez et al., 2024). In addition, age-associated differences in circulating concentrations of total cortisol and free cortisol have been reported in very young healthy foals (Hart et al., 2011a; Hart et al., 2009a; Murray & Luba, 1993). Total cortisol

in birth samples reported herein were lower than those found in previous studies and were more closely aligned with previous reports of foals at 1-2 days of age, however, the birth samples in the previous study were pre-suckle samples collected within 30 minutes after birth (Hart et al., 2011a). This difference may be attributed to breed differences, as our foals were all warmbloods, farm management practices, or the exact timing (post-suckle) of sample collection. However, the decrease from birth to 1 week of age in total cortisol concentrations is consistent with previous reports (Hart et al., 2011a; Hart et al., 2012). The %FC reported in our youngest foals corroborated previous reports showing the highest percentage of free cortisol in samples taken at birth (Hart et al., 2011a). Earlier studies only measured %FC through 1 week of age, our findings herein show that %FC remains higher for most foals (average 14.6%) through the first 20 weeks of life compared to the reported %FC found in normal healthy horses (average 7%) (Hart et al., 2011a). The higher %FC found in foals is believed to be attributed to a reduction in cortisol binding capacity in foals, but it is unclear when cortisol binding capacity in the foal is equivalent to adult horses. We did not measure cortisol-binding globulin concentrations, but measurements of this protein in the future may provide more information into the kinetics of HPA axis maturation and cortisol binding capacity that might have implications for immune function in growing foals.

We also did not see an effect of respiratory disease in between-group comparisons for either cortisol or vitamin D. Concentrations of both cortisol and vitamin D have been shown to be associated with bacterial infections in newborn foals, but this is the first report to measure them in foal pneumonia following naturally occurring

exposure to *R. equi* on a farm endemic for disease. While not statistically significant, likely because of the high degree of variability between individuals, foals in this small sample visually the concentration of 25(OH)D, 1, 25(OH)D, total cortisol and %FC were lower in the CLINICAL group at the 8-, 12- and 20-week timepoints. The majority of our foals (87%) were diagnosed with pneumonia between the 8 and 20 weeks of age, slightly older than we had originally anticipated. Therefore, we believe that the timing of our sample collection may have impacted our ability to detect more subtle temporal changes in these steroid hormone parameters during this period that might be associated with disease progression in CLINICAL foals. More strategic sampling strategies (i.e., at diagnosis (SUBCLINICAL or CLINICAL) and more frequent collections post diagnosis may have allowed us to better determine if there is utility in quantifying these steroid hormones to predict disease severity in foals with naturally occurring pneumonia.

A key limitation in the current study is the lack of confirmation of *R. equi*, or another organism, as the causative agent of pneumonia in our foals. In fact, only one foal in our CLINICAL group had a transtracheal wash submitted for culture and was positively identified as having *R. equi*. The farm in our study is also endemic for pneumonia associated with *Streptococcus equi subspecies zooepidemicus* (*S. zooepidemicus*), and it is possible that some pneumonic foals had disease associated with that bacterium rather than *R. equi*. However, previous work on this farm has confirmed the presence of *R. equi* in lung tissue at post-mortem exam in foals with ante-mortem ultrasonic lesions (24/24 foals), and *R. equi* was isolated from tracheal aspirates

of over 50% of the foals with ultrasonic evidence of pneumonia (118/217 foals) in previous years (Lammer, 2010; Weimar, 2006).

An additional limitation is that our samples were obtained from only one breed of foals from a single farm and may not be directly applicable to other breeds or different farm management practices. Importantly, infrequent sample collection timing may have impaired our ability to detect key disease-associated changes in circulating steroid hormones and cytokines in both our clinical and subclinical populations. Finally, as mentioned above, it is possible that measuring systemic cytokine concentrations does not accurately reflect cytokine availability and activity at the tissue level of the lung during active pulmonary infection, and key changes in concentrations in the microenvironment may differ from what is reported systematically here (Thakur et al., 2019).

Conclusion

This report provides a comprehensive investigation of systemic changes in inflammatory cytokines and steroid hormones in foals from birth to weaning. This is the first report of circulating cortisol, vitamin D, and cytokine concentrations in foals naturally exposed to *R. equi* on a farm endemic for *R. equi* pneumonia. The immunoregulators assessed herein do not appear to be viable candidate biomarkers to predict individual respiratory disease susceptibility at the ages sampled here. However, subsequent studies with more deliberate sample timing (i.e., the time of pneumonia and more frequent measurements) would be necessary to confirm this finding. Important

disease-associated changes in IFN-γ production were found and warrant further investigation in naturally occurring foal pneumonia and foal immune function assessment.

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Table 5.1. Enrolled foal numbers by health category with mean, median, and range of age (in weeks) at diagnosis. Health category was determined by evaluation of health records at weaning (20 weeks of age) and divided into three groupings. Foals were categorized HEALTHY, if they had no abnormal physical exam findings or evidence of pulmonary lesions via trans-thoracic ultrasonography on weekly physical exams. A foal was considered SUBCLINICAL, if lung abscess scores were < 15 cm, but the foal had no clinical signs of pneumonia or antimicrobial therapy. A foal was considered CLINICAL if presented with clinical signs of pneumonia and required antimicrobial therapy for resolution of symptoms. Fifteen foals from each group were selected from the total number of foals categorized by group and from each breeding season (n = 30 total/group) using Research Randomizer, randomizer.org. The average, median and range of age (in weeks) at the time of either subclinical or clinical diagnosis is shown below. *Indicates significant difference between group ages at $P < 0.05$ level. N/A = not applicable.

Health category	# 2021 foals (total enrolled)	# 2022 foals (total enrolled)	Age (in weeks) at SUBCLINICAL diagnosis [Mean, median (range)]	Age (in weeks) at CLINICAL diagnosis [Mean, median (range)]
HEALTHY	15 (15)	15 (21)	N/A	N/A
SUBCLINICAL	15 (43)	15 (27)	13.3, 13.6 (3.5-19.7)	N/A
CLINICAL	15 (41)	15 (52)	9.8, 9.6 (3.8-17.1) *	13.7, 15.4 (7.2-19.7)

Figures

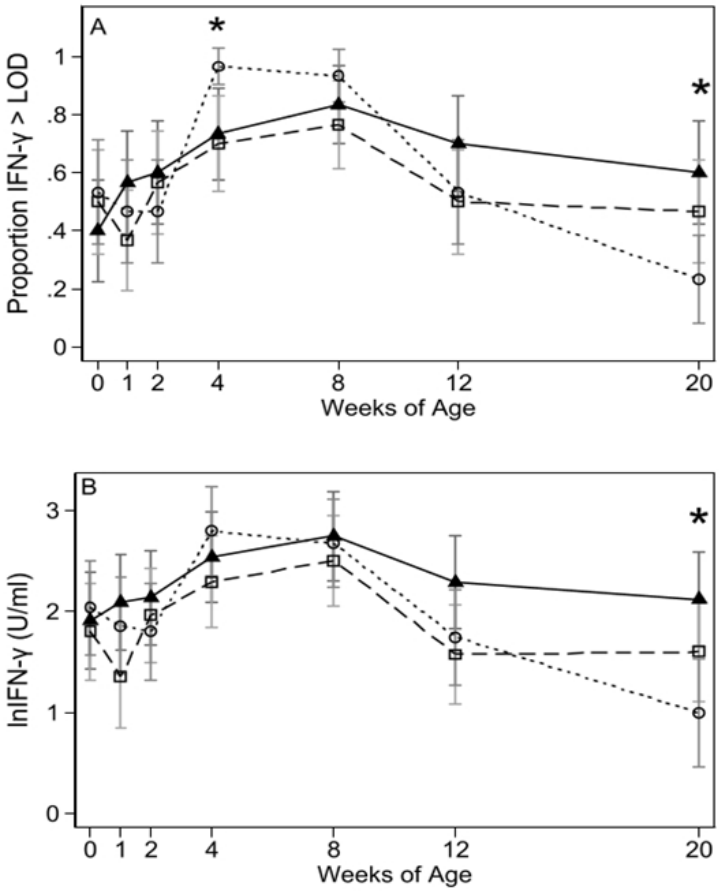
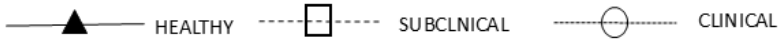


Figure 5.1. Interferon (IFN)-γ production in foals from birth to weaning. The proportion of foals producing interferon gamma (IFN-γ) above the limit of detection (> LOD) (A) and the mean natural log of the concentration of IFN-γ > LOD (B) in plasma of foals from birth to weaning on a farm endemic for *Rhodococcus equi* pneumonia as measured by validated bead-based immunoassay (5-plex, Animal Health Diagnostic Center, Cornell University, Ithaca, NY) as previously described (Wagner and Freer 2009). Between group comparisons were made using generalized estimating equation logistics (accounting for repeated measurements in the same foal) for the proportion of foals producing IFN-γ > LOD and a mixed effects TOBIT regression censored for the lower LOD was used for group comparisons of concentrations. The median with interquartile range for each age is shown by symbols and vertical lines. Groups are represented by closed triangle/solid line (HEALTHY), open square/dashed line (SUBCLINICAL), and open circle/dotted line (CLINICAL). Significant ($P < 0.05$) between-group-differences are indicated by *.



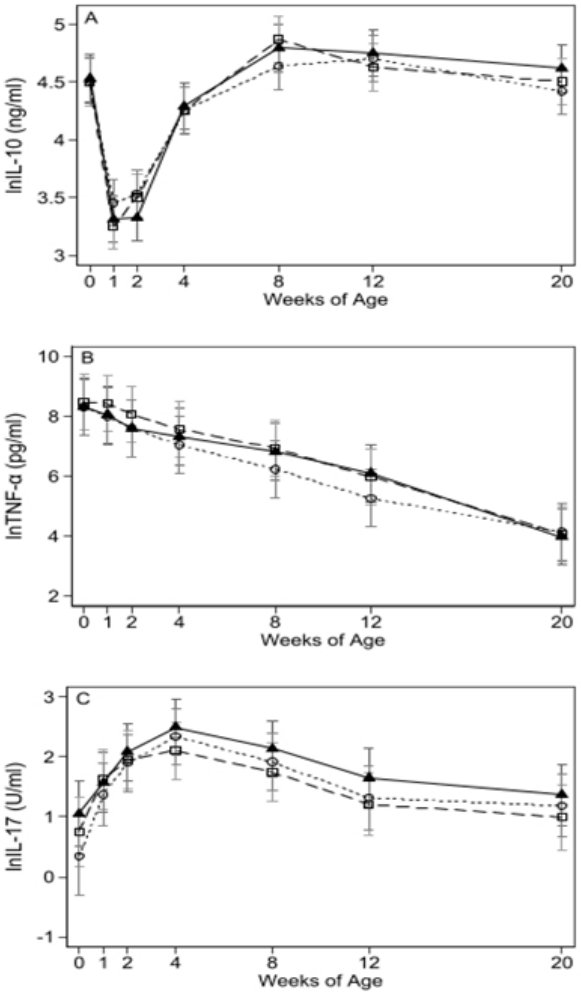
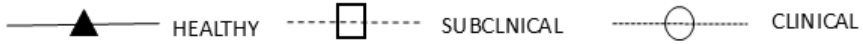


Figure 5.2. Interleukin (IL)-10, tumor-necrosis-factor- (TNF)-α, and IL-17 concentrations. The mean natural log of the cytokines interleukin (IL)-10 (A), tumor-necrosis-factor (TNF)-α (B), and IL-17 (C) in plasma as measured by a previously validated bead-based immunoassay (5-plex, Animal Health Diagnostic Center, Cornell University, Ithaca, NY) as previously described (Wagner and Freer 2009, Burton, Wagner et al. 2009) for IL-10 and IL-17 or commercial enzyme-linked immunosorbent assays (ELISA) kit (R&D Duoset, Minneapolis, MN) according to manufacturer directions for TNF-α, as previously described (Naskou et al., 2018, Berghaus et al. 2024). Between group comparisons were made using linear mixed models. The mean and 95% confidence interval for each age is shown by symbols and vertical lines Groups are represented by closed triangle/solid line (HEALTHY), open square/dashed line (SUBCLINICAL), and open circle/dotted line (CLINICAL). Significant ($P < 0.05$) age- but not health-associated effects were found for IL-10, TNF-α, and IL-17 ($P < 0.001$; $P > 0.425$).



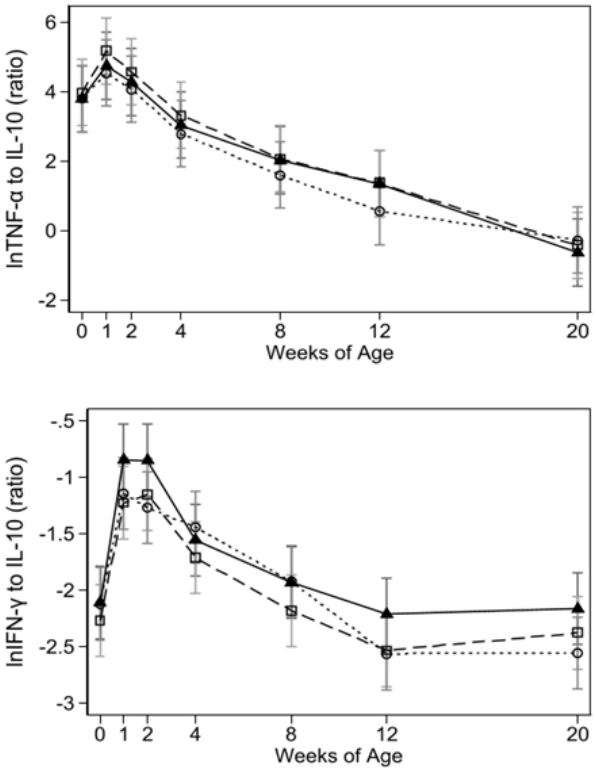
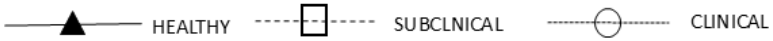


Figure 5.3. Interferon (IFN)-γ and tumor necrosis factor (TNF)-α to Interleukin-(IL)-10 concentration ratios. Group comparisons of pro- (TNF-α or IFN-γ) to anti-inflammatory (IL-10) cytokine ratios are shown A (IFN-γ/IL-10) and B (TNF-α/IL-10). The concentrations for TNF-α were determined by validated ELISA as previously described (Naskou et al., 2018, Berghaus et al. 2024) and the concentrations of IFN-γ and IL-10 by validated bead-based immunoassay (5-plex, Animal Health Diagnostic Center, Cornell University, Ithaca, NY) as previously described (Wagner and Freer 2009, Burton, Wagner et al. 2009). The ratios were determined by dividing the concentrations of TNF-α or IFN-γ by the concentration of IL-10 of each sample from foals at birth to weaning and comparing by mixed linear regression. The mean and 95% confidence interval for each age is shown by symbols and vertical lines. Groups are represented by closed triangle/solid line (HEALTHY), open square/dashed line (SUBCLINICAL), and open circle/dotted line (CLINICAL). There was a significant ($P < 0.05$) overall effect of age for both ratios ($P < 0.001$) but no effect of disease ($P > 0.500$).



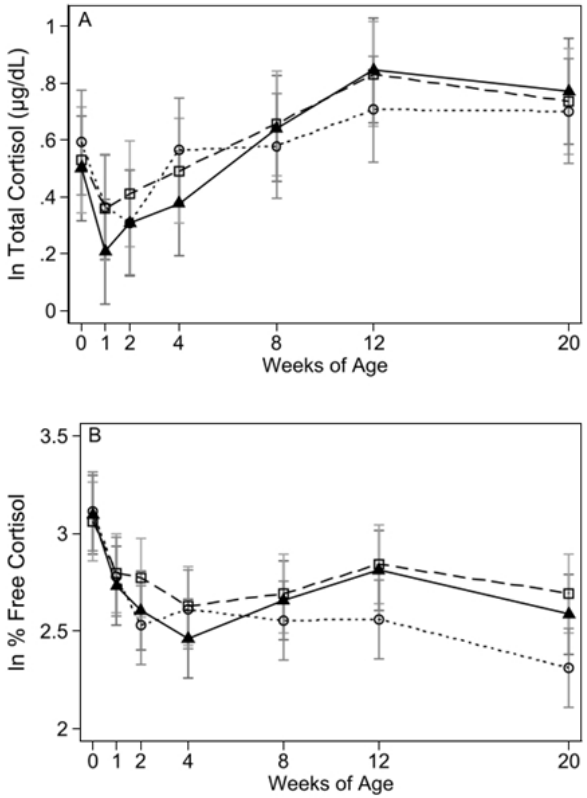
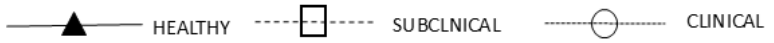


Figure 5.4. Serum total cortisol and % free cortisol (%FC). The mean natural log of total cortisol (A), and % FC (B) from serum of foals from birth to 20 weeks of age in foals on a farm endemic for *Rhodococcus equi* pneumonia. Concentrations of total cortisol were determined by previously validated chemiluminescent immunoassay (Reimers et al., 1996; Singh et al., 1997). % FC was determined by modification of ultracentrifugation method and validated ELISA (Salimetrics) as previously described (Hart et al., 2011a; Hart et al., 2016). Between group comparisons were made using linear mixed models. The mean and 95% confidence interval for each age is shown by symbols and vertical lines. Groups are represented by closed triangle/solid line (HEALTHY), open square/dashed line (SUBCLINICAL), and open circle/dotted line (CLINICAL). Age- but not health-associated changes were significant ($P < 0.05$) for total cortisol, and % FC ($P < 0.001$, $P = 0.310$).



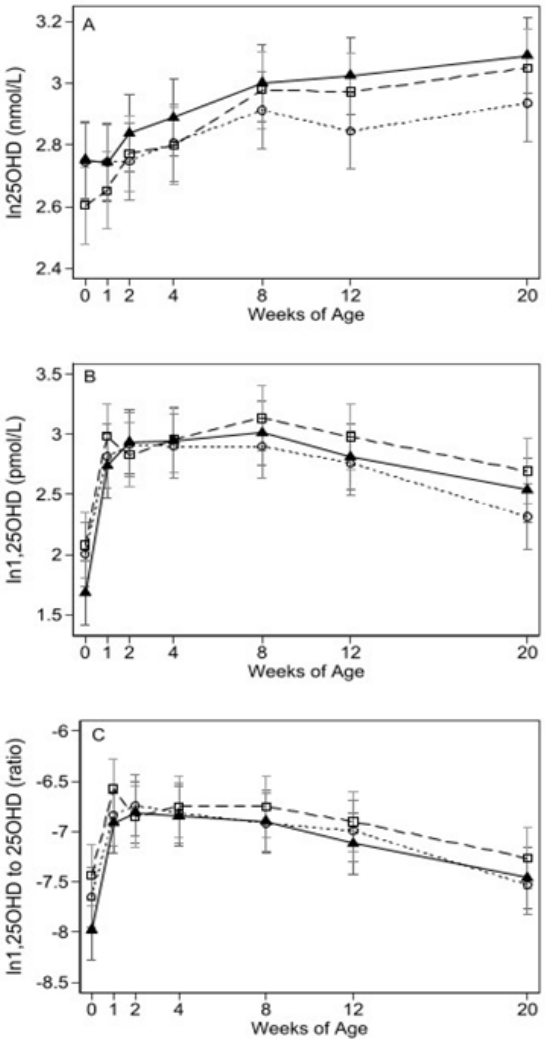
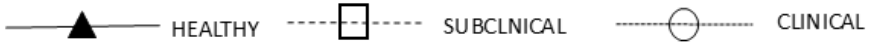


Figure 5.5. Vitamin D metabolite concentrations and ratios in plasma. The mean natural log of vitamin D metabolite concentrations, 25(OH)D (A) and 1,25(OH)D (B) and the ratio of 1,25(OH)D to 25(OH)D (C) in plasma as measured by validated enzyme immunoassay (Immunodiagnostic Systems, Tyne and Wear, UK) in foals from birth to 20 weeks of age in foals on a farm endemic for *Rhodococcus equi* pneumonia as previously described (Kamr, et al 2015). The ratio of 1.25(OH)D to 25(OH)D was calculated by dividing the concentration of 1,25(OH)D by the concentration of 25(OH)D. The median with interquartile range for each age is shown by symbols and vertical lines. Groups are represented by closed triangle/solid line (HEALTHY), open square/dashed line (SUBCLINICAL), and open circle/dotted line (CLINICAL). Age- but not health-associated changes were significant ($P < 0.05$) for 25(OH)D, 1,25(OH)D and the ratio of 1,25(OH)D to 25(OH)D ($P < 0.001$ and P = 0.386).



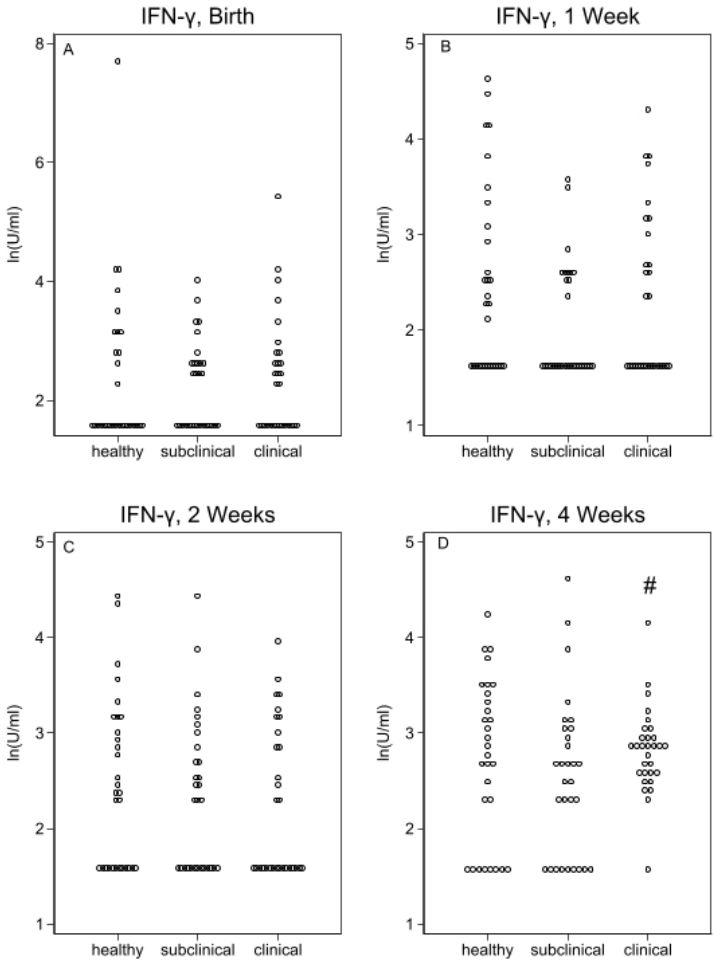


Figure 5.6. IFN-γ as a predictor of disease. To determine if interferon (IFN)-γ production could predict disease in foals naturally exposed to *Rhodococcus equi*, the mean natural log of IFN-γ [above the lower limit of detection (> LOD)] was compared between groups that remained healthy through weaning (HEALTHY) and foals that developed clinically apparent *R. equi* pneumonia after 8 weeks of age (CLINICAL IFN-γ concentrations were measured by previously validated bead-based immunoassay (5-plex, Animal Health Diagnostic Center, Cornell University, Ithaca, NY) as previously described (Wagner and Freer 2009). The concentration > LOD for individual foals (open circles) are represented by dot plots (Figure 6A-D). Between group comparisons were made using generalized estimating equation logistics and accounting for repeated measurements in the same foal for proportion of foals producing IFN-γ > LOD and a mixed effects TOBIT regression censored for the lower limit of detection was used for group comparisons of concentrations. Significance ($P < 0.05$) between number of individuals producing IFN-γ is represented by (Figure 5.6D) ($P < 0.003$).

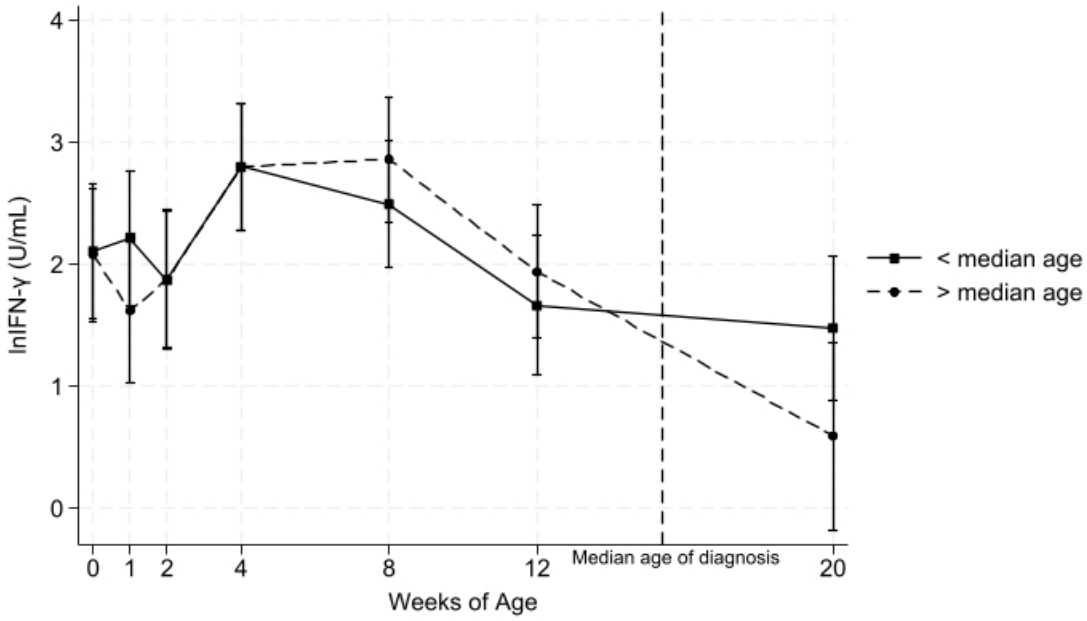


Figure 5.7. Association of IFN-γ production with age of respiratory disease diagnosis. To determine if IFN-γ concentration was associated with age of clinical diagnosis in CLINICAL foals, foals in the CLINICAL group were subdivided into two categories based on the median age of clinical diagnosis. Fifteen foals were diagnosed with clinical pneumonia younger than the median age of 15.4 weeks and fifteen foals were diagnosed with clinical pneumonia older than 15.4 weeks of age. Sample timepoints corresponding with foal age are shown on the x-axis and ln IFN-γ concentration (U/mL) is on the Y-axis. The median and interquartile range for foals with diagnosis younger than median age are represented by solid line and square symbol. The median and interquartile range for foals with diagnosis older than median age are represented by dashed line and solid circle. Median age of diagnosis is represented by a vertical dashed line crossing X-axis at 15.4 weeks of age. Subgroup comparisons were made by tobit censored regression models with fixed effects for health and age with a random effect for foal were used to compare concentrations with values below the lower limit of detection being censored. There were no significant ($P < 0.05$) differences for IFN-γ between diagnosis age at any timepoint ($P < 0.069$).

CHAPTER 6

CONCLUSIONS

The studies described in this dissertation explore the role of age-associated changes in immunoregulators in response to *ex vivo* and *in vivo R. equi* exposure. Two studies specifically focused on the role of the vitamin D/VDR pathway within equine leukocytes and a third study focused on assessing associations between circulating immune response modulators, including vitamin D and cortisol, and naturally occurring foal pneumonia disease susceptibility and severity. Though the individual studies varied in specific focus, the overarching goal was to determine if age-related differences in cortisol, vitamin D, and cytokines alter the immune response in respiratory disease caused by *R. equi* in the growing foal.

Prior to this body of work, the vitamin D/VDR pathway was unexplored in equine leukocytes. The VDR had previously been described in equine kidney and intestinal cells in studies investigating calcium regulation within the horse (Azarpeykan et al., 2016; Rourke et al., 2010). However, research in *Mycobacterium tuberculosis* (*Mtb*) has shown the importance of the vitamin D/VDR pathway for the clearance of intracellular bacteria (Bongiovanni et al., 2020; Kearns & Tangpricha, 2014). The similarities between *Mtb* and *R. equi* implicate a role for the vitamin D/VDR pathway during *R. equi* infection (von Bargen & Haas, 2009). In our initial study, we evaluated expression of the VDR within equine AMϕ. We chose to use AMϕ because they are the primary cells involved in the initial uptake following bacteria inhalation (Berghaus et al., 2014). Not only were we

able to measure VDR expression within equine AMϕ, but expression was higher in cells from foals at 2 weeks of age compared to expression in cells from older foals and adult horses.

The primary effect of the vitamin D/VDR pathway is suppression of the pro-inflammatory adaptive immune response (Bikle, 2009). Suppression of the pro-inflammatory response is common in newborn mammals as a regulatory mechanism to prevent uncontrolled inflammatory reactions and ensure cellular proliferation and growth are maintained (Butler et al., 2009; Perkins & Wagner, 2015). Thus, increased expression in cells from our youngest foals indicates that the vitamin D/VDR pathway may also suppress adaptive immune responses, similar to suppressive responses seen in other species (Baeke et al., 2010; Nelson et al., 2012). This finding also indicated that the vitamin D/VDR pathway may play a role in the age-window of susceptibility to *R. equi* disease by impacting the young foal's ability to mount pro-inflammatory responses during the initial stages of *R. equi* infection.

The goal of the second study was a deeper exploration of the vitamin D/VDR pathway in AMϕ, specifically evaluating the impact of *R. equi* infection on VDR expression and downstream immune protein production in an *ex vivo* model of infection. In this study, we specifically tried to mimic natural disease by developing a novel infection model characterized by a low multiplicity of infection (MOI) within cultured equine AMϕ. VDR expression in cultured AMϕ increased with age in foals, but the expression was not impacted by *R. equi* infection, in contrast to impacts we

observed in infected AMϕ from adult horses. These findings indicate that the vitamin D/VDR pathway may not be fully mature in foal AMϕs, and further research is needed to determine when VDR expression in foal cells would respond similarly to *R. equi* infection as seen in adult cells.

In human leukocytes, infection with intracellular bacteria results in upregulation of VDR expression, synthesis of the active vitamin D metabolite, and regulation of downstream immune proteins (Liu et al., 2006; Liu et al., 2007). Surprisingly, VDR expression decreased in *R. equi*-infected AMϕ from adult horses. It is possible that decreased expression of the VDR in response to intracellular infection is unique to the horse. Unique differences in vitamin D metabolism related to its classic role in calcium homeostasis in the horse have also been reported (Azarpeykan et al., 2022; Dittmer & Thompson, 2011; Hymoller, 2015). Additionally, our study lacked evidence of vitamin D synthesis within AMϕ from foals or adult cells. It is also possible that vitamin D synthesis does not occur within equine leukocytes, but limitations within our study design may also have impacted our ability to detect these differences if they do exist.

The vitamin D/VDR pathway regulates cytokine production in human macrophages during infection (Ismailova & White, 2022). Therefore, we also measured cytokine concentrations in the supernatants of our cultured infected/mock-infected AMϕ. Age-associated differences in cytokine mRNA from equine AMϕ infected with *R. equi* have been reported, but age-associated differences in cytokine protein production in *ex vivo* infection of AMϕ is lacking (Berghaus et al., 2014). Findings from our study

indicate that AMϕ from foals at 2 weeks of age did not produce IFN-γ and TNF-α in response to *R. equi* infection, but production in cells from 4-week-old foals was similar to production seen in cells from adult horses for both cytokines. Foals at 8 weeks of age made significantly more IFN-γ than foals at 2 weeks regardless of infection status, but quantities of IFN-γ did not increase in response to *R. equi*; however, there was a lot of variability between samples at that age that potentially impacted a significant finding. Additionally, age-associated differences were seen in production of IL-1β, IL-10, and IL-17, with cells from foals at 4 weeks of age producing IL-1β and IL-10 at levels greater than adult cell responses. Our findings corroborate cytokine production in one-month-old foals during an *R. equi* foal-challenge study (Jacks et al., 2007). Interestingly, a small proportion of uninfected control samples from foals had measurable concentrations of IL-17, but IL-17 was produced only in response to infection in cells from adult horses. If IL-17 production in response to infection is truly limited in foal cells, this could play a role in the age-window of susceptibility to *R. equi* pneumonia. The role of IL-17 during infection with *R. equi* is mostly uncharted, and further research is warranted in this area.

While *in vitro* studies and challenge models of experimental infection are useful research tools, they may not accurately reflect the immune response during natural infection with *R. equi*. Interestingly, prior to this body of work, there were no reports of circulating concentrations of cytokines during normal environmental exposure to *R. equi* and subsequent development of bronchopneumonia in foals on farms endemic for disease. Furthermore, the role that steroid hormones might play during *R. equi* infection

was completely unexplored, even though differences in both cortisol and vitamin D concentrations have been associated with other diseases in young foals and tuberculosis in people (Hart et al., 2011; Iftikhar et al., 2013; Kamr et al., 2015; Yareshko & Kulish, 2022). Therefore, our final study was an investigation into differences in circulating concentrations of these immunoregulators during naturally developing foal pneumonia, with the aim of determining if they could be useful parameters to forecast individual responses to *R. equi* exposure.

There was a significant effect of age for every cytokine and hormone measurement in our study, providing the most comprehensive report to date of systemic changes in the foal's maturing immune system and key immune regulators. While our findings are specific to a single horse breed on an individual farm, the data should provide a valuable foundation for assessment of immune changes measured in foals in future studies.

Potentially the most important finding of this third study was related to age- and disease-associated differences in the cytokine IFN-γ. The importance of IFN-γ in *R. equi* immunogenicity and bacterial clearance has been described for over a quarter of a century and been investigated extensively in both *R. equi* challenge models (murine and equine) and *in vitro* assays (Giguère et al., 1999; Jacks et al., 2007; Kasuga-Aoki et al., 1999). This study is the first to show an association between IFN-γ and naturally occurring bronchopneumonia following environmental exposure to *R. equi*. Our findings clearly indicate that foals can make endogenous IFN-γ when exposed to pneumonia-

causing bacteria by 1 month of age, but sustained production, which could result in bacterial clearance, falls short in foals that develop clinical disease. Currently it is unclear if this IFN-γ decline is related to delayed maturity of the adaptive immune response, waning of maternal immune factors, an increased bacterial exposure, or some other unknown parameter. This is, however, an important finding that suggests that both the mechanisms and kinetics of IFN-γ production may play a role in disease susceptibility and severity, and warrants further investigation.

Measuring systemic concentrations of immune proteins or hormones provides an important but potentially incomplete picture of the immune response at the tissue level. Unfortunately, at this time there are several limitations to gaining more detailed information on specific immune responses within the microenvironment of the lungs. First, we do not fully understand the timing from initial pulmonary infection to clinical disease in naturally occurring disease caused by *R. equi*, or if differences in bacterial load are associated with individual disease responses. Thus, mimicking natural disease in an animal model is extremely difficult. Secondly, repeated sampling of the lungs during natural disease development would be most informative; however, it would be expensive, invasive, and could potentially impact the normal immune responses. Furthermore, invasive lung sampling would be contraindicated and, therefore, undesirable to both veterinarians and clients. Therefore, at this time, we will have to rely on either circulating parameters as in the study herein, current challenge models, or

in vitro assays to further investigate the mechanisms and kinetics of IFN-γ in foal respiratory immune responses.

The study design and timing of sample collection was a key limitation and potential missed opportunity for more substantial disease-associated findings. Fortunately, it is an area that could be improved and changed for future studies. Specifically, sample collection at the time from foals diagnosed with subclinical and clinical pneumonia, with age-matched heathy foals, as well as more frequent sampling thereafter, might elicit more information about immune and immunoregulatory responses that may be critical for understanding differences between self-resolving and severe respiratory disease in the foal.

In sum, there is evidence that the vitamin D/VDR pathway is present in equine leukocytes, playing a role in equine immunity, but the functionality of this pathway is not fully mature within foal macrophages. Additionally, age-associated changes in systemic measurements of immunoregulators in the growing foal reported herein will be useful points of reference for future studies into foal immunity. Finally, this study confirmed the association between IFN-γ and disease severity in naturally occurring foal pneumonia following exposure to *R. equi* and can be used to support further research into the immune mechanisms and kinetics of IFN-γ production in the foal.

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