# OPSONIZATION OF RHODOCOCCUS EQUI DECREASES CYTOTOXIC EFFECTS AND MODULATES CYTOKINE EXPRESSION IN EQUINE MACROPHAGES

By

CRYSTAL LESLIE MONTOYA

A thesis submitted in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE IN VETERINARY SCIENCE

WASHINGTON STATE UNIVERSITY
College of Veterinary Medicine

May 2010

To the Faculty of Washington State University:

The members of the Committee appointed to examine the thesis of CRYSTAL L. MONTOYA find it satisfactory and recommend that it be accepted.

_____
Stephen Hines, Ph.D., Chair

_____
Donald Knowles, Ph.D.

_____
Kevin Lahmers, Ph.D.

_____
Robert Mealey, Ph.D.

ACKNOWLEDGMENTS

I would like to express my appreciation to my mentor Stephen Hines for his guidance, advice, and expertise, which has enabled me to become a better scientist. I am also grateful for the time he put into reviewing my thesis and helping me to become a better writer. I would also like to thank my other committee members, Donald Knowles, Kevin Lahmers, and Robert Mealey, for their time and effort in helping me to complete my Master of Science degree. In addition I would like to express gratitude towards Debby Alperin for providing technical assistance and guidance during this research. I would also like to thank Seth Harris and Robson Dossa for their assistance in collecting blood samples from the horses. Without their invaluable help, this research could not have been completed.

OPSONIZATION OF RHODOCOCCUS EQUI DECREASES CYTOTOXIC EFFECTS AND MODULATES CYTOKINE EXPRESSION IN EQUINE MACROPHAGES

By
Crystal Leslie Montoya, M.S.
Washington State University
May 2010

Chair: Stephen A. Hines

## ABSTRACT

The ability to survive and replicate in macrophages is considered the key event in the pathogenesis of *Rhodococcus equi* infection in foals. A Type 1 immune response, characterized by interferon gamma production and cytotoxic T lymphocytes, is considered the primary mechanism of immune clearance. However, there is evidence that antibody can also provide at least partial protection from disease. These effects presumably reflect effector mechanisms such as increased phagocytosis and enhanced phagosome-lysosomal fusion. In the present study, immunoglobulin G opsonization of *R. equi* resulted in increased bacterial uptake, decreased intracellular survival, and improved host cell viability. Antibody can also affect cellular responses, including Type 1 responses likely relevant to the outcome of *R. equi* infection in foals. IgG opsonization of *R. equi* resulted in increased transcription by macrophages of Type 1

cytokines interleukin (IL)-12p35, IL-12p40, IL-23p19 and tumor necrosis factor alpha (TNF-α). Moreover, *R. equi* infection resulted in IL-10 up-regulation and IL-18 down-regulation that was not affected by opsonizing antibody. The effects of antibody on developing cell mediated immune responses may be applicable to immune prevention of equine rhodococcal pneumonia in young horses.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

# CHAPTER ONE

## INTRODUCTION

*Rhodococcus equi* is a pathogenic nocardioform actinomycete bacteria closely related to *Mycobacterium tuberculosis* (Prescott, 1993). Both pathogens produce life threatening pyogranulomatous lesions in the lung. While *M. tuberculosis* infects humans, *R. equi* is an important cause of morbidity and mortality in foals between approximately 2 and 5 months of age. It is also an opportunistic infection in immunocompromised people. Like *M. tuberculosis,* survival and replication within macrophages is considered the key event in *R. equi* pathogenesis. In horses, virulent strains persist within membrane bound vacuoles that do not fuse with lysosomes. Moreover, *R. equi* has a cytotoxic effect that results in the necrotic death of macrophages (Luhrmann *et al*., 2004).

Virtually all foals are exposed to *R. equi* shortly after birth. Foals that remain asymptomatic or recover develop immune responses that protect them throughout life. A protective immune response is thought to reflect the development of antigen specific CD4+ Th1 lymphocytes that secrete IFN-γ and the induction of *R. equi* specific cytotoxic T lymphocytes (CTL) – i.e. a type 1 cell mediated immune (CMI) response. The unique susceptibility of foals is hypothesized to reflect a lack of pre-existing immunity at the time of challenge and the age-associated decreased ability of neonates and perinates to develop Type 1 immune responses (Boyd et al., 2003). In particular, foals may have a decreased capacity to produce IFN-γ, which activates macrophages and appears absolutely required for immune clearance (Breathnach et al., 2006).

Despite the requirement for cell mediated Type 1 immune responses, antibody also appears to play a role in immunity to *R. equi.* In one early study, opsonization with horse serum containing *R. equi* specific antibody increased phagosome-lysosome fusion and enhanced killing by foal alveolar macrophages (Hietala and Ardans, 1987). Likewise, immunosuppressed mice were partially or fully protected against *R. equi* intraperitoneal challenge after receiving IgG purified from horses immunized with the *R. equi* virulence associated protein A (VapA) (Fernandez *et al*., 1997). The apparent effector functions of *R. equi* specific antibodies, including opsonization and complement fixation, provide the theoretical basis for the passive administration of commercial hyperimmune plasma (HIP), which is widely used to decrease the incidence and severity of rhodococcal pneumonia in foals (Becu *et al*., 1997; Caston *et al*., 2006; Higuchi *et al*., 1999; Madigan *et al*., 1991; Martens *et al*., 1989). In one study, an equal degree of protection was observed when foals were administered HIP or purified IgG from horses immunized with recombinant VapA and VapC virulence proteins (Hooper-McGrevy *et al*., 2001).

In addition to its effector functions, antibody may also have regulatory effects that influence cell mediated responses. For example, human polyclonal antibodies to *Mycobacterium bovis* BCG increase IFN-γ production and stimulate CD4+ and CD8+ lymphocyte proliferation (de Valliere et al., 2005). Furthermore, Fc receptor gamma (FcγR) activation increases IL-12 production, indirectly resulting in up-regulation of the Type 1 cytokines TNF-α and IFN-γ (Igietseme *et al*., 2004). Likewise, immunoglobulin bound to FcγR also directly stimulates TNF-α production (Clynes *et al*., 1999). Human monocyte FcγR ligation results in up-regulation of IL-8 and Monocyte Chemoattractant

Protein-1cytokines, which recruits neutrophils, macrophages, and dendritic cells (Marsh *et al*., 1995; Marsh *et al*., 1997; Ralston *et al*., 1997). In summary, studies on the immunomodulatory effects of antibody suggest it decreases the dose of antigen needed to stimulate lymphocytes (Amigorena and Bonnerot, 1999; Chang, 1985; Gosselin *et al*., 1992; Guyre *et al*., 1997; Manca *et al*., 1988), alters the expression of key cytokines, and increases the recruitment and proliferation of lymphocytes. In the experiments described here, we hypothesized that antibody mediated opsonization of *R. equi* would increase bacterial uptake by equine monocyte derived macrophages, decrease intracellular survival of bacteria, decrease the cytotoxic effects of virulent *R. equi* on infected cells, and result in the up-regulation of Type 1 cytokines likely to play a role in immune clearance.

# CHAPTER TWO

# RESEARCH DESIGN AND METHODOLOGY

## Peripheral blood mononuclear cells

Venous blood was collected from the jugular vein of three randomly selected horses using 500 ml evacuated containers (Baxter, Deerfield, IL) containing 75 ml of anticoagulant citrate dextrose (Baxter, Deerfield, IL). The horses are housed at the Washington State University and used in accordance with IACUC mandates. Peripheral blood mononuclear cells (PBMC) were isolated using a Ficoll- Hypaque technique as previously described (Raabe *et al*., 1998).

## Monocyte derived macrophages

Monocyte derived macrophages (MDM), were isolated from PBMC as previously described (Patton *et al*., 2004). Briefly, 1.5 X $10^8$ PBMC in 25 ml RPMI media were allowed to adhere overnight to bovine gelatin and horse serum coated plates. To coat the plates, 150 $cm^2$ petri dishes were coated with 2% sterile gelatin (J.T. Baker Inc, Phillipsburg, NJ) and incubated for 2 hours at 37$^o$C with 5% $CO_2$, the gelatin was then removed, and the plates were air dried for 30 minutes. Next, 6 ml of heat inactivated horse serum (Invitrogen, Carlsbad, CA) was added to the dishes and incubated for 30 min at 37$^o$C with 5% $CO_2$ then removed and PBMC transferred to the plate. The non-adherent cells were removed after 18 to 24 hours by washing each plate with Hank's balanced salt solution (Hyclone Laboratories Inc., Logan, UT). Adherent cells were eluted with 20 ml antibiotic free- complete RPMI medium containing 20% heat inactivated horse serum and 5mM EDTA (Sigma-Aldrich, St Louis, MO) for 25 minutes at 37°C with 5% $CO_2$. Adherent cells were collected by vigorous pipetting, followed by

washing each plate three with 10 ml of Hank's balanced salt solution. Eluted cells were centrifuged at 700x*g* for 15 minutes, combined, and washed once with antibiotic free complete medium. The cells were stained with trypan blue then counted and suspended to a concentration of $4X10^6$ MDM/ml or $1X10^6$ MDM/ml. For real time PCR experiments, $4X10^6$ eluted MDM in 1000 µl RPMI were transferred and adhered overnight to wells in a 24 well plate coated with 2% bovine gelatin and heat inactivated horse serum at 37°C with 5% $CO_2$. For viability and intracellular *R. equi* CFU assays, $1X10^5$ eluted MDM in 100 µl RPMI were transferred and adhered overnight to wells in a 96 well plate coated with 2% bovine gelatin and heat inactivated horse serum at 37°C with 5% $CO_2$.

**Antibody purification and analysis**

Immunoglobulin G was purified from commercial Immuno-Glo equine plasma obtained from *R. equi* antigen vaccinated horses (MG biologics, serial no. 6330-113-08) and from plasma obtained from an EIAV persistently infected horse (Dr. Robert Mealy, Washington State University, Pullman, WA) using octanoic acid precipitation as previously described (Rojas et al., 1994). The latter was used as a negative control. Briefly, 500 ml aliquots of equine plasma were adjusted to pH 5.8 adding 1.76N acetic acid. Then, octanoic acid (Sigma-Aldrich, St Louis, MO) was slowly added to a final concentration of 5% (v/v) and mixed vigorously for 1 hour at room temperature. The suspension was then centrifuged for 30 minutes at 10,000x*g*. The supernatant was collected and centrifuged for 30 minutes at 10,000x*g,* this was repeated twice. The supernatant was then filtered by gravity filtration twice using Whatman 2V grade filter paper (Whatman International, Ltd, Maidstone, England). The filtrate was subsequently

filtered through a 0.45 µm vacuum filter (Nalgene MF75 vacuum filter, Rochester, NY) and dialyzed for 48 hours against distilled water using Spectra/Por Membrane 6 dialysis tubing with MWCO 50,000 (Spectrum Laboratories, Rancho Dominquez, CA. The dialyzed immunoglobulins were then filtered with a 0.22 µm bottle top filter (Millipore, Billerica, MA). Purified anti-EIAV (α- EIAV) IgG was subsequently mixed with live *R. equi* to absorb any *R. equi* specific antibodies by incubating for 60 minutes at room temperature then centrifuged at 2,100X*g* for 15 minutes. This was repeated four times, and the adsorbed product filtered with a 0.22 µm bottle top filter.

Total IgG concentration was determined using the Equine IgG RID kit (VMRD, Inc., Pullman, WA) as per the manufacturer's instructions.

*R. equi* specific antibodies were analyzed by ELISA as previously described (Lopez et al., 2002). Ninety-six-well Immulon II plates were coated overnight with 1 µg of *R. equi* soluble antigen per ml in carbonate-bicarbonate buffer (total volume, 50 µl/well) at 4°C. IgG was adjusted to fixed concentration of 3.5 mg/dl, then diluted to 1:400 in PBS containing 0.05% Tween and 2.5% sodium chloride (PBST) and serial two fold dilutions were analyzed. To detect the total α- *R. equi* IgG bound, plates were washed with PBST and incubated for 30 min with 50 µl of α-equine IgG peroxidase-conjugated caprine antibodies per well (1:10,000; Kirkegaard & Perry Laboratories, Gaithersburg, Md.) and analyzed by reading optical densities at 450 nm with an ELISA reader. The titer was determined when the absorbance equaled the control with no primary antibody calculating the mean plus or minus three standard deviations.

Isolated protein was visualized by SDS-PAGE using SimplyBlue SafeStain (Invitrogen, Carlsbad, CA). SimplyBlue SafeStain can detect as little as 7 ng reduced BSA, and 49 ug of protein was loaded into lane.

**Bacteria**

Virulent *R. equi* strain ATCC 33701 and avirulent plasmid cured bacteria (ATCC 33701-PC) was stored at -80° and reconstituted by incubation on a brain heart infusion (BHI) (Difco Laboratories, Detroit, MI) agar plate at 37°C for approximately 24 hours. A single colony was transferred from a BHI agar and cultivated overnight in BHI broth in a shaking incubator at 200 rpm at 37°C. The bacteria were then washed twice with phosphate buffered saline (PBS) by centrifugation at 3000 X *g* for 15 min. *R. equi was* suspended to a concentration of 1.5 X $10^8$ bacteria/ ml in antibiotic free complete RMPI medium. Heat killed bacteria were obtained by incubating bacteria at 90° C for 45 minutes (Giguère and Prescott, 1998). The bacterial concentration was confirmed by plating serial dilutions on BHI agar and calculating CFU/ml.

**Macrophage infection**

*R. equi (*1.5 X $10^8$ bacteria/ ml) was incubated with a final concentration of 0.28mg/ ml anti-*R .equi* (α- *R. equi*) IgG, 0.28mg/ml α-EIAV control IgG, or a corresponding volume of media for a no additional IgG control. Each sample was vortexed and incubated for 60 minutes at room temperature on a rocker. Bacteria were then added to MDM cell cultures at 30 to 60 MOI as determined by calculating CFU. One hour post infection (HPI) the media was removed, and the cells in 96 well plates were washed twice with 100 µl of RPMI media containing 0.2 mg/ml gentamicin sulfate, media was then replaced with 100 µl of fresh RPMI media containing 0.2 mg/ml

gentamicin sulfate to kill extracellular bacteria. The same procedure was followed with the MDM in 24 well plates, except 1 ml of RPMI media containing 0.2 mg/ml gentamicin sulfate was used instead of 100 µl. Each experiment was repeated three times on three different dates for each horse studied.

**Intracellular R. equi colony forming units**

Two and 24 hours post infection, triplicate samples were washed once with 100 µl of PBS. To lyse the MDM, PBS was removed and 100 µl of sterile water was transferred to each well and incubated for 40 minutes at 37°C. The resulting cell lysates were transferred to a PCR tube, then vortexed for 5 minutes, sonicated for 5 minutes, and vortexed again for 5 minutes. Serial dilutions were plated on BHI agar plates, incubated overnight, and then CFU were calculated.

**MDM viability**

Two and 24 hours post infection, triplicate MDM samples were eluted with 10 mM EDTA to elute adherent cells as described above. Trypan blue was added to each sample (v/v) and 200 MDM were counted from each sample.

**RNA and cDNA preparation**

Preliminary data was generated by collecting mRNA from infected cells at 1, 3, 5, 7, 9, 11, and 24 hours post infection and real time PCR was used to analyze cytokine transcripts. Based on the preliminary data, we determined that 5 and 11 HPI were the best time points to analyze cytokine production. RNA was extracted from triplicate samples of MDM at 5 HPI and 11 HPI using the RNeasy mini kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Genomic DNA was removed using the TURBO DNA-*free* kit (Ambion, Foster City, CA) by following the manufacturer's

instructions. The RNA concentration was determined using the Warburg-Christian method.

A two step PCR reaction was used for cDNA synthesis. For the first step, 1 µg of RNA was mixed with 3.3 µM of random hexamers (Applied Biosystems Foster City, CA), 0.67 mM dNTP (Applied Biosystems Foster City, CA), and nuclease free water to a total volume of 15 µl. The reaction was incubated at 70°C for 5 minutes and 10°C for 7 minutes. For the second reaction, 200 U M-MLV reverse transcriptase (M-MLV RT; Promega, Madison, WI), 5 µl M-MLV RT reaction buffer, 4 mM DTT (Invitrogen, Carlsbad, CA), 20 U RNase Inhibitor (Applied Biosystems, Foster City, CA), and 2 mM $MgCl_2$ (Applied Biosystems, Foster City, CA) were mixed with the first reaction, then incubated for 60 minutes at 37°C. Corresponding samples containing water in place of M-MLV RT (no reverse transcriptase control) were prepared in order to test for genomic DNA contamination.

**Real-time PCR conditions**

Real-time PCR was used to detect cytokine expression. RT-PCR was performed in a 25 µl reaction containing 12.5 µl SYBR Green (Bio-Rad, Hercules, CA), 6 µl water, 5 µl cDNA, 1 mM $MgCL_2$, 4 µM of each primer (see Table 1). Primers were either designed with Vector NTI (Invitrogen, Carlsbad, CA) or previous published. All samples were run in triplicate and amplified using an iCycler (Bio-Rad, Hercules, CA): 2 minutes at 50°C and 10 minutes at 95°C, then 45 cycles of 95°C for 30 seconds and 55°C for 60 seconds, followed by 72°C for 7 minutes The $\Delta\Delta$ CT method was used to calculate relative fold difference (Livak and Schmittgen, 2001). The mean of the triplicate samples was calculated.

| Gene | GenBank Accession No. | Primer | Sequence (5'→3)' | Reference |
|---|---|---|---|---|
| IL-10 | U38200 | Forward | GAT CTC CCA AAT CCC ATC CA | Sponseller et al., 2009 |
| | | Reverse | AGG AGA GAG GTA CCA CAG GGT TT | |
| IL-12p35 | Y11130 | Forward | CCC GGA AAG GCC TCT TCT | Nerren et al., 2009 |
| | | Reverse | ACC TGG TAC ATC TTC AAG GTC CTC AT | |
| IL-12p40 | Y11129 | Forward | GGA TGC TGT TCA CAA GCT CAA G | This Publication |
| | | Reverse | AAT GGC TTC AGC TGC AGG TTC | |
| IL-18 | Y11131 | Forward | AAT TGC ATT AGC TTG GTG GAA | This Publication |
| | | Reverse | GCA TAT CCT CAA ACA CAG GTT G | |
| IL-23P19 | AY704416 | Forward | TCC ACC AGG GCC TGG TTT | Ainsworth et al., 2007 |
| | | Reverse | AGT AGA GAA GGC TCC CCT GTG A | |
| TNF-α | AB035735 | Forward | AGC CCA TGT TGT AGC AAA CCC | This Publication |
| | | Reverse | ACC ATC TGG GTT GTC TGT CTA GC | |
| GAPDH | AF157626 | Forward | GTA ATC AAC GGA AAG GCC ATC | Lopez et al., 2002 |
| | | Reverse | CCT TTT GGC TCC ACC CTT CAA G | |

**Table 1:** Sequences for equine cytokine specific primers used in Real-time PCR

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism 4 Software (GraphPad Software, Inc., San Diego CA). Significant differences were determined using repeated measures two way ANOVA followed by Boneferroni's multiple comparison test. P values of less than 0.05 were considered significant.

# CHAPTER THREE

# RESULTS

## Characteristics of purified immunoglobulin

The purified immunoglobulin samples were tested for α- *R. equi* total IgG by ELISA (Fig.1). The α- *R. equi* IgG titer was 12,800 and the α-EIAV IgG was negative for *R. equi* specific antibodies.

Immunoglobulin G purification was determined using SDS-PAGE. Bands were visualized at 27,000 Da, 52,000 Da, and 77,000 Da under reducing conditions, correlating to the light chains and heavy chain of IgG. These bands correlate to light chain, heavy chain, and one light chain plus the heavy chain of IgG. Under non-reducing conditions, there was a single band just below 160,000 Da.

## The effects of α- R. equi IgG on uptake, cytotoxicity and intracellular survival

At 2 hours post infection (HPI) MDM infected with α- *R. equi* IgG opsonized bacteria had significantly higher uptake of bacteria compared to α- EIAV and no additional IgG controls (Fig. 2 a, b, c). There was no significant loss in host cell viability at 2 HPI between uninfected MDM and *R. equi* infected MDM, regardless of treatment (Fig. 2 d, e, f).

The increased bacterial uptake, at 24 HPI the α- *R. equi* IgG treatment resulted in significantly decreased intracellular survival of *R. equi* when compared to controls, presumably due to increased bacterial killing (Fig. 2 a, b, c). Compared to 2 HPI, there was significant increase in intracellular *R. equi* at 24 HPI for the controls (all 3 horses with no additional IgG control and 2 of 3 horses for the α- EIAV IgG control). The number of intracellular bacteria did not increase in cells infected with bacteria opsonized

with α- *R. equi* IgG. The viability of MDM infected with *R. equi* at 24 HPI was significantly decreased compared to uninfected MDM (Fig. 2 d, e, f). Opsonization with α- *R. equi* IgG significantly improved MDM viability compared to both controls. Viability was also significantly increased in MDM infected with *R. equi* incubated with α- EIAV IgG when compared to the no additional IgG control.

**The effects of α- R. equi IgG on cytokine expression**

As predicted, infection of equine MDM with *R. equi* resulted in significant changes in cytokine expression compared to uninfected controls at 5 and 11 HPI (Fig. 3). The changes were most dramatic at 11 HPI, when there were significant increases in IL-12p35, IL-12p40, IL-23p19, and TNF-α Fig. (3 a, b, c, d). These increases at 11 HPI were significant compared to uninfected MDM regardless of treatment.

Although statistically significant compared to uninfected cells, the increase in IL-10 expression at 11 HPI was consistently less than the increased observed for other cytokines (Fig. 3 e). Likewise, there was no significant increase in IL-10 expression at 5 HPI compared to uninfected MDM.

Opsonization of *R. equi* with α- *R. equi* IgG resulted in significantly increased transcription of IL-12p35, IL-12p40, IL-23p19 and TNF-α mRNA compared to both the α- EIAV IgG control and the no additional IgG controls ($p \leq 0.05$). The increase was most apparent at 11 HPI but was also present at 5 HPI. Interestingly compared to the no IgG control, the addition of purified IgG from the EIAV control also significantly increased IL-12p35, IL-12p40, IL-23p19, and TNF-α transcripts at 11 HPI and IL-23p19 and TNF-α transcripts at 5 HPI. There was no statistically significant difference of IL-10 mRNA production between the α- *R. equi* IgG opsonized samples and controls.

In contrast to *R. equi* infection up-regulating the cytokines IL-10, IL-12p35, IL-12p40, IL-23p19 and TNF-α, IL-18 expression was down regulated in response to *R. equi* infection of MDM. The IL-18 decrease was significant in all horses at 11 HPI and one horse at 5 HPI (Fig. 4 a, b, c). There was no significant difference in IL-18 expression attributed to α-*R. equi* or α- EIAV IgG.

## CHAPTER FOUR

## DISCUSSION

Clinical cases of rhodococcal pneumonia in horses typically begin to appear at 6-8 weeks of age – about the same time that passively acquired maternal antibody is waning. This observation provided the original justification for prophylactic administration of anti-*R. equi* hyperimmune plasma (HIP). The theory was that the age-related susceptibility of foals to *R. equi* infection reflects loss of the protection provided by maternal antibodies at a time when foals are only beginning to produce their own antibody. The provision of high levels of *R. equi* specific antibody in the form of HIP was intended to address what was viewed as the basic underlying problem. In most studies, HIP has been shown to have a beneficial effect – decreasing the number of cases and often times the severity of clinical signs and/or lesions (Becu et al., 1997; Caston et al., 2006; Higuchi et al., 1999; Madigan et al., 1991; Martens et al., 1989). Even where the protection was not statistically significant, there was as much as a 30% decrease in cases, a change that could have real life significance on endemic farms (Giguere et al., 2002). When the theory emerged that foals might actually be infected in the first week of life and then incubate the disease for multiple weeks, many clinicians began to administer HIP at 1-2 weeks of age – sometimes followed by a second dose closer to the more traditional age (Caston et al., 2006; Horowitz et al., 2001; Perkins et al., 2002).

*R. equi* is, however, an intracellular pathogen - closely related and behaving similarly to *M. tuberculosis* (Prescott, 1993). Subsequent research performed in mice and supported by studies in horses has indicated that immune clearance of *R. equi* is

cell mediated response. Clearance is achieved primarily by antigen-specific CD4+ Th1 lymphocytes secreting IFN-γ and thereby activating macrophages (Darrah et al., 2000; Hines et al., 2003; Kanaly et al., 1995, 1996). CD8+ cytotoxic T lymphocytes that recognize and lyse *R. equi*-infected cells are also thought to be involved (Patton et al., 2004; Patton et al., 2005). The partial protection of antibody demonstrated in vivo and in several in vitro studies were thought to reflect effector functions of immunoglobulin, notably the ability of equine isotypes like IgGa (IgG1) and IgGb (IgG4 and IgG7) to opsonize and fix complement (Lewis et al., 2008). The apparent inability of HIP to provide more robust protection was attributed to the failure of individual foals to develop the required cellular responses. Like neonates and perinates of other species, young horses appear to have a decreased ability to mount the protective Type 1 immune responses seen in adult horses (i.e. the protective phenotype) (Boyd et al., 2003; Breathnach et al., 2006; Giguere and Polkes, 2005; Merant et al., 2009). In conjunction with their lack of immunologic memory, this age-associated defect predisposes foals to rhodococcal pneumonia and explains their unique susceptibility.

In this study, we first provide further evidence in support of the effector functions of *R. equi*-specific antibodies. As predicted, opsonization increased the uptake of virulent *R. equi* bacteria by equine macrophages and decreased intracellular survival. As *R. equi* typically enters equine macrophages via the Mac-1 type 3 complement receptor, we hypothesize that these effects likely reflect increased entry of bacteria via the Fc receptor (Hondalus et al., 1993). FcR mediated phagocytosis has been shown to, increase phagosome-lysosomal fusion, increase reactive oxygen species, and increase reactive nitrogen molecules (Dasgupta et al., 2000; Gaikwad and Sinha, 2008;

Hietala and Ardans, 1987; Martinez et al., 2009). By changing the receptor mediating phagocytosis, the route of entry is also changed, potentially altering the outcome (Martinez et al., 2009). Similarly, these data show for the first time that opsonization improves the viability of *R. equi* infected cells – i.e. anti-*R. equi* IgG decreases the cytotoxic effects that result in the necrotic death of infected macrophages (Luhrmann et al., 2004). As necrosis is, in contrast to apoptosis, a pro-inflammatory response that likely contributes to development of pyogranulomatous lesions in the lung, this effect is also likely to be beneficial to the host (Russell et al., 2009; Saunders and Britton, 2007).

In addition to its effector functions, antibody can influence cellular responses. For example, antibody can decrease the dose of antigen needed to stimulate lymphocytes, increase the recruitment and proliferation of lymphocytes, and alter the expression of cytokines (Amigorena and Bonnerot, 1999; Chang, 1985; Clynes et al., 1999; Dasgupta et al., 2000; Gosselin et al., 1992; Guyre et al., 1997; Igietseme et al., 2004; Manca et al., 1988; Marsh et al., 1995; Marsh et al., 1997; Ralston et al., 1997). Polyclonal antibody to a related actinomycete bacteria *M. bovis* Bacillus Calmette-Guérin (BCG) increases IFN-$\gamma$ production in humans and enhances proliferation of CD4+ and CD8+ T lymphocytes (de Valliere et al., 2005). Studies in knock-out strains of mice have provided strong evidence that the effects of antibody on cell mediated responses reflect engagement of activating FcR on antigen presenting cells (Moore et al., 2002; Pleass and Woof, 2001).

In most naturally occurring in vivo situations, antibody is present as a result of a previous primary immune response. Therefore, it would affect cell mediated responses in the case of a subsequent secondary immune response (e.g. challenge) and/or an

ongoing infection. The scenario is different with *R. equi* infection in foals. Because of the passive acquisition of maternal antibody via colostrum and/or administration of HIP, foals may already have significant levels of *R. equi* specific antibody during initiation of the primary immune response. Beyond its expected effector functions, passively acquired antibody might influence alter the outcome of infection through its effects on professional phagocytes, notably macrophages and dendritic cells, and the subsequent effects of those cells on developing T cell responses. This is potentially important as the quantity and quality of antibody in neonatal and perinatal foals is manipulable. For example, strategic immunization of the pregnant mare and/or administration of an improved anti-*R. equi* IgG product foals could be part of an integrated immunologic approach that also involved active immunization of foals (Cauchard et al., 2004).

In this study, we addressed the initial question of whether anti-*R. equi* IgG alters in vitro expression of cytokines with potential relevance to immune clearance. The expression of five Type 1 cytokines (IL-12p35, IL-12p40, IL-23p19, TNF-α, and IL-18) and the Type 2 cytokine IL-10 was measured in equine macrophages (MDM) using real time RT-PCR at 5 and 11 hours post infection. These two time points were selected based on the results of preliminary experiments and differ somewhat from earlier studies with murine macrophages in which cytokine mRNA was measured at 4 HPI (Giguere and Prescott, 1998).

As previously reported, *R. equi* infection of equine macrophages results in significant up-regulation of cytokine expression (Flaminio et al., 2009; Garton et al., 2002). However, opsonization of *R. equi* with IgG resulted in further increases in transcription of IL-12 (both p35 and p40 subunits), IL-23p19, and TNF-α when

compared to controls. In other species, IL-12 plays at least three important roles that could affect the outcome of *R. equi* infection in horses. It induces naïve T cells to differentiate into Th1 cells, activates NK and T lymphocytes, and stimulates IFN-γ production which is required for clearance of *R. equi* (Langrish et al., 2004; Stobie et al., 2000). The IL-23p19 subunit forms a heterodimer with the p40 subunit of IL-12 to produce bioactive IL-23. In other species, heterodimeric IL-23 stimulates the production of IL-17, a potent inflammatory mediator that increases IL-1, IL-6, TNF-α and IFN-γ (Langrish et al., 2004). Because these cytokines can bias immunity to a Type 1 cell mediated immune response, upregulation of IL-23 with opsonization is likely also relevant to outcome. In addition, infection of equine MDM with opsonized *R. equi* increased expression of TNF-α at 5 and 11 HPI. TNF-α induces local inflammation, endothelial cell activation, macrophage activation, and IFN-γ production (Herbein and O'Brien, 2000). Previous studies have shown that TNF-α plays a role in the course of *R. equi* infection in mice; TNF-α-depleted mice given a sub-lethal dose of R, equi developed lethal rhodococcal pneumonia infections (Kasuga-Aoki et al., 1999; Nordmann et al., 1993).

In contrast, IL-10 is an anti-inflammatory Type 2 cytokine that potently inhibits Th1 cells and the production of Type 1 cytokines (Moore et al., 2001). Increased expression of IL-10 would be expected to detrimental to the host. In this study, we detected more modest changes in IL-10 transcripts compared to other cytokines and expression was not increased by opsonization. Although it is possible that IL-10 expression had not peaked at the time points we examined, another study also reported

less than 10 fold differences compared to uninfected equine macrophages over a 24 hour period (Garton et al., 2002).

Although the effects of anti-*R. equi* IgG cytokine expression were significant compared to both controls, we often also observed an effect of the anti-EIAV IgG control when compared to the *R. equi* infected MDM to which no additional IgG had been added. There are several potential explanations for this finding. One is the presence of residual anti-*R. equi* antibodies in the control. As might be expected, the equine plasma from which this IgG was prepared had low levels of *R. equi* specific antibody at the start. However, the ELISA titer following multiple absorptions against whole *R. equi* bacteria was neglible. Another possible explanation is the interaction of IgG aggregates in the control with activating Fcγ receptor FcγRIIa (CD32) and associated cross-linking that could result in activation (Hart et al., 2004). A third possibility is small amounts of contaminating cytokines.

An unexpected finding that has not previously been reported was the down-regulation of IL-18 transcription produced by *R. equi* infection. At 11 HPI, *R. equi* infected cells from all horses produced significantly less IL-18 mRNA than non-infected MDM. Neither opsonization with anti-*R. equi* IgG nor addition of the anti-EIAV control IgG had an additional effect. In other species, IL-18 is a pro-inflammatory cytokine that induces the expression of IFN-γ, IL-2, and GM-CSF (O'Donovan et al., 2004). Down-regulation of IL-18 due to *R. equi* infection would be expected to dampen a protective Type 1 response and enhance survival of the bacteria. This may be a mechanism by which the bacterium modulates the host response in its favor.

In summary, these in vitro studies provide evidence that the previously described benefits of anti-*R. equi* antibody might be attributable, at least in part, to its effects on developing cellular responses. An expected argument against the use of passively acquired anti-*R. equi* antibodies are the potential for antibody to interfere with primary immune responses in foals. Maternal antibody interference is a well known phenomenon in infants and animals (Crowe, 2001; Jeffcott, 1974; Siegrist, 2003; Wilson et al., 2001). However, the inhibition is B cell specific and T cells are typically not significantly altered (Endsley et al., 2003; Siegrist, 2003). Uptake of antigen: maternal antibody complexes by antigen presenting cells typically promotes antigen processing and presentation, leading to T cell priming even in the face of B cell interference. Additional experiments that would further support the use of anti-*R. equi* antibody include testing the effect of opsonization on equine dendritic cells, determining whether cells from foals respond similarly to cells from adult horses, and adding T lymphocytes to the cultures to confirm the expected outcomes (e.g. increased production of IFN-γ) (Orme et al., 1993). Ultimately, confirmation of enhanced Type 1 cytokine responses in vivo will also be required. However, these experiments suggest that passive transfer of anti-*R. equi* may be useful as part of an integrated immunoprophylactic strategy in horses - especially considering the likely need to begin immunization of foals during the first weeks of life.

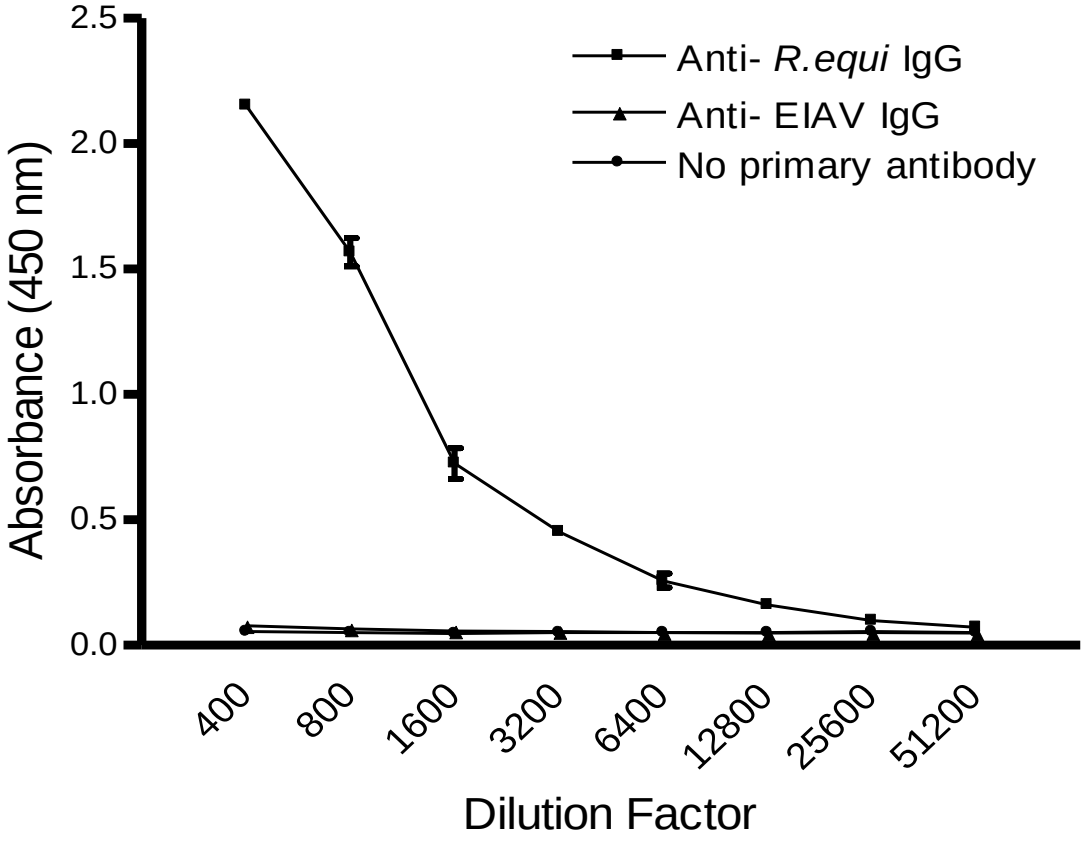


**Fig. 1:** Anti- *R. equi* IgG and Anti- EIAV IgG titers determined by ELISA. Values represent the titer plus the standard deviation at each dilution.

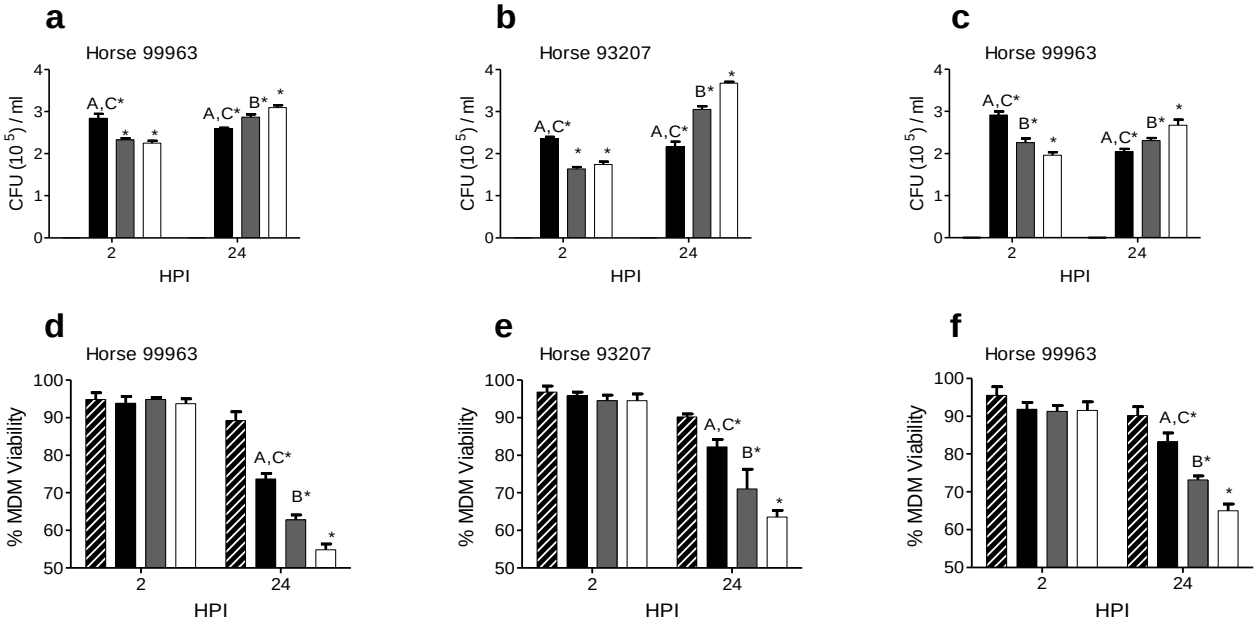


**Fig. 2**: Effect of IgG on *R. equi* uptake, intracellular replication, and host cell viability. Uninfected MDM (▨) are compared to *R. equi* infected MDM. The three treatments are opsonization with α- *R. equi* IgG (■), incubation with α-EIAV IgG control that has been absorbed to remove α- *R. equi* antibodies (▩), and no additional IgG control (□). The intracellular *R. equi* CFU (a, b, c) and host cell viability (d, e, f) were observed at 2 and 24 hours post infection. Each graph depicts one representative outcome of the three independent experiments that were completed with each animal. Within each experiment, each test was run in triplicate; each bar shows the mean and standard deviation. (*) Statistically significant ($P<0.05$) compared to uninfected MDM; (A) α- *R. equi* IgG opsonization statistically significant ($P<0.05$) compared to α-EIAV IgG control; (B) α-EIAV IgG control statistically significant ($P<0.05$) compared to no additional IgG control; (C) α- *R. equi* IgG opsonization statistically significant ($P<0.05$) compared no additional IgG control

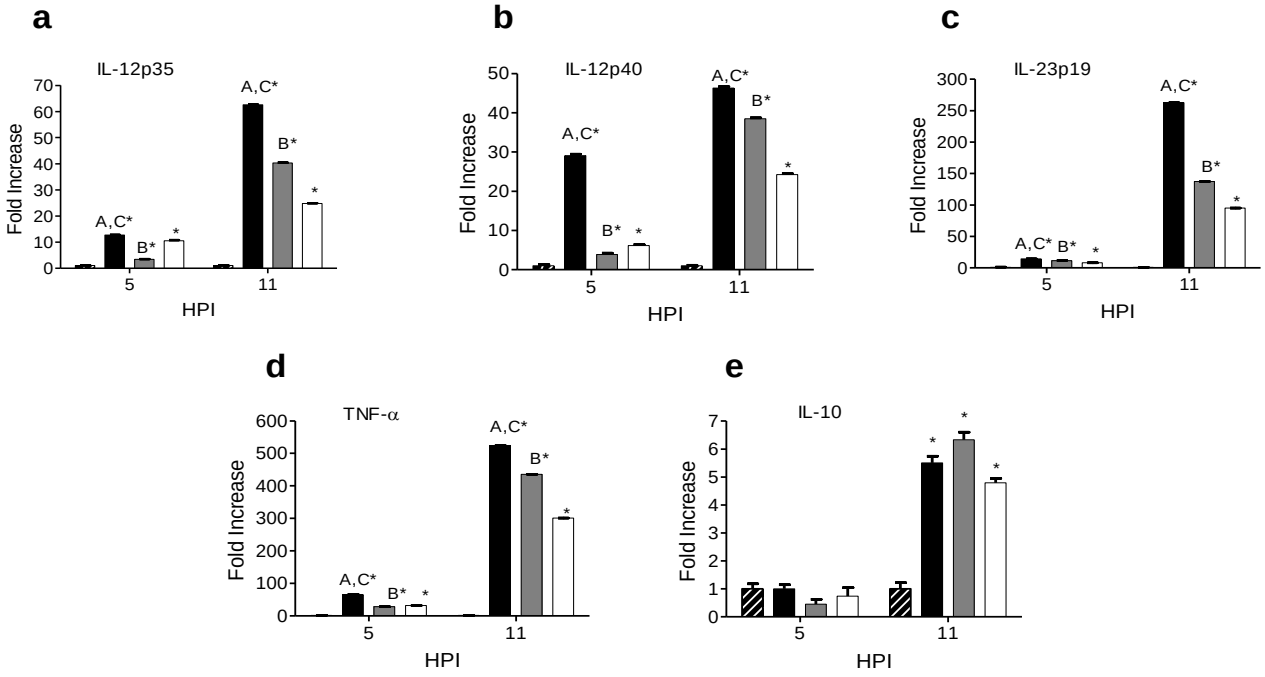


**Fig. 3**: Effect of IgG on cytokine expression. (a) IL-12p35, (b) IL-12p40, (c) IL-23, (d)TNF-α, and (e) IL-10 mRNA are up-regulated in MDM infected with *R. equi*. *R. equi* was opsonized with α- *R. equi* IgG (■), incubated with α-EIAV IgG control that has been absorbed to remove α- *R. equi* antibodies (▭), or incubated with a no additional IgG control (□). The ΔΔ CT method was used to calculate the mean fold increase in expression relative to uninfected MDM (▨) at 5 and 11 HPI. Each graph from horse 93207 depicts one representative outcome of the three independent experiments completed with each animal. Similar results were seen in all animals. Within each experiment, each test was run in triplicate; each bar shows the mean and standard deviation. (*) Statistically significant ($P<0.05$) compared to uninfected MDM; (A) α- *R. equi* IgG opsonization statistically significant ($P<0.05$) compared to α-EIAV IgG control; (B) α-EIAV IgG control statistically significant ($P<0.05$) compared to no additional IgG control; (C) α- *R. equi* IgG opsonization statistically significant ($P<0.05$) compared no additional IgG control

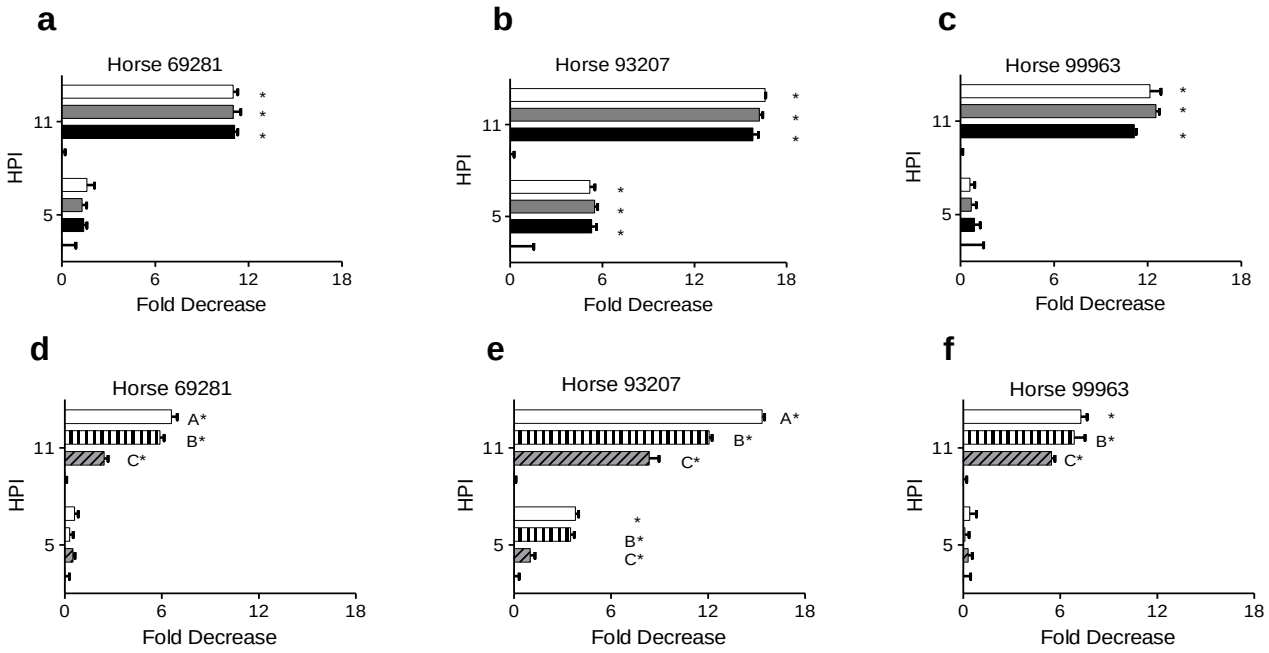


**Fig. 4:** IL-18 expression is down-regulated in adult equine MDM infected with *R. equi.* MDM infected *R. equi* opsonized with α- *R. equi* IgG (■), incubated with α –EIAV control (▭), and a no additional IgG control (□) compared to non-infected MDM (▨) at 5 and 11 HPI (a, b, c,). IL-18 expression by MDM infected with virulent *R. equi* (□), avirulent *R. equi* (▥), stimulated with virulent heat killed *R. equi* (▨), and non-infected MDM (▨). Values represent the mean fold changes in expression relative to virulent *R. equi* infected MDM plus standard deviation at 5 and 11 HPI. Each graph depicts one representative outcome of the three independent experiments that were completed with each animal. Within each experiment, each test was run in triplicate; each bar shows the mean standard deviation. (*) Statistically significant (P<0.05) compared to uninfected MDM; (A) Virulent *R. equi* infected MDM statistically significant (P<0.05) compared to virulent heat killed stimulated MDM; (B) virulent heat killed stimulated MDM statistically significant (P<0.05) compared to avirulent *R. equi* infected MDM; (C) avirulent *R. equi* infected MDM statistically significant (P<0.05) compared virulent *R. equi* infected MDM

## REFERENCES


Amigorena, S., Bonnerot, C., 1999, Fc receptor signaling and trafficking: a connection for antigen processing. Immunol Rev 172, 279-284.

Becu, T., Polledo, G., Gaskin, J.M., 1997, Immunoprophylaxis of *Rhodococcus equi* pneumonia in foals. Vet Microbiol 56, 193-204.

Boyd, N.K., Cohen, N.D., Lim, W.S., Martens, R.J., Chaffin, M.K., Ball, J.M., 2003, Temporal changes in cytokine expression of foals during the first month of life. Veterinary Immunology and Immunopathology 92, 75-85.

Breathnach, C.C., Sturgill-Wright, T., Stiltner, J.L., Adams, A.A., Lunn, D.P., Horohov, D.W., 2006, Foals are interferon gamma-deficient at birth. Veterinary Immunology and Immunopathology 112, 199-209.

Caston, S.S., McClure, S.R., Martens, R.J., Chaffin, M.K., Miles, K.G., Griffith, R.W., Cohen, N.D., 2006, Effect of hyperimmune plasma on the severity of pneumonia caused by *Rhodococcus equi* in experimentally infected foals. Vet Ther 7, 361-375.

Cauchard, J., Sevin, C., Ballet, J.-J., Taouji, S., 2004, Foal IgG and opsonizing anti-*Rhodococcus equi* antibodies after immunization of pregnant mares with a protective VapA candidate vaccine. Veterinary Microbiology 104, 73-81.

Chang, T.W., 1985, Regulation of immune response by antibodies: the importance of antibody and monocyte Fc receptor interaction in T-cell activation. Immunology Today 6, 245-249.

Clynes, R., Maizes, J.S., Guinamard, R., Ono, M., Takai, T., Ravetch, J.V., 1999, Modulation of immune complex-induced inflammation in vivo by the coordinate expression of activation and inhibitory Fc receptors. J Exp Med 189, 179-185.

Crowe, J.E., Jr., 2001, Influence of maternal antibodies on neonatal immunization against respiratory viruses. Clin Infect Dis 33, 1720-1727.

Darrah, P.A., Hondalus, M.K., Chen, Q., Ischiropoulos, H., Mosser, D.M., 2000, Cooperation between reactive oxygen and nitrogen intermediates in killing of *Rhodococcus equi* by activated macrophages. Infect Immun 68, 3587-3593.

Dasgupta, D., Chakraborty, P., Basu, M.K., 2000, Ligation of Fc receptor of macrophages stimulates protein kinase C and anti-leishmanial activity. Mol Cell Biochem 209, 1-8.

de Valliere, S., Abate, G., Blazevic, A., Heuertz, R.M., Hoft, D.F., 2005, Enhancement of innate and cell-mediated immunity by antimycobacterial antibodies. Infect Immun 73, 6711-6720.

Endsley, J.J., Roth, J.A., Ridpath, J., Neill, J., 2003, Maternal antibody blocks humoral but not T cell responses to BVDV. Biologicals 31, 123-125.

Fernandez, A.S., Prescott, J.F., Nicholson, V.M., 1997, Protective effect against *Rhodococcus equi* infection in mice of IgG purified from horses vaccinated with virulence associated protein (VapA)-enriched antigens. Vet Microbiol 56, 187-192.

Flaminio, M.J., Nydam, D.V., Marquis, H., Matychak, M.B., Giguere, S., 2009, Foal monocyte-derived dendritic cells become activated upon *Rhodococcus equi* infection. Clin Vaccine Immunol 16, 176-183.

Gaikwad, A.N., Sinha, S., 2008, Determinants of natural immunity against tuberculosis in an endemic setting: factors operating at the level of macrophage-*Mycobacterium tuberculosis* interaction. Clin Exp Immunol 151, 414-422.

Garton, N.J., Gilleron, M., Brando, T., Dan, H.H., Giguere, S., Puzo, G., Prescott, J.F., Sutcliffe, I.C., 2002, A novel lipoarabinomannan from the equine pathogen *Rhodococcus equi*. Structure and effect on macrophage cytokine production. J Biol Chem 277, 31722-31733.

Giguere, S., Gaskin, J.M., Miller, C., Bowman, J.L., 2002, Evaluation of a commercially available hyperimmune plasma product for prevention of naturally acquired pneumonia caused by *Rhodococcus equi* in foals. J Am Vet Med Assoc 220, 59-63.

Giguere, S., Polkes, A.C., 2005, Immunologic disorders in neonatal foals. Vet Clin North Am Equine Pract 21, 241-272, v.

Giguere, S., Prescott, J.F., 1998, Cytokine induction in murine macrophages infected with virulent and avirulent *Rhodococcus equi*. Infect Immun 66, 1848-1854.

Gosselin, E.J., Wardwell, K., Gosselin, D.R., Alter, N., Fisher, J.L., Guyre, P.M., 1992, Enhanced antigen presentation using human Fc gamma receptor (monocyte/macrophage)-specific immunogens. J Immunol 149, 3477-3481.

Guyre, P.M., Graziano, R.F., Goldstein, J., Wallace, P.K., Morganelli, P.M., Wardwell, K., Howell, A.L., 1997, Increased potency of Fc-receptor-targeted antigens. Cancer Immunol Immunother 45, 146-148.

Hart, S.P., Alexander, K.M., Dransfield, I., 2004, Immune complexes bind preferentially to Fc gamma RIIA (CD32) on apoptotic neutrophils, leading to augmented

phagocytosis by macrophages and release of proinflammatory cytokines. J Immunol 172, 1882-1887.

Herbein, G., O'Brien, W.A., 2000, Tumor necrosis factor (TNF)-alpha and TNF receptors in viral pathogenesis. Proc Soc Exp Biol Med 223, 241-257.

Hietala, S.K., Ardans, A.A., 1987, Interaction of *Rhodococcus equi* with phagocytic cells from *R. equi*-exposed and non-exposed foals. Vet Microbiol 14, 307-320.

Higuchi, T., Arakawa, T., Hashikura, S., Inui, T., Senba, H., Takai, S., 1999, Effect of prophylactic administration of hyperimmune plasma to prevent *Rhodococcus equi* infection on foals from endemically affected farms. Zentralbl Veterinarmed B 46, 641-648.

Hines, S.A., Stone, D.M., Hines, M.T., Alperin, D.C., Knowles, D.P., Norton, L.K., Hamilton, M.J., Davis, W.C., McGuire, T.C., 2003, Clearance of virulent but not avirulent *Rhodococcus equi* from the lungs of adult horses is associated with intracytoplasmic gamma interferon production by CD4+ and CD8+ T lymphocytes. Clin Diagn Lab Immunol 10, 208-215.

Hondalus, M.K., Diamond, M.S., Rosenthal, L.A., Springer, T.A., Mosser, D.M., 1993, The intracellular bacterium *Rhodococcus equi* requires Mac-1 to bind to mammalian cells. Infect Immun 61, 2919-2929.

Hooper-McGrevy, K.E., Giguere, S., Wilkie, B.N., Prescott, J.F., 2001, Evaluation of equine immunoglobulin specific for *Rhodococcus equi* virulence-associated proteins A and C for use in protecting foals against *Rhodococcus equi*-induced pneumonia. Am J Vet Res 62, 1307-1313.

Horowitz, M.L., Cohen, N.D., Takai, S., Becu, T., Chaffin, M.K., Chu, K.K., Magdesian, K.G., Martens, R.J., 2001, Application of Sartwell's model (lognormal distribution of incubation periods) to age at onset and age at death of foals with *Rhodococcus equi* pneumonia as evidence of perinatal infection. J Vet Intern Med 15, 171-175.

Igietseme, J.U., Eko, F.O., He, Q., Black, C.M., 2004, Antibody regulation of Tcell immunity: implications for vaccine strategies against intracellular pathogens. Expert Rev Vaccines 3, 23-34.

Jeffcott, L.B., 1974, Studies on passive immunity in the foal. 1. Gamma-globulin and antibody variations associated with the maternal transfer of immunity and the onset of active immunity. J Comp Pathol 84, 93-101.

Kanaly, S.T., Hines, S.A., Palmer, G.H., 1995, Cytokine modulation alters pulmonary clearance of *Rhodococcus equi* and development of granulomatous pneumonia. Infect Immun 63, 3037-3041.

Kanaly, S.T., Hines, S.A., Palmer, G.H., 1996, Transfer of a CD4+ Th1 cell line to nude mice effects clearance of *Rhodococcus equi* from the lung. Infect Immun 64, 1126-1132.

Kasuga-Aoki, H., Takai, S., Sasaki, Y., Tsubaki, S., Madarame, H., Nakane, A., 1999, Tumour necrosis factor and interferon-gamma are required in host resistance against virulent *Rhodococcus equi* infection in mice: cytokine production depends on the virulence levels of *R. equi*. Immunology 96, 122-127.

Langrish, C.L., McKenzie, B.S., Wilson, N.J., de Waal Malefyt, R., Kastelein, R.A., Cua, D.J., 2004, IL-12 and IL-23: master regulators of innate and adaptive immunity. Immunol Rev 202, 96-105.

Lewis, M.J., Wagner, B., Woof, J.M., 2008, The different effector function capabilities of the seven equine IgG subclasses have implications for vaccine strategies. Mol Immunol 45, 818-827.

Livak, K.J., Schmittgen, T.D., 2001, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402-408.

Lopez, A.M., Hines, M.T., Palmer, G.H., Alperin, D.C., Hines, S.A., 2002, Identification of pulmonary T-lymphocyte and serum antibody isotype responses associated with protection against *Rhodococcus equi*. Clin Diagn Lab Immunol 9, 1270-1276.

Luhrmann, A., Mauder, N., Sydor, T., Fernandez-Mora, E., Schulze-Luehrmann, J., Takai, S., Haas, A., 2004, Necrotic death of *Rhodococcus equi*-infected macrophages is regulated by virulence-associated plasmids. Infect Immun 72, 853-862.

Madigan, J.E., Hietala, S., Muller, N., 1991, Protection against naturally acquired *Rhodococcus equi* pneumonia in foals by administration of hyperimmune plasma. J Reprod Fertil Suppl 44, 571-578.

Manca, F., Fenoglio, D., Kunkl, A., Cambiaggi, C., Sasso, M., Celada, F., 1988, Differential activation of T cell clones stimulated by macrophages exposed to

antigen complexed with monoclonal antibodies. A possible influence of paratope specificity on the mode of antigen processing. J Immunol 140, 2893-2898.

Marsh, C.B., Gadek, J.E., Kindt, G.C., Moore, S.A., Wewers, M.D., 1995, Monocyte Fc gamma receptor cross-linking induces IL-8 production. J Immunol 155, 3161-3167.

Marsh, C.B., Wewers, M.D., Tan, L.C., Rovin, B.H., 1997, Fc(gamma) receptor cross-linking induces peripheral blood mononuclear cell monocyte chemoattractant protein-1 expression: role of lymphocyte Fc(gamma)RIII. J Immunol 158, 1078-1084.

Martens, R.J., Martens, J.G., Fiske, R.A., Hietala, S.K., 1989, *Rhodococcus equi* foal pneumonia: protective effects of immune plasma in experimentally infected foals. Equine Vet J 21, 249-255.

Martinez, F.O., Helming, L., Gordon, S., 2009, Alternative activation of macrophages: an immunologic functional perspective. Annu Rev Immunol 27, 451-483.

Merant, C., Breathnach, C.C., Kohler, K., Rashid, C., Van Meter, P., Horohov, D.W., 2009, Young foal and adult horse monocyte-derived dendritic cells differ by their degree of phenotypic maturity. Vet Immunol Immunopathol 131, 1-8.

Moore, K.W., de Waal Malefyt, R., Coffman, R.L., O'Garra, A., 2001, Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol 19, 683-765.

Moore, T., Ananaba, G.A., Bolier, J., Bowers, S., Belay, T., Eko, F.O., Igietseme, J.U., 2002, Fc receptor regulation of protective immunity against Chlamydia trachomatis. Immunology 105, 213-221.

Nordmann, P., Ronco, E., Guenounou, M., 1993, Involvement of interferon-gamma and tumor necrosis factor-alpha in host defense against *Rhodococcus equi*. J Infect Dis 167, 1456-1459.

O'Donovan, L.H., McMonagle, E.L., Taylor, S., Argyle, D.J., Nicolson, L., 2004, Bioactivity and secretion of interleukin-18 (IL-18) generated by equine and feline IL-18 expression constructs. Vet Immunol Immunopathol 102, 421-428.

Orme, I.M., Roberts, A.D., Griffin, J.P., Abrams, J.S., 1993, Cytokine secretion by CD4 T lymphocytes acquired in response to *Mycobacterium tuberculosis* infection. J Immunol 151, 518-525.

Patton, K.M., McGuire, T.C., Fraser, D.G., Hines, S.A., 2004, *Rhodococcus equi*-infected macrophages are recognized and killed by CD8+ T lymphocytes in a major histocompatibility complex class I-unrestricted fashion. Infect Immun 72, 7073-7083.

Patton, K.M., McGuire, T.C., Hines, M.T., Mealey, R.H., Hines, S.A., 2005, *Rhodococcus equi*-specific cytotoxic T lymphocytes in immune horses and development in asymptomatic foals. Infect Immun 73, 2083-2093.

Perkins, G.A., Yeager, A., Erb, H.N., Nydam, D.V., Divers, T.J., Bowman, J.L., 2002, Survival of foals with experimentally induced *Rhodococcus equi* infection given either hyperimmune plasma containing *R. equi* antibody or normal equine plasma. Vet Ther 3, 334-346.

Pleass, R.J., Woof, J.M., 2001, Fc receptors and immunity to parasites. Trends Parasitol 17, 545-551.

Prescott, J.F., 1993, Immunodeficiency and serious pneumonia in foals: the plot thickens. Equine Vet J 25, 88-89.

Raabe, T., Bukrinsky, M., Currie, R.A., 1998, Relative contribution of transcription and translation to the induction of tumor necrosis factor-alpha by lipopolysaccharide. J Biol Chem 273, 974-980.

Ralston, D.R., Marsh, C.B., Lowe, M.P., Wewers, M.D., 1997, Antineutrophil cytoplasmic antibodies induce monocyte IL-8 release. Role of surface proteinase-3, alpha1-antitrypsin, and Fcgamma receptors. J Clin Invest 100, 1416-1424.

Rojas, G., Jimenez, J.M., Gutierrez, J.M., 1994, Caprylic acid fractionation of hyperimmune horse plasma: description of a simple procedure for antivenom production. Toxicon 32, 351-363.

Russell, D.G., Cardona, P.J., Kim, M.J., Allain, S., Altare, F., 2009, Foamy macrophages and the progression of the human tuberculosis granuloma. Nat Immunol 10, 943-948.

Saunders, B.M., Britton, W.J., 2007, Life and death in the granuloma: immunopathology of tuberculosis. Immunol Cell Biol 85, 103-111.

Siegrist, C.A., 2003, Mechanisms by which maternal antibodies influence infant vaccine responses: review of hypotheses and definition of main determinants. Vaccine 21, 3406-3412.

Stobie, L., Gurunathan, S., Prussin, C., Sacks, D.L., Glaichenhaus, N., Wu, C.Y., Seder, R.A., 2000, The role of antigen and IL-12 in sustaining Th1 memory cells in vivo:

IL-12 is required to maintain memory/effector Th1 cells sufficient to mediate protection to an infectious parasite challenge. Proc Natl Acad Sci U S A 97, 8427-8432.

Wilson, W.D., Mihalyi, J.E., Hussey, S., Lunn, D.P., 2001, Passive transfer of maternal immunoglobulin isotype antibodies against tetanus and influenza and their effect on the response of foals to vaccination. Equine Vet J 33, 644-650.

## APPENDIX

List of commonly used abbreviations

| | |
|---|---|
| APC | Antigen Presenting Cells |
| CMI | Cell Mediated Immunity |
| CTL | Cytotoxic T Lymphocytes |
| FcγR | Fc Receptor Gamma |
| IFN-γ | Interferon Gamma |
| IL | Interleukin |
| MDM | Monocyte Derived Macrophage |
| MHC | Major Histocompatibility Receptor |
| PBMC | Peripheral Blood Mononuclear Cells |
| Th | T helper cells |
| TNF-α | Tumor Necrosis Factor Alpha |