ANTIBODIES TARGETING POLY-*N*-ACETYL GLUCOSAMINE PROTECT

AGAINST THE INTRACELLULAR PATHOGEN, *RHODOCOCCUS EQUI*

A Dissertation

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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December 2018

Major Subject: Biomedical Sciences

ABSTRACT

Rhodococcus equi is a facultative intracellular pathogen that causes

pyogranulomatous pneumonia in foals <6 months of age. Why foals are highly susceptible

to R. equi is unknown. R. equi pneumonia is a leading cause of morbidity and mortality

for foals and continues to be an economic burden for the horse industry world-wide. A

commercial vaccine against R. equi is lacking. We determined the immunogenicity and

efficacy of maternal vaccination with the highly conserved microbial surface

polysaccharide, β-1$\rightarrow$6-linked poly-N-acetyl glucosamine (PNAG) in foals against

intrabronchial infection with R. equi and characterized the functional properties associated

with antibodies to R. equi.

We vaccinated 19 pregnant mares 6 and 3 weeks prior to foaling with PNAG and

experimentally infected their foals at ~4 weeks of age with ~10^6 cfu of R. equi. Eleven of

12 (92%) foals born to immunized mares remained healthy, whereas 6 of 7 (86%) foals

born to unvaccinated controls developed pneumonia (P=0.0017). Antibodies to PNAG

mediated killing of extracellular and intracellular R. equi in the presence of complement

and neutrophils by PNAG recognition on infected cells. Peripheral blood mononuclear

cells from immune and protected foals released higher levels of interferon-γ in response to

PNAG compared to controls, indicating vaccination also induced an antibody-dependent

cellular release of immune cytokines. To determine the relative function in immunity to

R. equi, sub-isotypes IgG_1 and $IgG_{4/7}$ were enriched using a protein G column from PNAG

hyperimmune or nonimmune plasmas. They were compared in their ability to deposit

complement component 1 (C1) on to PNAG and to mediate opsonophagocytic killing

(OPK) by neutrophils. Sub-isotype IgG_1 from PNAG hyperimmune plasma had the

highest ability to deposit C1 onto PNAG ($P<0.05$) and to elicit OPK by neutrophils

($P<0.05$). These results show maternal vaccination with PNAG generates antibodies that

are transferred via colostrum that protect foals against the intracellular pathogen, *R. equi*

by facilitating antibody-mediated opsonic killing, and that sub-isotype IgG_1 targeting

PNAG appears to be a correlate of protective immunity. Additionally, vaccination

appeared to modulate interferon-γ release in response to PNAG on *R. equi*, suggesting that

PNAG antibodies derived from colostrum might mediate cell immunity.

DEDICATION

I would like to dedicate this dissertation to my family: my mom, dad, sister, and husband. You are the people most dear to me in the world. You have unknowingly given me the strength and confidence I needed to persevere and to follow my ambition. Thank you for your support and for being a very important part of my life.

ACKNOWLEDGEMENTS

Finally, thanks to my mother and father for their encouragement and to my husband for his patience and love.

Thank you to all of you. Without you none of this would have been possible.

CONTRIBUTORS AND FUNDING SOURCES

Veterinary Large Animal Clinical Sciences and were submitted in 2018 to Infection and Immunity. All other work conducted for the dissertation was completed by the student independently.

NOMENCLATURE

ADCC   Antibody-dependent cellular cytotoxicity

APC   Antigen presenting cell

BAL   Bronchoalveolar lavage

BALF   Bronchoalveolar lavage fluid

BCG   Bacillus Calmette Guerin vaccine

C1   Complement component 1

CBC   Complete blood count

CFU   Colony forming unit

CMI   Cell-mediated immunity

DC   Dendritic cell

DEAE   Diethylaminoethyl

ELISA   Enzyme-linked immunosorbent assay

GFP   Green fluorescent protein

IFNγ Interferon gamma

IgG   Immunoglobulin G

MAb   Monoclonal antibody

MDM   Monocyte-derived macrophage

OD   Optical density

OPK   Opsonophagocytic killing

PBMC   Peripheral blood mononuclear cells

PMN   Polymorphonuclear cell

PNAG   β-1→6-linked poly-*N*-acetylglucosamine

SDS-PAGE   Sodium dodecyl sulfate polyacrylamide gel electrophoresis

TB   Tuberculosis

TRIM21   Tripartite motif-containing 21

TT   Tetanus toxoid

T-TBA   Transendoscopic tracheobronchial aspiration

VapA   Virulence-associated protein A

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INRODUCTION: ANTIBODY-MEDIATED PROTECTION AGAINST THE

INTRACELLULAR PATHOGEN RHODOCOCCUS EQUI

Introduction

Background on Rhodococcus equi *(R. equi) and* R. equi *pneumonia*

Rhodococcus equi (*R. equi*) is a ubiquitous facultative intracellular bacterial

pathogen that causes severe pyogranulomatous pneumonia in foals less than 6 months of

age, and is among the leading causes of morbidity and mortality for foals (1-3). Why

foals but not adult horses are highly susceptible to *R. equi* is unknown and there is no

commercial vaccine available. Infection occurs when environmental *R. equi* found in

the soil (4) or in feces of herbivores (3) is aerosolized and inhaled into the lungs of foals

(5, 6), presumably during the first few weeks after birth (7). Virulent *R. equi* that are

inhaled into the lower airways of foals can enter alveolar macrophages, interfere with

phagosome maturation and phagolysosome fusion (8), and replicate intracellularly (9,

10), ultimately causing pneumonia (11).

Virulence

Virulent *R. equi* are characterized by expression of the virulence-associated

protein A (VapA), encoded by an 85-kilobase plasmid (1, 12-14). Avirulent *R. equi* or

R. equi cured of *vapA* plasmids are unable to cause disease in foals (15) and mice (16) or

to multiply intracellularly within murine macrophages *in vitro* (17). In contrast, *R. equi*

containing *vapA* plasmids successfully evade intracellular killing and replicate inside

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macrophages (17, 18), preventing phagosome maturation and its fusion with lysosomes (19) and leading to persistent infection in mice (14). The continuous replication of *R. equi* eventually kills the host cell by necrosis (20), characterized in foals by a proinflammatory response (12, 19) and pneumonia (20). Evidence exists that *vapA* is necessary but not sufficient for virulence in macrophages (14, 17).

Respiratory infections in young foals caused by *R. equi* pneumonia continue to be a major problem for the horse industry world-wide and an economic burden due to mortality rates, cost of treatment and prophylaxis, and decreased performance and earnings because recovered foals are less likely to race as adults than their birth-cohort (21, 22). The cumulative incidence data is sparse and varies among farms. Generally, cumulative incidence of clinical signs is between 5-15% but cumulative incidences greater than 20% are not unusual with case fatality rates typically 10-30% (23-25). Thus, the purpose of this paper is to review immunity to *R. equi* with an emphasis on current knowledge about antibody-mediated protection and possible mechanisms for this protection.

Immunity to R. equi pneumonia

Role of cell-mediated immunity

Historically, intracellular pathogens were considered to be killed by cell-mediated immunity (CMI) whereas humoral immunity was thought to be responsible for killing extracellular bacteria (26, 27). This dogma arose because it was assumed that once inside of a cell, pathogens were shielded from antibodies that could only eradicate pathogens extracellularly (28). In addition, diseases caused by intracellular pathogens such as *R. equi* have been associated with individuals with impaired CMI (such as

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persons with AIDS) (27, 29). Most evidence regarding the role of CMI against *R. equi*

has come from experiments in mice demonstrating that functional T lymphocytes are

necessary for intracellular clearance of virulent *R. equi* (30-32). Although cluster of

differentiation 8-positive (CD8+) cytotoxic T lymphocytes (CTLs) can recognize and

kill *R. equi*-infected cells, CD4+ lymphocytes have been demonstrated to be the

principal determinant for complete pulmonary clearance of *R. equi* by producing

interferon-gamma (IFNγ) and activating macrophages (32-34). The importance of the

role of CD4+ T lymphocytes in the clearance of *R. equi* has been demonstrated in mice

in which enriched CD4+ or CD8+ spleen lymphocytes from previously immunized mice

were transferred to naïve mice prior to experimental infection with *R. equi* (32). By day

6 post-infection, bacterial numbers in lungs of CD4+-transferred mice were significantly

lower compared to CD8+ mice (32). Interestingly, on day 13 post-infection both groups

cleared *R. equi* from all organs; however, CD4+ cells were found in organs of CD8+

recipients while there were no CD8+ lymphocytes in the organs of CD4+ recipients (32).

Similarly, Kanaly *et al*. showed CD4+ Th1 lymphocytes and IFNγ production were

responsible for the clearance of *R. equi* infection in transgenic mice that were deficient

in either CD8+ or CD4+ T lymphocytes (33) using monoclonal antibodies against

cytokines (30). Additionally, in another study adoptive transfer of *R. equi*-specific

CD4+ Th1 cells cleared *R. equi* infection from the lungs of *R. equi*-susceptible nude

mice, but transfusion of an *R. equi*-specific CD4+ Th2 cell line expressing IL-4 failed to

clear pulmonary infection (35). This Th2 response has been proposed to contribute to

the unique susceptibility of young foals to *R. equi* infection (30, 35, 36).

The relevance of findings in mice to the pathogenesis of disease in foals is unknown. Foals are generally deficient in the ability to produce IFNγ but they are able to produce a protective Th1-type immune response to natural infection (37-39) and low-dose experimental infections of *R. equi* pneumonia (40, 41). Jacks *et al.* demonstrated that foals experimentally infected with *R. equi* at 7 to 10 days of age had increased IFNγ expression from *R. equi*-stimulated lymphocytes and increased CD4+ T lymphocytes in bronchoalveolar lavage fluid (BALF) (42), confirming the ability of young foals to produce a Th1 response against *R. equi* (40). Evidence exists that CMI is crucial for immunity to *R. equi* infections and is the protective immune response of adult horses. Adult horses rapidly clear virulent *R. equi* infection without showing clinical signs of pneumonia due to significant increases in pulmonary CD4+ and CD8+ T-lymphocytes producing IFNγ and marked increases in lymphoproliferative responses to soluble *R. equi* antigen and VapA (43). This rapid clearance of *R. equi* infections is associated with a Th1 pulmonary recall response and is considered to confer the immunity seen in adult horses (43-45). This recall response is characterized by significant proliferation and increase of antigen-specific memory CD4+ T lymphocytes in the lung and the production of IFNγ in response to infection (44-46). Virulent *R. equi* in the lungs of adult horses activate memory T-cells bearing receptors specific for *R. equi* antigens (43). Then, the memory cells expand and recruit activated effector cells to the affected lung (*i.e.*, a recall response) to secrete type 1 cytokines such as IFNγ to clear pulmonary infection by *R. equi* (43, 44). The clearance of *R. equi* infections in adult horses by CD4+ T cells is associated with IFNγ expression and subsequent macrophage activation (46). CD4+ T lymphocytes may provide help for CD8+ T lymphocytes to secrete IFNγ

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or to recognize and kill *R. equi*-infected macrophages as cytotoxic effector cells (44). Lopez *et al.* demonstrated *R. equi*-infected adult horses develop VapA-specific immune responses that predominantly stimulate IFNγ expression, showing that VapA is an antigen targeted by protective pulmonary T-lymphocyte responses in horses (46). It is probable that the time-lag required for CMI responses to develop and the apparent deficiencies in adaptive immune responses of foals render young foals more susceptible to *R. equi* pneumonia (39, 47).

Role of humoral immunity against intracellular infections

Recently, the viewpoint that CMI is the only mechanism for controlling intercellular pathogen infections has been challenged by evidence that antibodies can also play an important role in protection. For example, acetone-killed vaccines have been demonstrated to be highly effective at protecting mice and people against the intracellular pathogen *Salmonella typhi* (48). Furthermore, all viruses are intracellular pathogens, and specific antibodies are known to confer protection against many viral infections, such as smallpox, yellow fever, measles, varicella, and rabies (49-51). *Mycobacterium tuberculosis* (Mtb) is arguably the most important intracellular pathogen of humans as one-third of the world's population is infected with latent Mtb (52). Individuals with active or latent tuberculosis (TB) produce different humoral signatures and antibody glycosylation pattern, and antibodies from people with latent TB drive macrophages to kill intracellular Mtb (53). Chen *et al.* demonstrated that antibodies specific to the mycobacterial capsular polysaccharide arabinomannan in sera of individuals vaccinated with bacillus Calmette-Guerin (BCG) vaccine were able to

opsonize BCG and Mtb, and enhance phagocytosis and phagolysosome fusion in human

macrophages (54). Prados-Rosales *et al.* developed a capsular polysaccharide conjugate

vaccine against Mtb that decreased bacterial numbers in lungs and spleen in infected

mice and prolonged their life (55). These results indicate that antibody can play a role in

protective immunity against pathogens residing intracellularly.

Antibody-mediated protection against R. equi

Antibodies are glycoprotein molecules, called immunoglobulins (Ig), produced

by B lymphocytes that bind antigens with high specificity and affinity at their antigen-

binding sites (variable regions), and simultaneously interact with other molecules of the

immune system with their constant region to carry out the necessary effector functions to

clear infection (56). In particular, IgG is the most common type of antibody found in

blood and extracellular fluid (56). Understanding the role of antibody-mediated

immunity against intracellular infection with *R. equi* is important for fundamental

immunology, vaccine design, and development of immunotherapy against *R. equi*

infections (26). There are several lines of evidence indicating a role for antibodies in

protecting against *R. equi* infection.

Serological evidence from natural exposure

Serological evidence from foals indicates that natural exposure to *R. equi*

generally results in a robust humoral response against *R. equi* antigens (39, 57, 58).

Serum IgG against *R. equi* develops following natural exposure in most foals (healthy or

pneumonic) and adult horses such that titers against *R. equi* are not exclusively

associated with disease (24, 37, 39, 59-62). Rising titers of anti-*R equi* antibodies

detected by enzyme-linked immunosorbent assay (ELISA) in foals were indicative of development of R $equi$ pneumonia (60, 63). Foals with naturally occurring $R. equi$ pneumonia have been shown to have higher concentrations of VapA-specific IgA in their tracheal aspirates, and VapA-specific $IgG_{4/7}$ and $IgG_{3/5}$ sub-isotypes in their serum (37, 64). This response was not seen in naturally exposed adult horses or healthy foals, who produce an IgG_1 response (37). The increase in $R. equi$-specific $IgG_{3/5}$ in naturally infected foals, a response associated to a Th2-biased ineffective response (37), may be a good predictor of $R. equi$ pneumonia and possible marker for disease (64). However, systematic evaluation of previously described serological tests for antibodies against $R. equi$ such as the serum hemolysis inhibition test (SHI test) indicate that these tests do not accurately identify foals with disease or predict foals that will develop disease (65, 66).

Humoral immune responses to experimental infection

Experimental infection induces rapid humoral IgG responses in foals and adult horses (67). Seven equine IgG sub-isotypes have been identified: IgG_1, IgG_2, IgG_3, IgG_4, IgG_5, IgG_6 and IgG_7 (67, 68). There are scant and conflicting data regarding the role of IgG sub-isotypes in immunity to $R. equi$. In adult horses experimentally infected with $R. equi$ there seems to be an increase in IgG_1(37), $IgG_{4/7}$ (40), or both IgG_1 and $IgG_{4/7}$ (46). Experimental challenge of neonatal foals using a low dose of $R. equi$, which has been shown to result in disease progression similar to that of natural infection (69), resulted in a significant increase in VapA-specific IgG_1 and $IgG_{4/7}$ (40). An IgG_1-biased profile has been reported to be a reliable measure of protection against $R. equi$

pneumonia (67, 70) and $IgG_{4/7}$- and $IgG_{3/5}$-biased responses are more prevalent among

pneumonic foals (37). It is suggested that $IgG_{4/7}$ and $IgG_{3/5}$ reflect a Th2-type (non-

protective) response, whereas IgG_1 represents a (protective) Th1-type response (37, 71).

Other studies, however, show $IgG_{4/7}$ has a protective role against *R. equi* in both

naturally (38) and experimentally infected foals (40). Moreover, an $IgG_{3/5}$-specific

R. equi response was found in foals that were protected against *R. equi* infection by oral

administration of $1x10^8$ colony forming unit (cfu) of live, virulent *R. equi* (72) while our

laboratory has shown oral administration of $1x10^{10}$ cfu of live virulent *R. equi* to foals

increased serum IgG_1 more strongly than $IgG_{4/7}$ (73). Additional studies of serological

responses following natural and experimental infection of foals with *R. equi* are greatly

needed.

Plasma transfusion

      The efficacy of humoral immunity against a pathogen can be demonstrated by

acquiring protection after passive transfer of antigen-specific antibody. Several studies

evaluating the impact of transfusion of plasma derived from immunized donor horses (so

called hyper-immune plasma (74)) on development of *R. equi* pneumonia following

natural or experimental infections have been conducted, yielding conflicting results.

Transfusion of HIP was first demonstrated to protect pony foals from experimental

infection with nebulized *R. equi* (75). Subsequently, field studies in California

demonstrated efficacy of protection in endemic farms (76). Ensuing field studies,

however, have yielded evidence either of protection that was not statistically significant

(61, 77) or of failure to protect (78). Recent experimental infectious challenge studies,

however, demonstrate that HIP from a commercial plasma manufacturer was able to reduce severity of pneumonia caused by *R. equi* (79, 80). Epidemiological data also indicate that transfusion reduces the cumulative incidence of *R. equi* pneumonia (81, 82). Most recently, our laboratory has demonstrated that plasma hyperimmune against the highly-conserved bacterial polysaccharide β-1→6-linked poly-*N*-acetylgucosamine (PNAG) protected foals against experimental infection with virulent *R. equi* (70).

A number of explanations exist for the variable findings regarding the efficacy of prophylactic transfusion of *R. equi* HIP to protect foals. Evidence exists that commercial plasma products vary in their concentrations of antibodies targeting *R. equi*, as well as in the distribution of sub-isotypes induced (80). The various reports used different products of plasma and foals were transfused at varying ages (61, 75-77, 79, 83); these factors could have contributed to efficacy of the transfused products. Moreover, the volume of plasma transfused also was variable among studies (25, 61, 75-77). The original report demonstrating efficacy in pony foals used a dose of 4 ml of plasma per kg body-weight of foals (75). This corresponds to 2 liters for a 50 kg foal; however, most studies used only 1 liter of plasma for transfusion of foals (76, 77). Some of the studies were under-powered because clinically useful relative risk reductions of > 33% were observed, yet statistical significance was not achieved (61, 77).

Collectively, the available data indicate that transfusion of HIP reduces the severity and cumulative incidence of *R. equi* pneumonia at farms. This provides compelling evidence for a role for antibody in mediating protection against the intracellular pathogen *R. equi*. Discovering the characteristics and functions of antibodies that protect against *R. equi* pneumonia will allow us to elucidate their role in

mediating protection against intracellular pathogens. Further studies are warranted to determine the type(s) of antibodies responsible for this protection, the dose (concentration and volume) of antibodies necessary, and optimal age(s) at which a foal should be transfused in order to maximize benefits of transfusion of HIP.

Vaccines

Evidence exists that antibodies appear capable of mediating protection against *R. equi*. Several vaccines have been tested against *R. equi* such as subunit vaccines (84), genetically-modified organisms (85, 86), and DNA vaccines (87-89); however, none has proven to be protective (84, 86, 87). A recent promising candidate vaccine comprised of a mutant strain of *R. equi* with an impaired steroid catabolic pathway administered intra-tracheally to 2- to 4-week-old foals provided partial protection against subsequent experimental infection with virulent *R. equi* at 6- to 8-weeks of age (90). Oral administration of live virulent *R. equi* to foals during the first weeks of life has been documented to protect against intra-bronchial infection with *R. equi* (72, 91). However, widespread administration of large numbers of live, virulent *R. equi* as a vaccine is considered infeasible because of potential to cause disease in the host and concerns for widespread environmental contamination.

Challenges of developing a vaccine against *R. equi* pneumonia arise because foals mount less effective immune responses to vaccines. This is due to their naive or immature immune system (1, 92, 93). These challenges are further complicated by the fact that foals are infected very early in life (7) when they are more susceptible to infection (69). For this reason, maternal vaccination has been evaluated for protecting

foals against *R. equi* pneumonia. The rationale for maternal vaccination targeting

R. equi is to provide passive transfer of antibodies via colostrum to foals to protect them

against infection with *R. equi*, recapitulating the protection derived by transfusion of

HIP. Early studies of maternal vaccination failed to protect foals despite evidence that

antibodies recognizing *R. equi* were transferred to foals (94, 95). A subsequent study in

France, however, documented protection of foals by maternal vaccination with

supernatant from cell culture of virulent *R. equi* (71). Recently, our laboratory

demonstrated that vaccination of mares against PNAG protected their foals against

intrabronchial infection with virulent *R. equi* (70). We further demonstrated that the

anti-PNAG antibodies were transferred to their foals via colostrum, and that these anti-

PNAG antibodies were capable of fixing complement component 1 (C1) and mediating

opsonophagocytic killing (OPK) *ex vivo* (63). These data provide compelling evidence

that antibody-mediated protection is possible against this intracellular pathogen.

Mechanisms of antibody-mediated protection

 The mechanism by which anti-PNAG antibodies mediate immunity to *R. equi* in

foals is unknown. Antibody-mediated protection against intracellular pathogens is

achieved through a variety of different mechanisms that have been characterized as

either classical or non-classical (27, 28, 96).

Classical mechanisms

 Classical mechanisms of antibody-mediated killing of intracellular bacteria

include: 1) opsonization; 2) neutralization of bacteria or bacterial toxins; 3) antibody-

dependent cellular cytotoxicity (ADCC); and, 4) interaction of the constant region (Fc) of antibodies with Fc receptors (FcR) of effector cells (26, 28).

<u>Opsonization</u>

Antibody-mediated opsonization is the coating of IgG on the surface of pathogens which enhances uptake and killing of the pathogen by phagocytic cells. Antibody opsonization of pathogens (when they are extracellular) can prevent infection of target cells either by interfering with pathogen motility (97-99), by neutralizing critical surface epitopes required for host cell entry (100-103), by complement-mediated bacterial degradation (104-107), or by promoting intracellular degradation (26). Some of these functions are accomplished by antibodies without additional mediators or cells, while others require other components of the immune system such as complement, phagocytic cells, or effector cells such as natural killer cells (28, 96). For example, when IgG opsonizes a pathogen by recognizing and binding to specific epitopes by its Fab portion (variable region), its Fc portion (constant region) can be simultaneously recognized by phagocyte FcR which in turn activates ingestion and killing of the opsonized pathogen (108).

Examples of opsonizing antibodies mediating protection against *R. equi* in foals can be found in a study by Cauchard *et al.* demonstrating that the opsonizing activity of maternally-derived antibodies protected foals against natural infection of *R. equi* at an endemic horse-breeding farm (71). The opsonizing activity of these antibodies was determined *in vitro* by the increased phagocytosis of *R. equi* by neutrophils (71). Other studies have also shown *R. equi* opsonized with antibodies from HIP increased activation and cytokine production of neutrophils and monocyte-derived macrophages, and

oxidative burst in macrophages (92). In our anti-PNAG vaccine study, *in vitro* opsonization of *R. equi* with anti-PNAG IgG increased the deposition of C1 onto PNAG and the opsonic killing of *R. equi* by equine neutrophils (70).

Neutralization

Neutralization is the ability of an antibody by itself to inhibit infection of susceptible cells or to inhibit an initial step in pathogenesis (109). Antibodies can inhibit or "neutralize" microbes and their toxins by blocking the binding of these microbes and toxins to cellular receptors. Binding of antibodies to microbial structures can result in conformational changes that interfere with the ability of the microbes to interact with cellular receptors, thus preventing infection (110). Many microbial toxins also bind to specific cellular receptors to mediate their pathologic effects. Examples include the tetanus toxin known as tetanospasmin which causes paralysis by binding to and inhibiting receptors in the motor end plate of neuromuscular junctions (111), and exotoxins produced by *Staphylococcus aureus* that overstimulate T cells to produce large quantities of cytokines leading to acute toxic shock (112). Antibodies against such toxins hinder the interactions of toxins and host cells and thus prevent the toxins from causing tissue injury and disease. Antibody-mediated neutralization of microbes and toxins is independent of complement and leukocytes *in vitro*; however, the mechanism of *in vivo* neutralization is not known (113). As it seems to require only the antigen-binding regions of the antibodies, it can be mediated by antibodies of any isotype in the circulation (IgG; IgM; IgA; IgE) and in mucosal secretions (IgA). Evidence exists that neutralizing antibodies are transferred from *R. equi* HIP to foals (75). It has been

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suggested that passively transferred antibodies neutralize *R. equi* before it invades

macrophages, and that opsonization could impair intracellular replication of *R. equi* if

internalized via FcR on neutrophils and macrophages by increasing phagolysosome

formation and increasing oxidative burst activity of phagocytes (114-116). If high

concentrations of *R. equi*–specific antibodies are present in the pulmonary epithelial

lining fluid at the time of infection, bacterial viability may be reduced and phagocytosis

and intracellular bacterial killing may be improved (75, 115).

ADCC

Another classical method of antibody-mediated killing of intracellular infections

is ADCC, in which antibody (typically IgG) forms a bridge between an infected target

cell (or directly with some pathogens) and an FcR-bearing effector leukocyte (*i.e.*,

monocytes, neutrophils, eosinophils, or natural killer cells) (109). The engagement of

FcR by antibody-coated pathogens activates the effector leukocyte resulting in either

lysis or apoptosis of the target cell/pathogen (96, 109). Although ADCC can readily be

demonstrated *in vitro*, its role *in vivo* for host defense against microbes is not definitively

established. ADCC seems to be important in protection against tumors, but its role in

infections is less clear and complicated by the multiple functions of antibody (117-119).

Gorander *et al.* showed that protection of mice vaccinated against the herpes virus HSV-

2 was associated with non-neutralizing antibodies that mediated ADCC as well as the

production of CD4+ T cell IFNγ responses (120). Complement-independent killing of

bacteria *in vitro* in the presence of cytotoxic T lymphocytes has been described for

bacterial pathogens (121, 122). IgA and IgG, in combination with intestinal-derived

murine lymphocytes, is reported to mediate ADCC against *S. flexneri* and *Salmonella* spp. (123, 124) and IgA in combination with murine lung lymphocytes also has antibacterial activity against *Streptococcus pneumoniae* (125). ADCC protection has been documented *in vitro* for nematode parasites (126-130) and *in vivo* against parasitic flatworms (*Schistosoma mansoni*) (131), roundworms (*Brugia malayi*), microfilaria (132), and trypanosomes (133, 134). It is important to note that many of the effector cells mediating ADCC are also capable of antibody-mediated phagocytosis (109). Finally, antibodies may also inhibit infections using the components of ADCC (*i.e.*, infected target cells, antibody, and FcR-bearing effector cells) for cell-mediated inhibition. In such examples, development of intracellular parasites is blocked by triggering of both FcγRIIa and FcγRIIIa but the host cell is not killed (135, 136).

Interaction of antibodies with FcR of effector cells

As mentioned above, FcR of effector cells can be triggered by antigen-bound antibodies by non-specific binding of the Fc portion of the immune complex. The FcR most responsible for the induction of phagocytosis of opsonized (marked) microbes is the FcγR family of receptors that recognize IgG isotypes (137). This family includes FcγRI (CD64), FcγRIIA (CD32), FcγRIIB (CD32), FcγRIIIA (CD16a), and FcγRIIIB (CD16b), each with different affinity for IgG and their sub-isotypes. Non-specific binding of antigen-bound antibodies to FcR can trigger this receptor to contribute to protection against intracellular pathogens (27, 96, 138, 139) by: altering the inflammatory response via cytokine production (96); activating complement and neutralizing immunomodulatory microbial products (113); stimulating a respiratory

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burst (114); and, enhancing production of microbial oxidants (114), antigen presentation (115, 116), phagocytosis (61), and antibody-dependent cellular cytotoxicity.

Non-classical mechanisms

Non-classical mechanisms by which antibodies mediate protection against infection by intracellular pathogens include: 1) intracellular antibodies, 2) intracellular FcR, and, 3) non-specific engagement of FcRs.

Intracellular antibodies

Antibodies can enter infected cells through pinocytosis and be transported intracellularly to mediate their effects in the intracellular space (26, 140). In the bacterial phagosome they may neutralize toxins or viruses (9, 27, 141-143), and in the nucleus they may bind to eukaryotic chromatin and trigger transcriptional responses which could interfere with the course of infection of intracellular pathogens. Incubation of Mtb ingested by macrophages with specific antibodies inhibits intracellular replication (144), possibly by the same intracellular transport of neutralizing antibodies described above (145). Antibodies can bind to intracellular pathogens and alter transcriptional expression of cell wall constituents (*e.g.*, reduced polysaccharide release in *C. neoformans* (146) and reduced mycolic acid content in Mtb (147)) which interferes with microbial pathogenesis by reducing biofilm formation and changing bacterial biochemistry (55, 148).

The tripartite motif-containing 21 (TRIM21) is an intracellular FcR that binds

IgGs bound to pathogens (mainly viruses) from both humans and rodents (149). The

interaction between TRIM21 and IgG is highly conserved in mammals. It allows

phagocytic and nonphagocytic cells to resolve intracellular pathogen infection and viral

replication by binding to internalized antibody-coated cytosolic viruses and bacteria and

targeting them for proteasomal degradation by auto-ubiquitination that targets proteins to

the proteasome for degradation (150). Other than targeting apoptotic cells for

phagocytosis (149), TRIM21 predominantly neutralizes viruses, such as adenovirus

(150-152), nonenveloped viruses (150, 152), but also neutralizes certain bacteria,

(*Salmonella enterica*) (153). Interestingly, the number of antibodies required for

neutralization by TRIM21 are low: 5–6 antibodies per virus particle are sufficient to

neutralize rhinovirus (154) and 1–4 for poliovirus (155, 156). Another intracellular FcR

is the neonatal Fc receptor (FcRn) which ensures protection of the neonate and adult

hosts against a variety of pathogens for which antibodies are protective (157). The FcRn

structurally resembles the major histocompatibility class I (MHC-I) molecules and

enables transportation of IgG molecules across cells without targeting them to lysosomes

(157). This is possible due to its ability to bind with high affinity to IgG molecules only

at acidic pH (<6.5) (such as in acidic environment of endocytic vacuoles) but not at

neutral or higher pH (physiological pH) (157). This is necessary, for example, for FcRns

found in the intestinal epithelium of neonates for efficient transportation of IgG from the

slightly acidic intestinal lumen to the basolateral side of the epithelium where the pH is

neutral to slightly basic (158). During the short period of time during which neonates

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have FcRns, antibodies derived from colostrum are transported from the intestinal lumen by transcytosis across epithelial cells into the circulation of the neonate to protect them from infections (159). FcRns located in the epithelium of the intestinal lumen can also mount effective immune responses against epithelial pathogens by: 1) transporting the pathogen-specific IgG from the systemic circulation across the epithelial barrier into the intestinal lumen; 2) inhibiting the adhesion and/or invasion of the bacterium of the invading pathogen; 3) mediating transcytosis of IgG/bacteria complexes into the lamina propria; and, 4) induction of an antigen-specific immune response induced by DCs that are able to activate antigen-specific acquired immune responses by CD4+ T cells and their expansion within regional lymphoid structures and associated peripheral tissues (157). FcRn expression in the intestinal, genitourinary, and respiratory tract epithelium enables the transmucosal transportation of pathogen-specific IgG to prevent colonization and invasion of pathogenic bacteria and viruses infections (160). FcRns have also been found in human neutrophils where they facilitate IgG-mediated bacterial phagocytosis by translocating IgG-opsonized bacteria from neutrophil granules to nascent phagosomes upon phagocytosis(161).

Non-specific engagement of FcRs

Engagement of FcRs mediates intracellular killing by altering microbial physiology (55, 146), or by targeting immune complexes to lysosomes for degradation (FcR-mediated lysososmal targeting) (92, 162). FcR-mediated lysosmal targeting of intracellular pathogens modifies intracellular signaling leading to phagolysosomal fusion of intracellular pathogens that would otherwise evade lysosomal fusion for its

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degradation (163). There is evidence that equine IgG_1, $IgG_{4/7}$, and $IgG_{3/5}$ likely interact

with FcRs on effector cells (67, 164) and contribute to protection against *R. equi* (46,

165, 166). The interaction of IgG_1 and $IgG_{4/7}$ with FcγR elicit a strong respiratory burst

from equine lymphocytes (67), and mediate efficient phagocytosis of

R. equi by equine neutrophils and alveolar macrophages (71, 114, 167). Studies show

when antibody-opsonized Mtb interacts with FcR (FcεRII-CD23 or FcγIIIa) on

macrophages, it mediates controlled bacterial replication within mature phagosomes (9,

139). Although the role of FcR-mediated protection against *R. equi* is not fully

understood, bacterial entry of antibody-opsonized *R. equi* via the FcR could potentially

alter its fate by inducing intracellular killing (67).

A novel non-classical mechanism

Recently, the laboratory of our collaborator, Gerald Pier has found evidence for

the possibility of another non-classical mechanism by which antibodies mediate

protection against intracellular *R. equi* (70). PNAG antigen was identified on the surface

of infected macrophages but not uninfected macrophages, indicating the antigen

originated from the intracellular bacteria (70). The Pier laboratory proposes that when

R. equi infects macrophages, the polysaccharide PNAG from the *R. equi* is released in

the form of extracellular vesicles from infected cellular compartments and becomes

embedded in the surface of the infected macrophage. These PNAG antigens serve as

targets for anti-PNAG antibodies to identify infected cells and, along with complement

and neutrophils, to lyse the cells, thereby releasing the intracellular microbes, which are

then killed by classic opsonic killing involving complement and neutrophils (70).

However, direct evidence is lacking that these PNAG molecules are associated with rhodococcal membranes embedded in the equine macrophage membrane, or that the cells are targeted by antibodies for destruction by equine neutrophils. Nor is it established whether this mechanism occurs *in vivo* to control infection of *R. equi* in the lungs of foals.

It thus will be necessary to conduct experiments showing PNAG to be associated with bacterial membranes found embedded in the membrane of *R. equi*-infected, but not uninfected, foal alveolar macrophages *in vitro*. Foal alveolar macrophages infected *in vitro* with live, virulent *R. equi* could be used for the microscopic identification of PNAG on *R. equi* vesicles embedded on the surface of foal alveolar macrophages using monoclonal antibodies against PNAG and membrane dyes of different absorbent wavelengths and as a target cell for opsonic killing by equine neutrophils. It also will be necessary to demonstrate that anti-PNAG antibodies induce significantly greater opsonic killing of *R. equi*-infected alveolar macrophages than of uninfected alveolar macrophages in the presence of equine complement and neutrophils; macrophages could be infected either *in vitro* or recovered from infected (and uninfected control) foals. Correlation of *in vitro* effects with *in vivo* protection against *R. equi* would provide compelling evidence of the existence of this proposed new mechanism of antibody-mediated protection against intracellular infection.

Conclusions

A full understanding of immunity of foals to *R. equi* remains elusive. Nevertheless, it appears that antibodies can mediate protection in foals. Having a fuller

understanding of the mechanisms of this protection is greatly needed to improve control

and prevention of this disease. Moreover, lessons learned by studying *R. equi* should

have relevance to understanding the role of antibodies in mediating protection against

other intracellular bacteria.

CHAPTER II

ANTIBODY TO POLY-*N*-ACETYL GLUCOSAMINE PROVIDES PROTECTION
AGAINST INTRACELLULAR PATHOGENS: MECHANISM OF ACTION AND
VALIDATION IN HORSE FOALS CHALLENGED WITH *RHODOCOCCUS EQUI*[*]

Synopsis

Immune correlates of protection against intracellular bacterial pathogens are
largely thought to be cell-mediated, although a reasonable amount of data supports a role
for antibody mediated protection. To define a role for antibody-mediated immunity
against an intracellular pathogen, *Rhodococcus equi*, that causes granulomatous
pneumonia in horse foals, we devised and tested an experimental system relying solely
on antibody-mediated protection against this host-specific etiologic agent. Immunity
was induced by vaccinating pregnant mares 6 and 3 weeks prior to predicted parturition
with a conjugate vaccine targeting the highly conserved microbial surface
polysaccharide, poly-*N*-acetyl glucosamine (PNAG). We ascertained antibody was
transferred to foals via colostrum, the only means for foals to acquire maternal antibody.
Horses lack transplacental antibody transfer. Next, a randomized, controlled, blinded
challenge was conducted by inoculating at ~4 weeks of age ~10^6 cfu of *R. equi* via
intrabronchial challenge. Eleven of 12 (91%) foals born to immune mares did not
develop clinical *R. equi* pneumonia, whereas 6 of 7 (86%) foals born to unvaccinated

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controls developed pneumonia (P = 0.0017). In a confirmatory passive immunization

study, infusion of PNAG-hyperimmune plasma protected 100% of 5 foals against

R. equi pneumonia whereas all 4 recipients of normal horse plasma developed clinical

disease (P = 0.0079). Antibodies to PNAG mediated killing of extracellular and

intracellular *R. equi* and other intracellular pathogens. Killing of intracellular organisms

depended on antibody recognition of surface expression of PNAG on infected cells,

along with complement deposition and PMN assisted lysis of infected macrophages.

Peripheral blood mononuclear cells from immune and protected foals released higher

levels of interferon-γ in response to PNAG compared to controls, indicating vaccination

also induced an antibody-dependent cellular release of this critical immune cytokine.

Overall, antibody-mediated opsonic killing and interferon-γ release in response to PNAG

may protect against diseases caused by intracellular bacterial pathogens.

Introduction

Correlates of cellular and humoral immunity to major intracellular, non-viral

pathogens capable of informing vaccine development are incompletely understood. It is

unknown which ones can form the basis of a highly effective vaccine to prevent diseases

such as tuberculosis (TB). Protection studies conducted to date, primarily in laboratory

rodents and non-human primates, have not led to an effective human vaccine for such

pathogens (168, 169) outside of the limited efficacy of the live Bacillus Calmette-Guerin

whole-cell vaccine against TB (169-171). *Rhodococcus equi* is a Gram-positive,

facultative intracellular pathogen carrying an essential virulence plasmid that primarily

infects alveolar macrophages of horse foals following inhalation. *R. equi* replicates

within a modified phagocytic vacuole, with survival dependent on the virulence plasmid preventing phagosome-lysosome fusion, resulting in a granulomatous pneumonia that is pathologically similar to that caused by *Mycobacterium tuberculosis* infection in humans (2). *R. equi* also causes extrapulmonary disorders including osseous and intra-abdominal lymphadenitis (1, 2, 172). The disease is of considerable importance to the equine industry (1, 2, 172), and while some reports indicate vaccination and/or passive transfer of hyperimmune plasma using bactrin-based or virulence associated protein A vaccines can reduce the severity of *R. equi* pneumonia (80, 173), it is generally felt that most attempts to date to create an effective *R. equi* vaccine have been unsuccessful (89, 174). There is no approved vaccine for *R. equi* in any animal species. Presently, it can be solidly reasoned that cell-mediated immune (CMI) responses underlay the basis for natural immunity to *R. equi*. Disease occurs almost exclusively in foals less than 6 months of age, but by ~9 months of age most young horses become highly resistant to this pathogen (1, 2, 172, 175). This acquired natural resistance is obviously not antibody-mediated inasmuch as the solid immunity to infection in healthy horses >9 months of age, which obviously includes pregnant mares, is not transferred to susceptible foals via antibody in the colostrum. Colostrum is the only source of maternal antibody in foals and the offspring of other animals producing an epitheliochorial placenta. Therefore, an effectual vaccine trial can be designed to test whether an antibody-eliciting immunogen is efficacious by immunization of pregnant mares that should lead to colostral transfer of vaccine-induced antibody to their offspring, with a subsequent evaluation of protective efficacy following challenge of these foals with virulent *R. equi*. *R. equi* synthesizes the conserved surface capsule-like polysaccharide,

poly-*N*-acetyl glucosamine (PNAG), wherein this antigen is intercalated into the same extracellular space as classical bacterial capsules (176) or serves as a single, encapsulating antigen on the surface of organisms such as *Neisseria gonorrhoeae* and non-typable *Hemophilus influenzae* (176). PNAG is also expressed by fungal and protozoan pathogens (176). As such, PNAG is a target for the development of a vaccine potentially protective against many pathogens (176, 177). Since numerous microbes produce this antigen, there is natural IgG antibody in most human and animal sera (178, 179). But natural antibody is generally ineffective at eliciting protection against infection. Natural antibodies usually poorly activate the complement pathway and thus ineffectively mediate microbial killing (178-180). By removing most of the acetate substituents from the *N*-acetyl-glucosamine sugars comprising PNAG (181, 182), or using synthetic oligosaccharides composed of only β-1→6-linked glucosamine conjugated to a carrier protein such as tetanus toxoid (TT), (176, 180, 183, 184) complement-fixing, microbial-killing, and protective antibody to PNAG can be induced. A final premise justifying immunizing pregnant mares to evaluate vaccine-induced immunity to *R. equi* is that foals are considered to be infected soon after birth (7) when they are more susceptible to infection (69) and when their immune system is less effective in responding to vaccines (174, 185-187). This precludes active immunization of very young foals as a strategy for vaccine evaluation against *R. equi*. Indeed, as part of our clinical evaluations of a PNAG vaccine for *R. equi*, we attempted to immunize foals starting at two days of age and were unsuccessful at inducing antibody. Therefore, in order to ascertain if *R. equi* pneumonia could be prevented by antibody to PNAG, pregnant mares were vaccinated with the 5GlcNH2-TT vaccine, the transfer of

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functional opsonic antibodies via colostrum to foals verified, and foals were challenged

at 25-28 days of life with virulent *R. equi*. The primary hypothesis of this randomized,

controlled, blinded challenge study was that induction of complement-fixing, functional

antibody to PNAG would prevent the development of clinical *R. equi* pneumonia in

challenged foals.

Results

Maternal vaccination induces serum and colostral antibody to PNAG that is orally transferred to foals

Mares were immunized twice approximately 6 and 3 weeks prior to their

estimated date of parturition (based on last known breeding date) with 125 or 200 μg of

the 5GIcNH2 vaccine conjugated to TT (AV0328 from Alopexx Vaccine, LLC)

adjuvanted with 100 μl of Specol. Immunization of mares resulted in no detectable local

or systemic reaction following either 1 or 2 vaccine doses except for a slightly swollen

muscle 24 h after the first vaccination followed at day 2 by a small dependent edema that

resolved by day 3 in a single mare. Serum samples from mares immunized in 2015 were

only collected on the day of foaling, so statistical comparisons with immunized mare

titers were only made between all 7 control samples collected on the day of foaling (D0

post-foaling (PF)) with 12 vaccinated samples collected pre-immunization, on day 21

prior to the booster dose, and on D0 PF (S1 Fig). When compared with IgG titers to

PNAG in non-immune controls obtained on D0 PF, immunization of mares against

PNAG gave rise to significant ($P < 0.05$) increases in total serum IgG titers as well as

increases in the titers of equine IgG subisotypes IgG_1, $IgG_{3/5}$, and $IgG_{4/7}$ (S1 Fig) on day

21 after a single immunization, and on D0 PF after the booster dose. Similarly, total IgG

and IgG subisotype titers were significantly higher in the colostrum obtained on the day of foaling from vaccinated mares compared with controls (S2 Fig). Notably, non-immunized mares had antibody titers to PNAG, representative of the natural response to this antigen commonly seen in normal animal and human sera. Successful oral delivery of antibody to the blood of foals born to vaccinated mares (hereafter termed vaccinated foals) was shown by the significantly higher titers of serum IgG to PNAG compared with foals from control mares at ages 2, 28, and 56 days, but not 84 days (Fig 1A). Foal serum concentrations of subisotypes IgG_1, $IgG_{3/5}$, and $IgG_{4/7}$ to PNAG were significantly higher at 2, 28, and 42 days of age in the vaccinated group compared with the control group, and subisotype IgG_1 titers remained significantly higher through age 56 days (Fig 1B-1D). The pattern in vaccinated foals of decreasing titers to PNAG with increasing age was consistent with the decay of maternally-transferred immunoglobulins.
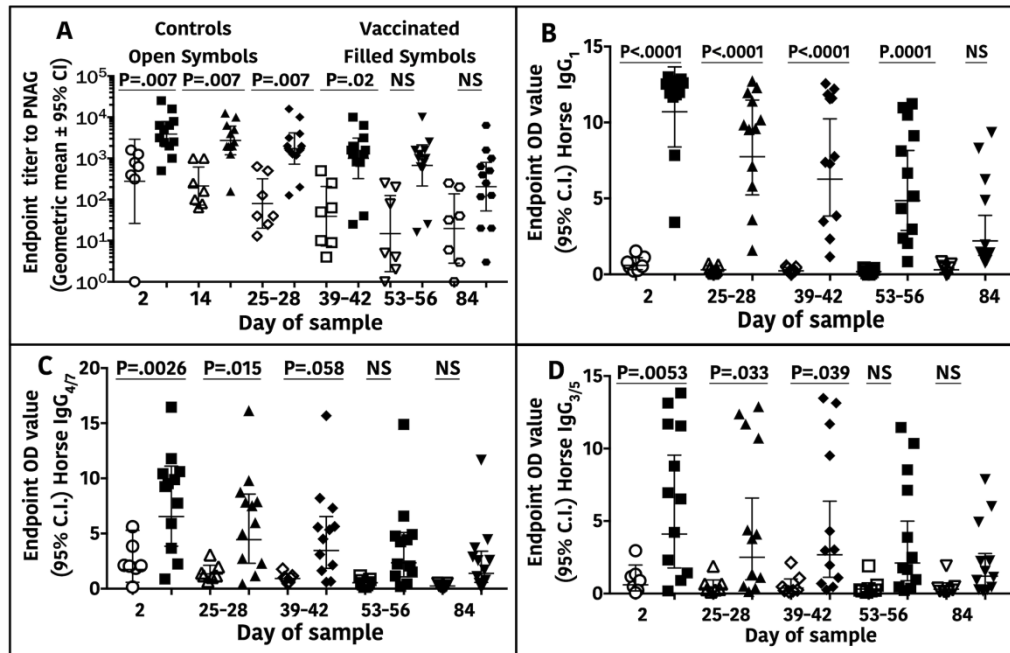
Fig 1. Total IgG and IgG subisotype antibody titers to PNAG in sera of horse foals.
Endpoint serum titers (N = 7 controls, 12 vaccinated) of IgG or IgG subisotypes are plotted
by vaccine group as a function of age in days. **A**: IgG antibody end-point titers to PNAG
were significantly higher in an age-dependent matter between foals from mares that were
vaccinated (filled symbols n = 12) compared with titers in sera of foals from unvaccinated,
control mares (open symbols n = 7) through Days 39-42 of life. **B-D**: Concentrations of
IgG_1, $IgG_{4/7}$, and $IgG_{3/5}$ to PNAG were significantly higher in foals in the vaccinated group
than the unvaccinated, control group through the day indicated on the figure. Statistical
comparisons made using linear mixed-effects modeling with individual foal as a random
effect; NS = not significant.

Orally obtained colostral antibody to PNAG protects foals against intrabronchial

infection with <u>R. equi</u>

Protection studies were undertaken using a randomized, controlled, blinded
experimental trial design. At days 25-28 of life, foals in the study were challenged with
~10^6 cfu of live *R. equi* contained in 40 ml of vehicle, with half of the challenge
delivered to each lung by intrabronchial dosing with 20 ml. Foals were followed for
development of clinical *R. equi* pneumonia (Table 1) for 8 weeks. The proportion of

vaccinated foals that developed *R. equi* pneumonia (8%; 1/12) was significantly (P = 0.0017; Fisher's exact test) less than that of unvaccinated control foals (86%; 6/7), representing a relative risk reduction or protected fraction of 84% (95% C.I. 42% to 97%, Koopman asymptotic score analysis (188)). The duration of clinical signs indicative of *R. equi* pneumonia was significantly (P ≤ 0.027, Wilcoxon rank-sum tests) longer for foals from control than vaccinated mares (Table 2). Thoracic ultrasonographic examination is the standard clinical technique for monitoring areas of pulmonary abscessation or consolidation attributed to *R. equi* infection. The severity and duration of ultrasonographic lesions were significantly greater in foals born to controls than vaccinated mares (Fig 2). Vaccinated foals that were protected against pneumonia had less severe clinical signs and smaller and fewer ultrasonographic lesions compared with control foals. Thus, maternal vaccination against PNAG demonstrated successful protection against clinical *R. equi* pneumonia, a disease for which there is no current vaccine (89), using a randomized, blinded experimental challenge model.

Table 1. Case definition for diagnosis of *R. equi* clinical pneumonia.

Cases had all of the following clinical signs within 3 weeks of challenge:
Coughing
Depressed attitude (subjective evidence of increased recumbency, lethargy, reluctance to rise)
Fever > 103.5˚F
Tachypnea (respiratory rate > 60 breaths/min)
Increased respiratory effort (abdominal lift, flaring nostrils)
Sonographic evidence of pulmonary abscessation or consolidation
Cytologic evidence of septic pneumonia from tracheal aspirate
Positive culture for *R. equi* from tracheal aspirate
Lack of diagnosis with clinical *R. equi* pneumonia was based on lack of any of the following clinical signs within 8 weeks post-challenge:
Coughing
Depressed attitude (subjective evidence of increased recumbency, lethargy, reluctance to rise)
Fever > 103.5˚F
Tachypnea (respiratory rate ≥ 60 breaths/min)
Increased respiratory effort (abdominal lift, flaring nostrils)

Table 2. Duration of clinical signs in foals from vaccinated or unvaccinated mares.

Variable	Mare Unvaccinated (n = 7)	Mare Vaccinated (n = 12)	P value[a]
Days meeting case definition (range)	10[b] (0 to 26)	0 (0 to 41)	0.0046
Days from first to last day meeting case definition (range)	20 (0 to 32)	0 (0 to 48)	0.0046
Days temperature > 103.0˚F (range)	8 (1 to 32)	2 (0 to 12)	0.0263
Days temperature > 103.0˚F and coughing (range)	4 (0 to 8)	0 (0 to 13)	0.0220

[a]P values derived using the Wilcoxon rank-sum test.
[b]Median (range)
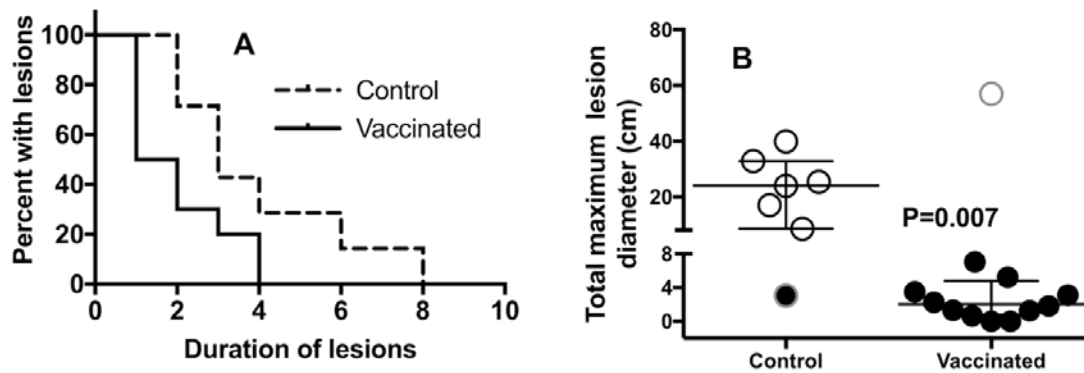
Fig 2. Comparison of induction and regression of ultrasonographic lesions in foals from vaccinated or unvaccinated mares following *R. equi* challenge.
A: Kaplan-Meier survival plot comparing duration of detectable ultrasonographic lesions as evidence of pulmonary abscessation. Duration of pulmonary lesions identified by ultrasound was significantly (P = 0.008; Log-rank test) shorter for foals of vaccinated mares (solid line) versus those of foals from control mares (hatched line). **B**: Cumulative sum of maximum diameters of thoracic ultrasonography lesions (N = 7 Controls, 12 Vaccinated). The sums of the cumulative maximum diameters were significantly (P = 0.007; Wilcoxon rank-sum test) lower for foals from vaccinated mares (n = 12) than for unvaccinated control mares (n = 7). Open circles indicate foals diagnosed with pneumonia, filled circles indicate foals that did not develop pneumonia. Symbols with outer gray rings indicate the unvaccinated foal that did not get pneumonia and the vaccinated foal that did develop pneumonia.

Passive infusion with hyperimmune plasma to PNAG protects foals against R. equi

pneumonia

To substantiate that vaccination-mediated protection was attributable to antibody to PNAG, hyperimmune plasma was prepared from the blood of 5GlcNH2-TT-immunized adult horses and 2 L (approximately 40 ml/kg) infused into 5 foals at 18-24 hours of age. Four controls were transfused at the same age with 2 L of standard commercial horse plasma. Titers of control and hyperimmune plasma IgG subisotypes and IgA antibody to PNAG and OPK activity against *R. equi* (S3 Fig) documented significantly higher titers of functional antibody to PNAG in the plasma from vaccinated donors and in foals transfused with the plasma from vaccinated donors compared to foals

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transfused with standard plasma. After challenge with *R. equi* as described above, there was a significant reduction in clinical signs in the foals receiving PNAG-hyperimmune plasma, compared to controls, except for the duration of ultrasound lesions (Table 3). None of the 5 foals receiving PNAG-hyperimmune plasma were diagnosed with *R. equi* pneumonia, whereas 4 of 4 recipients of normal plasma had a diagnosis of clinical pneumonia for at least 1 day (P = 0.0079, Fisher's exact test; relative risk reduction or protected fraction 100%, 95% C.I. 51%-100%, Koopman asymptotic score (188)).

Table 3. Duration of clinical signs in foals infused with control or PNAG-hyperimmune plasma.

Variable	Standard plasma (n = 4)	PNAG plasma (n = 5)	P value[a]
Days meeting case definition (range)	10.5[b] (1 to 14)	0 (0 for all)	0.0108
Days from first to last day meeting case definition (range)	11.5 (1 to 14)	0 (0 for all)	0.0104
Days temperature > 103.0°F (range)	11.5 (1 to 15)	2 (0 to 4)	0.0811
Days temperature > 103.0°F and coughing (range)	9.5 (0 to 11)	0 (0 to 3)	0.0072
Duration of ultrasound lesions in weeks (range)	2 (1 to 4)	0 (0 to 3)	0.2029
Maximum of TMD[c] (cm)	10.1 (4.0 to 29.8)	0 (0 to 3.4)	0.0179
Sum of TMDs (cm)	13.2 (4.0 to 50.5)	0 (0 to 7.9)	0.0342

[a]P values derived using the Mann-Whitney test.
[b]Mean (range)
[c]TMD = total (sum) of maximum diameters of lesions observed on a given day

https://doi.org/10.1371/journal.ppat.1007160.t003

R. equi expression of PNAG in vitro and in vivo

Using immunofluorescence microscopy, we demonstrated that 100% of 14 virulent strains of *R. equi* tested express PNAG (S4 Fig). Moreover, we found that PNAG was expressed in the lungs of foals naturally infected with *R. equi* (S5A Fig), similar to our prior demonstration of PNAG expression in the lung of a human infected

with Mtb (176). PNAG was detected within apparent vacuoles inside *R. equi*-infected

horse macrophages *in vivo* (S5B Fig).

PNAG vaccine-induced opsonic antibodies mediate killing of both extracellular and

intracellular R. equi

Testing of the functional activity of the antibodies induced in the pregnant mares

and in foal sera on the day of challenge demonstrated the antibodies could fix equine

complement component C1q onto the PNAG antigen (Fig 3A). Notably, the natural

antibody to PNAG in sera of non-vaccinated, control mares and their foals did not

deposit C1q onto the PNAG antigen, consistent with prior findings that natural

antibodies are immunologically inert in these assays (178, 179, 189). Sera from

vaccinated foals on the day of *R. equi* infection mediated high levels of opsonic killing

of extracellular *R. equi* whereas control foals with only natural maternal antibody to

PNAG had no killing activity (Fig 3B), again demonstrating the lack of functional

activity of these natural antibodies to PNAG. As some of the vaccinated foals developed

small subclinical lung lesions that resolved rapidly (Table 1, Fig 2) it appeared the bolus

challenge did lead to some uptake of *R. equi* by alveolar macrophages but without

development of detectable clinical signs of disease. This observation suggested that

antibody to PNAG led to resolution of these lesions and prevented the emergence of

clinical disease. Based on the finding that *R. equi*-infected foal lung cells expressed

PNAG *in vivo* (S5 Fig), we determined if macrophages infected with *R. equi in vitro*

similarly expressed PNAG, and also determined if this antigen was on the infected cell

surface, intracellular, or both. We infected cultured human monocyte-derived

macrophages (MDM) for 30 min with live *R. equi* then cultured them overnight in antibiotics to prevent extracellular bacterial survival. To detect PNAG on the infected cell surface we used the human IgG_1 MAb to PNAG (MAb F598) conjugated to the green fluorophore Alexa Fluor 488. To detect intracellular PNAG, we next permeabilized the cells with ice-cold methanol and added either unlabeled MAb F598 or control MAb, F429 (190) followed by donkey anti-human IgG conjugated to Alex Fluor 555 (red color). These experiments showed there was no binding of the MAb to uninfected cells (S6A Fig) nor binding of the control MAb to infected cells (S6B Fig). However, we found strong expression of PNAG both on the infected MDM surface and within infected cells (S6C Fig). Similarly, using a GFP-labeled Mtb strain (S6D and S6E Fig) and a GFP-labeled strain of *Listeria monocytogenes* (S6F Fig) we also visualized intense surface expression of PNAG on infected human MDMs in culture, even when the bacterial burden in the infected cell was apparently low. Importantly, within infected cultures, only cells with internalized bacteria had PNAG on their surface (S6G Fig), indicating the antigen originated from the intracellular bacteria. Thus, cells in infected cultures that did not ingest bacteria did not obtain PNAG from shed antigen or lysed infected cells.
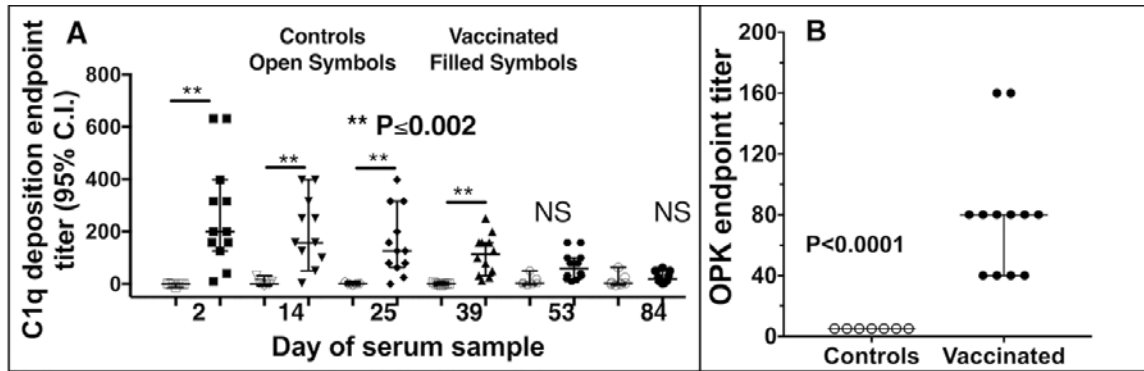
Fig 3. Functional activity of antibody in foal sera on day of challenge with *R. equi.*
A: Serum endpoint titer (N = 7 Controls, 12 Vaccinated) of deposition of equine C1q onto purified PNAG. P values determined by non-parametric ANOVA and pairwise comparisons by Dunn's procedure. NS, not significant. **B:** Serum endpoint titer (reciprocal of serum dilution achieving killing $\geq$ 30% of input bacteria) for opsonic killing of *R. equi* in suspension along with horse complement and human PMN. Values indicate individual titer in foal sera on day of challenge with *R. equi*, black bars the group median and error bars the 95% C.I. (upper 95% C.I for vaccinated foals same as median). P value by Wilcoxon rank-sum test.

This finding is consistent with published reports of intracellular bacterial release of surface vesicles that are transported among different compartments of an infected host cell (191). Next, we examined if the surface PNAG on infected cells provided the antigenic target needed by antibody to both identify infected cells and, along with complement and PMN, lyse the cells, release the intracellular microbes, and kill them by classic opsonic killing. Human MDM cultures were established *in vitro*, infected for 30 min with live *R. equi*, and then cells were washed and incubated for 24 h in the presence of 100 µg gentamicin/ml to kill extracellular bacteria and allow for intracellular bacterial growth. Then, various combinations of the human IgG$_1$ MAb to PNAG or the control MAb F429 along with human complement and human PMN were added to the cultures, and viable *R. equi* determined after 90 min. While a low level of killing ($\leq$ 30%) of intracellular *R. equi* was obtained with PMN and complement in the presence of the

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control MAb, there was a high level of killing of the intracellular *R. equi* when the full compendium of immune effectors encompassing MAb to PNAG, complement, and PMN were present (Fig 4A). Similarly, testing of sera from vaccinated foals on the day of challenge, representing animals with a low, medium, or high titer of IgG to PNAG, showed they also mediated titer-dependent killing of intracellular *R. equi* (Fig 4B). Measurement of the release of lactate dehydrogenase as an indicator of lysis of the macrophages showed that the combination of antibody to PNAG, complement, and PMN mediated lysis of the infected human cells (Fig 4°C), presumably releasing the intracellular bacteria for further opsonic killing. PNAG can be digested with the enzyme dispersin B that specifically recognizes the β-1→6-linked *N*-acetyl glucosamine residues (192, 193) but is unaffected by chitinase, which degrades the β-1→4-linked *N*-acetyl glucosamines in chitin. Thus, we treated human macrophages infected for 24 h with *R. equi* with either dispersin B or chitinase to determine if the presence of surface PNAG was critical for killing of intracellular bacteria. Dispersin B treatment markedly reduced the presence of PNAG on the infected cell surface (S7 Fig) as well as killing of intracellular *R. equi* by antibody, complement, and PMN (Fig 4D). Chitinase treatment had no effect on PNAG expression (S7 Fig) or killing, indicating a critical role for PNAG intercalated into the macrophage membrane for antibody-mediated killing of intracellular *R. equi*.
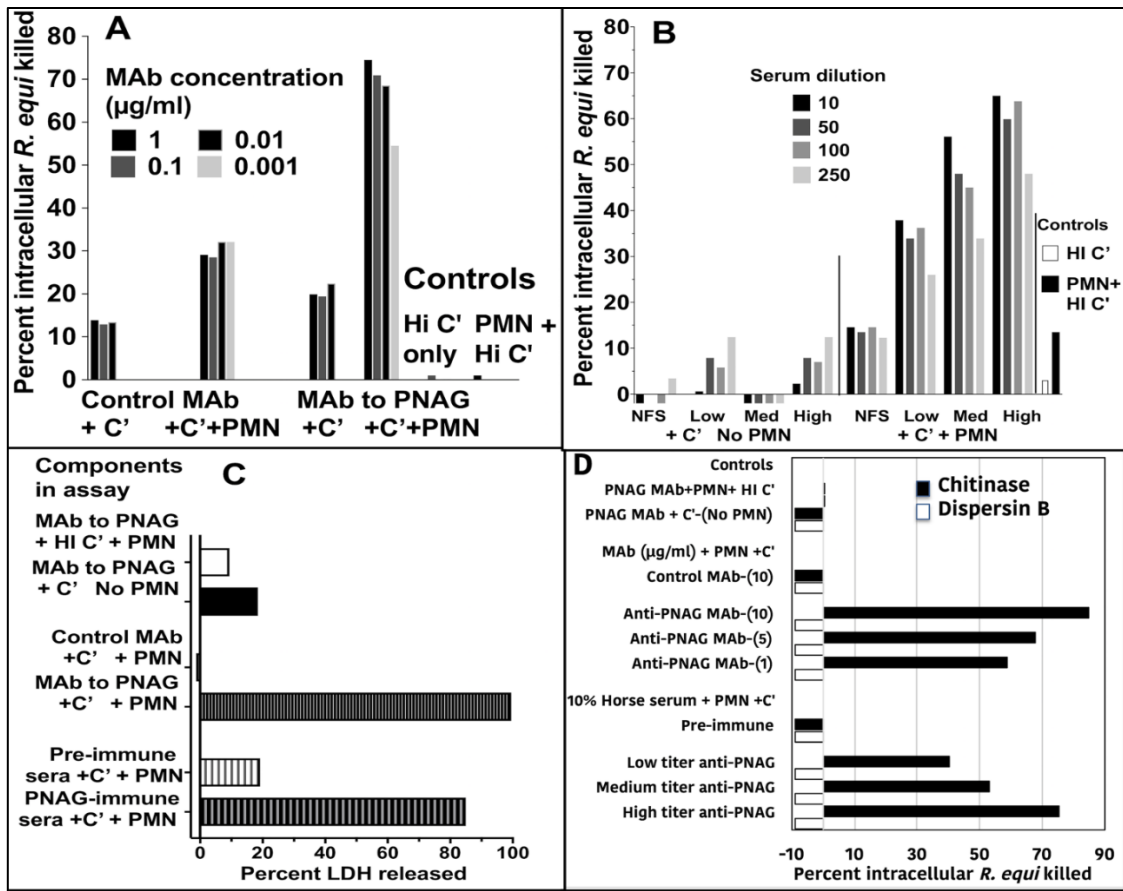
Fig 4. Opsonic killing of intracellular *R. equi*.
A: Maximal killing of intracellular *R. equi* mediated by MAb to PNAG requires both complement (C') and PMN (C'+PMN). Background killing <5% is achieved with heat-inactivated C' (HI C') or PMN + HI C'. **B**: Pre-immune, normal foal sera (NFS) or representative immune foal sera with low, medium (Med) or high titers to PNAG obtained on the day of challenge with *R. equi* mediate killing of intracellular *R. equi* along with C' and PMN. **C**: Measurement of percent cytotoxicity by LDH release shows MAb to PNAG or PNAG-immune sera plus C' and PMN mediate lysis of infected cells. **D**: Opsonic killing of intracellular *R. equi* requires recognition of cell surface PNAG. Treatment of infected macrophage cultures with dispersin B to digest surface PNAG eliminates killing whereas treatment with the control enzyme, chitinase, has no effect on opsonic killing. Bars represent means of 4-6 technical replicates. Depicted data are representative of 2-3 independent experiments. Bars showing <0% kill represent data wherein the cfu counts were greater than the control of PNAG MAb + PMN + HI C'.

Antibody to PNAG mediates intracellular killing of other intracellular pathogens

To show that antibody to PNAG, complement, and PMN represent a general

mechanism for killing of disparate intracellular pathogenic bacteria that express PNAG,

we used the above described system of infected human macrophages to test killing of

Mycobacterium avium, *Staphylococcus aureus*, *Neisseria gonorrhoeae*, *Listeria*

monocytogenes and *Bordetella pertussis* by the human MAb to PNAG or horse serum

from a foal protected from *R. equi* pneumonia. Human MDM infected with these

organisms expressed PNAG on the surface that was not detectable after treatment with

dispersin B (S7 Fig). When present intracellularly, all of these organisms were killed in

the presence of MAb to PNAG or anti-PNAG immune horse serum, complement, and

PMN following treatment of the infected cells with the control enzyme, chitinase, but

killing was markedly reduced in infected cells treated with dispersin B (Figs 5A and S8).

As with *R. equi*, maximal lysis of infected cells occurred when antibody to PNAG plus

complement and PMN were present (Fig 5B and S9 Fig), although when analyzing data

from all 5 of these experiments combined there was a modest but significant release of

LDH with antibody to PNAG and complement alone (Fig 5B and S9 Fig).
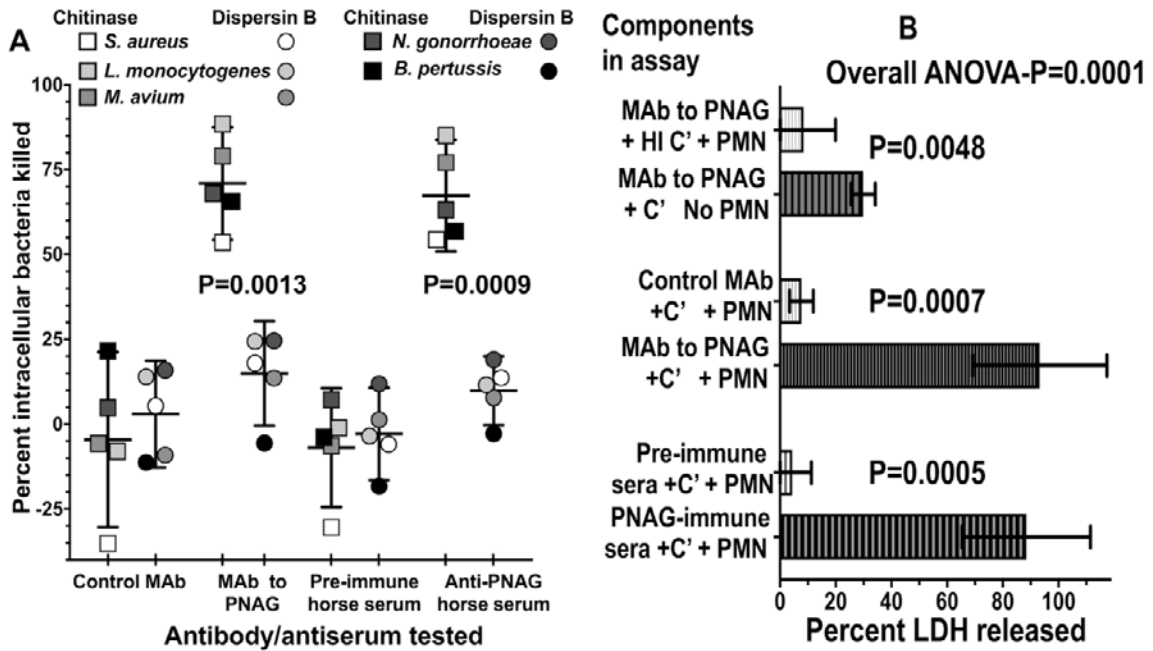
Fig 5. Opsonic killing of multiple intracellular pathogens by antibody to PNAG, complement (C') and PMN depends on infected-cell surface expression of PNAG and is associated with release of LDH.

A: Killing of 5 different intracellular bacterial pathogens by monoclonal or polyclonal antibody (10% concentration) to PNAG plus PMN and C' was markedly reduced following treatment of infected cells with Dispersin B (circles) to digest surface PNAG compared to treatment with the control enzyme, Chitinase (squares). Symbols represent indicated bacterial target strain. Horizontal bars represent means, and error bars show the 95% C.I. Symbols showing <0% kill represent data wherein the cfu counts were greater than the control of PNAG MAb + PMN + HI C'. P values: paired t-tests comparing percent intracellular bacteria killed with each antibody/antiserum tested after Chitinase or Dispersin B treatment. **B:** Opsonic killing is associated with maximal LDH release from infected cells in the presence of antibody to PNAG, C' and PMN. Bars represent means from 5 different intracellular pathogens, error bars the 95% C.I., overall ANOVA P value by one-way repeated measures ANOVA, pair wise comparisons determined by two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli.

Maternal PNAG vaccination and antibody transfer to foals enhances in vitro cell-mediated immune responses against <u>R. equi</u>

Cell-mediated immune (CMI) responses in vaccinated and unvaccinated, control foals were assessed by detecting production of IFNγ from peripheral blood mononuclear cells (PBMC) stimulated with a lysate of virulent *R. equi*. IFNγ production at 2 days of age was significantly ($P < 0.05$; linear mixed-effects modeling) lower than levels at all other days for both the control and vaccinated groups (Fig 6A, P value not on graph). There was no difference in IFNγ production between vaccinated and control foals at day 2 of age. Vaccinated foals had significantly higher (~10-fold) production of IFNγ in response to *R. equi* stimulation (Fig 6A) from cells obtained just prior to challenge on days 25-28 of life compared to unvaccinated controls. By 56 days of age, and 4 weeks post *R. equi* infection, the controls likely made a CMI response to the lysate antigens as they were infected at day 25-28 of life, accounting for the lack of differences between vaccinates and controls in PBMC IFNγ production at day 56. To substantiate the specificity of this CMI reaction from the PBMC of vaccinated foals, we demonstrated that stimulation of their PBMC with an *R. equi* lysate treated with the enzyme dispersin B diminished IFNγ responses by ~90% (Fig 6B). We did not test PBMC from control foals for specificity of their responses to PNAG. We also made a *post hoc* comparison of CMI responses between foals that remained healthy and foals that developed pneumonia. In this analysis (Fig 6C), foals that remained healthy (11 vaccinates and 1 control) had significantly ($P < 0.05$; linear mixed-effects modeling) higher CMI responses at all ages, including age 2 days, than foals that became ill (1 vaccinate and 6 controls), suggesting that both innate and acquired cellular immunity contribute to

resistance to *R. equi* pneumonia. Overall, it appears the maternally derived antibody to

PNAG sensitizes foal PBMC to recognize the PNAG antigen and release IFNγ, which is

a known effector of immunity to intracellular pathogens.
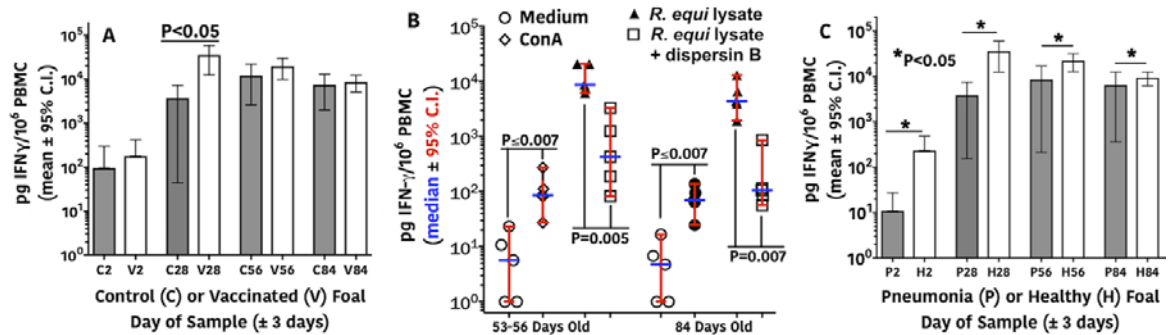
Fig 6. Cell-mediated immune responses of foal PBMCs.
A: Foals (N = 7 controls, 12 vaccinated) from vaccinated mares (V) had significantly (P <0.05; linear mixed effects modeling) higher concentrations of IFNγ produced at 28 days of age (prior to challenge) than control (C) foals in response to stimulation by a lysate of *R. equi*. IFNγ production at 2 days of age was significantly (P <0.05; linear mixed-effects modeling) lower than those at all other days for both the control and vaccine groups (P values not shown on graph). **B**: IFNγ production from PBMC from 5 vaccinated foals at 56 and 84 days of age following intrabronchial infection with virulent *R. equi*. Stimuli included media only (negative control), Concanavilin A (ConA; positive control), lysate of virulent *R. equi* strain used to infect the foals (*R. equi* lysate); and the same lysate treated with dispersin B to digest PNAG. All 3 stimulated groups were significantly different from the medium control at both day 56 and 84 (Overall ANOVA for repeated measures (P < 0.0001); P 0.0070 for all pairwise comparisons to media only and for pairwise comparison for *R. equi* lysate vs. lysate plus dispersin B (indicated on top of graph), Holm-Sidak's multiple comparisons test. **C:** Foals (N = 7 controls, 12 vaccinated) that developed pneumonia (P) had significantly (P < 0.05; linear-mixed effects modeling) lower concentrations of IFNγ expression at each day relative to foals that remained healthy (H).

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Discussion

In this study we tested the hypotheses that antibody to the conserved surface microbial polysaccharide, PNAG, could mediate protection against a significant intracellular pathogen of horse foals, *R. equi*. Overall we supported this hypothesis by showing maternal immunization against the deacetylated glycoform PNAG induced antibodies that protected ~4-week-old foals from challenge with live, virulent *R. equi*. Mechanistically we found that vaccine-induced antibody to PNAG deposited complement component C1q onto the purified PNAG antigen, mediated opsonic killing of both extracellular and intracellular *R. equi*, and sensitized PBMC from vaccinated foals to release IFNγ in response to PNAG. It appears that this spectrum of antibody activity induced by the 5GlcNH2-TT vaccine were all critical to the protective efficacy observed. While immunization-challenge studies such as those performed here are often correlative with protective efficacy against infection and disease, such studies can have limitations in their ability to predict efficacy in the natural setting. Bolus challenges provide an acute insult and immunologic stimulus that mobilizes immune effectors and clears infectious organisms, whereas in a field setting, such as natural acquisition of *R. equi* by foals, infection likely occurs early in life with onset of disease signs taking several weeks to months to develop (1, 2). Thus, it cannot be predicted with certainty that the protective efficacy of antibody to PNAG manifest in the setting of acute, bolus challenge will also be effective when a lower infectious inoculum and more insidious course of disease develops. In the context of acute challenge, we noted that many of the protected, vaccinated foals developed small lung lesions after challenge that rapidly resolved and no disease signs were seen. Finding such lesions by routine ultrasound

examination of foals that occurs on farms (194) might instigate treatment of subclinical

pneumonia if equine veterinarians are either unwilling to monitor foals until clinical

signs appear or unconvinced that disease would not ensue in vaccinated foals. This

approach could obviate the benefit of vaccination. The protection studies described here

for *R. equi* disease in foals has led to the implementation of a human trial evaluating the

impact of infusion of the fully human IgG$_1$ MAb to PNAG on latent and new onset TB.

The MAb has been successfully tested for safety, pharmacokinetic, and

pharmacodynamic properties in a human phase I test (195). The trial in TB patients

began in September 2017 (South African Clinical Trials Register:

http://www.sanctr.gov.za/SAClinicalbrnbspTrials/tabid/169/Default.aspx, then link to

respiratory tract then link to tuberculosis, pulmonary; and TASK Applied Sciences

Clinical Trials, AP-TB-201-16 (ALOPEXX): https://task.org.za/clinical-trials/). The

MAb was chosen for initial evaluation to avoid issues of variable immunogenicity that

might arise if a vaccine were tried in a TB-infected population, and to have a greater

margin of safety in case of untoward effects of immunity to PNAG in the human setting.

It is expected the half-life of the MAb will lead to its reduction to pre-infusion levels

over 9 to 15 months whereas this might not be the case following vaccination. A

successful effect of the MAb on treatment or disease course in TB will lead to an

evaluation of immunogenicity and efficacy of a PNAG targeting vaccine in this patient

population. The vaccine used here in horse mares was part of a batch of material

produced for human phase 1 safety and immunogenicity testing (ClinicalTrials.gov

Identifier: NCT02853617), wherein early results indicate that among a small number of

vaccinates there were no serious adverse events and high titers of functional antibody

elicited in 7 of 8 volunteers given either 75 μg or 150 μg doses twice 28 days apart. As

part of the safety evaluation, vaccinates kept daily logs of health status, which focused

on potential signs or symptoms of disease resulting from disruption of normal microbial

flora. This is not only a well-known consequence of antibiotic treatments (196), but also

can occur from many licensed and experimental drugs (197) across all major drug

classes. No adverse events attributable to microflora changes were reported. In addition,

we have previously published an extensive analysis of the low potential of antibody to

PNAG to impact the normal microbial flora (176). Numerous investigators have studied

how antibodies can mediate protection against intracellular bacterial pathogens (198-

200), although specific mechanisms of immunity are not well defined. The *in vitro*

results we derived indicated that a cell infected with a PNAG-producing pathogen has

prominent surface display of this antigen that serves as a target for antibody,

complement and PMN to lyse the infected cell and release the intracellular organisms for

subsequent opsonic killing. Likely other bacterial antigens are displayed on the infected

host cell as well, and thus this system could be used to evaluate the protective efficacy

and mechanism of killing by antibodies to other antigens produced by intracellular

organisms. Although we have not investigated the basis for the appearance of PNAG in

the plasma membrane of infected host cells, we suspect that microbial extracellular

vesicles, known to be released by many microbes (201), are a likely source of the plasma

membrane antigen due to trafficking from infected cellular compartments (191). A

notable component of the immune response in the foals associated with the protective

efficacy of the maternally derived antibody was the release of IFNγ from PBMC in

response to a *R. equi* cell lysate. The response to the lysate significantly dropped after

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treatment of the lysate with the PNAG-degrading enzyme dispersin B, indicating that an antibody-dependent cellular response to PNAG underlay the IFNγ response. As this cytokine is well known to be an important component of resistance to intracellular pathogens (202), it was notable that the maternal immunization strategy led to an antibody-dependent IFNγ response from the PBMC of the vaccinated foals. After challenge with *R. equi*, the control foals also developed an IFNγ-PBMC response. It also appears that the reliance on traditional T-cell effectors recognizing MHC-restricted microbial antigens to provide components of cellular immunity can potentially be achieved with an antibody-dependent mechanism of cellular responses, further emphasizing how antibody can provide immunity to intracellular pathogens. This study addressed many important issues related to vaccine development, including the utility of maternal immunization to provide protection against an intracellular pathogen via colostrum to immunologically immature offspring, the efficacy and mechanism of action of antibody to PNAG in protective efficacy, and identification of a role for antibody-dependent IFNγ release in the response to immunization that likely contributed to full immunity to challenge. The success of immunization in protecting against *R. equi* challenge in foals targeting the broadly synthesized PNAG antigen raises the possibility that this single vaccine could engender protection against many microbial pathogens. While the potential to protect against multiple microbial targets is encouraging, the findings do raise issues as to whether antibody to PNAG will be protective against many microbes or potentially manifest some toxicities or unanticipated enhancements of infection caused by some organisms. Thus, continued monitoring and collection of safety data among animals and humans vaccinated against PNAG is paramount until the

safety profile of antibody to PNAG becomes firmly established. Overall, the protective efficacy study in foals against *R. equi* has initiated the pathway to development of PNAG as a vaccine for significant human and animal pathogens, and barring unacceptable toxicity, the ability to raise protective antibodies to PNAG with the 5GlcNH2-TT conjugate vaccine portends effective vaccination against a very broad range of microbial pathogens.

Materials and methods

Experimental design

The objective of the research was to test the ability of maternal vaccination of horse mares with a conjugate vaccine targeting the PNAG antigen to deliver, via colostral transfer, antibody to their offspring that would prevent disease due to intrabronchial *R. equi* challenge at ~4 weeks of life. A confirmatory study using passive infusion of immune or control horse plasma to foals in the first 24 hours of life was also undertaken. The main research subjects were the foals; the secondary subjects were the mares and their immune responses. The experimental design was a randomized, controlled, experimental immunization-challenge trial in horses, with pregnant mares and their foals randomly assigned to the vaccine or control group. Group assignment was made using a randomized, block design for each year. Data were obtained and processed randomly then pooled after unblinding for analysis. Investigators with the responsibility for clinical diagnosis were blinded to the immune status of the foals. An unblinded investigator monitored the data collected to ascertain lack of efficacy and stopping of the infections if 5 or more vaccinated foals developed pneumonia. A similar design was used for the transfusion/passive immunization study, except for the stopping rule.

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Samples size determination

The sample size for the foal protection study was based on prior experience with this model (2, 41, 174) indicating a dose of 10^6 cfu of *R. equi* delivered in half-portions to the left and right lungs via intrabronchial instillation would cause disease in ~85% of foals. Thus, a control group of 7 foals, anticipating 6 illnesses, and a vaccinated group of 12 foals, would have the ability to detect a significant effect at a P value of <0.05 if 75% of vaccinated foals were disease free using a Fisher's exact test, based on the use of the hypergeometric distribution that underlies the experimental design wherein there is no replacement of a subject into the potential experimental outcomes once it is diagnosed as ill. All clinical and immunological data to be collected were defined prior to the trial in mares and foals, and no outliers were excluded from the analysis. The primary endpoint was development of clinical *R. equi* pneumonia as defined under Clinical Monitoring below. Experiments were performed over 3 foaling seasons: 2015 and 2016 for the active immunization of pregnant mares, with results from the 2 years of study combined, and 2017 for the passive infusion study.

Ethics statement

All procedures for this study were reviewed and approved by the Texas A&M Institutional Animal Care and Use Committee (protocol number AUP# IACUC 2014-0374 and IACUC 2016-0233) and the University Institutional Biosafety Committee (permit number IBC2014-112). The foals used in this study were university-owned, and permission for their use was provided in compliance with the Institutional Animal Care

and Use Committee procedures. No foals died or were euthanized as a result of this study.

Vaccine

Mares in the vaccine group received 125 µg (during 2015) or 200 µg (2016) of synthetic pentamers of β-1→6-linked glucosamine conjugated to tetanus toxoid (ratio of oligosaccharide to protein 35-39:1; AV0328, Alopexx Enterprises, LLC, Concord, MA) diluted to 900 µl in sterile medical grade physiological (*i.e.*, 0.9% NaCl) saline solution (PSS) combined with 100 µl of Specol (Stimune Immunogenic Adjuvant, Prionics, Lelystad, Netherlands, now part of Thermo-Fischer Scientific), a water-in-oil adjuvant. The rationale for increasing the dose in 2016 was that some vaccinated mares had relatively low titers, although all foals of vaccinated mares born in 2015 were protected. Mares in the unvaccinated group were sham injected with an equivalent volume (1 ml) of sterile PSS. All pregnant mares were vaccinated or sham vaccinated 6 and 3 weeks prior to their estimated due dates. For the transfusion of hyperimmune plasma, adult horses (not pregnant) were immunized as above, blood obtained, and hyperimmune plasma produced from the blood by the standard commercial techniques used by Mg Biologics, Ames, Iowa for horse plasma products. Controls received commercially available normal equine plasma prepared from a pool of healthy horses.

Study populations and experimental infection

Twenty healthy Quarter Horse mare/foal pairs were initially included in this study; 1 unvaccinated mare and her foal were excluded when the foal was stillborn. The unvaccinated group consisted of 7 mare/foal pairs (n = 4 in 2015 and n = 3 in 2016) and

the vaccinated group consisted of 12 mare/foal pairs (n = 5 in 2015 and n = 7 in 2016).

For the passive infusion of hyperimmune plasma, 9 foals were used, 4 infused with 2 L

of commercial normal horse plasma (Immunoglo Serial 1700, Mg Biologics, Ames, IA,

USA) and 5 were infused with 2 L of PNAG-hyperimmune plasma produced using

standard methods by Mg Biologics. Group assignment was made using a randomized,

block design for each year. All foals were healthy at birth and had total serum IgG

concentrations >800 mg/dl at 48 h of life using the SNAP Foal IgG test (IDEXX, Inc.,

Westbrook, Maine, USA), and remained healthy through the day of experimental

challenge. Immediately prior to experimental infection with *R. equi*, each foal's lungs

were evaluated by thoracic auscultation and thoracic ultrasonography to document

absence of pre-existing lung disease. To study vaccine efficacy, foals were

experimentally infected with 1 x 10^6 of live *R. equi* strain EIDL 5-331 (a virulent, *vapA*-

gene-positive isolate recovered from a pneumonic foal). This strain was streaked onto a

brain-heart infusion (BHI) agar plate (Bacto Brain Heart Infusion, BD, Becton,

Dickinson and Company, Sparks, MD, USA). One cfu was incubated overnight at 37°C

in 50 ml of BHI broth on an orbital shaker at approximately 240 rpm. The bacterial cells

were washed 3 times with 1 X phosphate-buffered saline (PBS) by centrifugation for 10

min, 3000 x g at 4ºC. The final washed pellet was resuspended in 40 ml of sterile

medical grade PBS to a final concentration of 2.5 x 10^4 cfu/ml, yielding a total cfu count

of 1 x 10^6 in 40 ml. Half of this challenge dose (20 ml with 5 x 10^5) was administered

transendoscopically to the left mainstem bronchus and the other half (20 ml with 5 x 10^5)

was administered to the right mainstem bronchus. Approximately 200 µl of challenge

dose was saved to confirm the concentration (dose) administered, and to verify virulence of the isolate using multiplex PCR (23).

For transendoscopic infection, foals were sedated using intravenous (IV) injection of romifidine (0.8 mg/kg; Sedivet, Boehringer-Ingelheim Vetmedica, Inc., St. Joseph, MO, USA) and IV butorphanol (0.02 mg/kg; Zoetis, Florham Park, New Jersey, USA). An aseptically-prepared video-endoscope with outer diameter of 9-mm was inserted via the nares into the trachea and passed to the bifurcation of the main-stem bronchus. A 40-mL suspension of virulent EIDL 5-331 *R. equi* containing approximately 1×10^6 viable bacteria was administered transendoscopically, with 20 ml infused into the right mainstem bronchus and 20 ml into the left mainstem bronchus via a sterilized silastic tube inserted into the endoscope channel. The silastic tube was flushed twice with 20 ml of air after each 20-ml bacterial infusion. Foals and their mares were housed individually and separately from other mare and foal pairs following experimental infection.

Sample collections from mares and foals

Colostrum was collected (approx. 15 ml) within 8 hours of foaling. Blood samples were collected from immunized mares 6 weeks and 3 weeks before their predicted dates of foaling, and on the day of foaling. Blood samples from 4 non-vaccinated mares in the 2015 study were only collected on the day of foaling, whereas blood was collected from the 3 non-vaccinated mares in the 2016 study at the same time-points as those for vaccinated mares. Blood for preparation of hyperimmune plasma was collected from immunized adult horses 2 weeks after the second injection of 200 µg of

the 5GlcNH2-TT vaccine plus 0.1 ml of Specol in a total volume of 1 ml. Blood samples were drawn from foals on day 2 (the day after foaling), and at 4, 6, 8, and 12 weeks of age. Samples at 4 weeks (25-28 days of life) were collected prior to infection. Blood was collected in EDTA tubes for complete blood count (CBC) testing, in lithium heparinized tubes for PBMC isolation, and in clot tubes for serum collection. Transendoscopic tracheobronchial aspiration (T-TBA) was performed at the time of onset of clinical signs for any foals developing pneumonia and at age 12 weeks for all foals (end of study) by washing the tracheobronchial tree with sterile PBS solution delivered through a triple-lumen, double-guarded sterile tubing system (MILA International, Inc. Erlanger, KY, USA).

Clinical monitoring

From birth until the day prior to infection, foals were observed twice daily for signs of disease. Beginning the day prior to infection, rectal temperature, heart rate, respiratory rate, signs of increased respiratory effort (abdominal lift, flaring nostrils), presence of abnormal lung sounds (crackles or wheezes, evaluated for both hemithoraces), coughing, signs of depressed attitude (subjective evidence of increased recumbence, lethargy, reluctance to rise), and nasal discharges were monitored and results recorded twice daily through 12 weeks (end of study). Thoracic ultrasonography was performed weekly to identify evidence of peripheral pulmonary consolidation or abscess formation consistent with *R. equi* pneumonia. Foals were considered to have pneumonia if they demonstrated ≥ 3 of the following clinical signs: coughing at rest; depressed attitude (reluctance to rise, lethargic attitude, increased recumbency); rectal

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temperature >39.4ºC; respiratory rate ≥ 60 breaths/min; or, increased respiratory effort (manifested by abdominal lift and nostril flaring). Foals were diagnosed with *R. equi* pneumonia if they had ultrasonographic evidence of pulmonary abscessation or consolidation with a maximal diameter of ≥ 2.0 cm, positive culture of *R. equi* from T-TBA fluid, and cytologic evidence of septic pneumonia from T-TBA fluid. The primary outcome was the proportion of foals diagnosed with *R. equi* pneumonia. Secondary outcomes included the duration of days meeting the case definition, and the sum of the total maximum diameter (TMD) of ultrasonography lesions over the study period. The TMD was determined by summing the maximum diameters of each lesion recorded in the 4th to the 17th intercostal spaces from each foal at every examination; the sum of the TMDs incorporates both the duration and severity of lesions. Foals diagnosed with *R. equi* pneumonia were treated with a combination of clarithromycin (7.5 mg/kg; PO; q 12 hour) and rifampin (7.5 mg/kg; PO; q 12 hour) until both clinical signs and thoracic ultrasonography lesions had resolved. Foals also were treated as deemed necessary by attending veterinarians (AIB; NDC) with flunixin meglumine (0.6 to 1.1 mg/kg; PO; q 12-24 hour) for inflammation and fever.

Immunoglobulin ELISAs

Systemic humoral responses were assessed among foals by indirectly quantifying concentrations in serum by ELISA from absorbance values of PNAG-specific total IgG and by IgG subisotypes IgG_1, $IgG_{4/7}$, and $IgG_{3/5}$. ELISA plates (Maxisorp, Nalge Nunc International, Rochester, NY, USA) were coated with 0.6 µg/ml of purified PNAG (203) diluted in sensitization buffer (0.04M PO4, pH 7.2) overnight at 4ºC. Plates were washed

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3 times with PBS with 0.05% Tween 20, blocked with 120 μl PBS containing 1% skim milk for 1 hour at 37°C, and washed again. Mare and foal serum samples were added at 100 μl in duplicate to the ELISA plate and incubated for 1 hour at 37ºC. Serum samples were initially diluted in incubation buffer (PBS with 1% skim milk and 0.05% Tween 20) to 1:100 for total IgG titers, 1:64 for IgG_1 and $IgG_{4/7}$ detection, and to 1:256 for $IgG_{3/5}$ detection. A positive control from a horse previously immunized with the 5GlcNH2-TT vaccine and known to have a high titer, along with normal horse serum known to have a low titer, were included in each assay for total IgG titers. For the subisotype assays, immune rabbit serum (rabbit anti-5GLcNH2-TT) was diluted to a concentration of 1:102,400 as a positive control and used as the denominator to calculate the endpoint OD ratio of the experimental OD values. The immune rabbit serum was used to account for inter-plate variability and negative control of normal rabbit serum were included with the equine serum samples. After 1 hour incubation at 37°C, the plates were washed 3 times as described above. For total IgG titers, rabbit anti-horse IgG whole molecule conjugated to alkaline phosphatase (Sigma-Aldrich, St. Louis, MO, USA) was used to detect binding. For IgG subisotype detection, 100 μl of goat-anti-horse $IgG_{4/7}$ (Lifespan Biosciences, Seattle, WA, diluted at 1:90,000), or goat anti-horse $IgG_{3/5}$ (Bethyl Laboratories, Montgomery, TX, USA, diluted at 1:30,000) conjugated to horseradish peroxidase, or mouse anti-horse IgG_1 (AbD Serotec, Raleigh, NC, USA), diluted at 1:25,000) were added to the wells and incubated for 1 hour at room temperature. For the IgG_1 subisotype, goat antibody to mouse IgG (Bio-Rad, Oxford, England, diluted at 1:1000) conjugated to peroxidase was used for detection. Plates were washed again, and for the total IgG titers pNPP substrate (1 mg/ml) was added while for

peroxidase-conjugated antibody to mouse IgG, SureBlue Reserve One Component TMB Microwell Peroxidase Substrate (SeraCare, Gaithersburg, MD, USA) was added to the wells. Plates were incubated for 15 to 60 minutes at 22ºC in the dark. The reaction was stopped by adding sulfuric acid solution to the wells. Optical densities were determined at 450 nm by using microplate readers. Equine subisotype concentrations of PNAG-specific IgG_1, $IgG_{4/7}$, and $IgG_{3/5}$ were also quantified in colostrum of each mare using the same protocol described above for serum. Colostral samples were diluted in incubation buffer (PBS with 1% skim milk and 0.05% Tween 20) to 1:8,192 for IgG_1, 1:4096 for $IgG_{4/7}$ detection, and at 1:64 for $IgG_{3/5}$ detection. For total IgG endpoint titers were calculated by linear regression using a final OD405nm value of 0.5 to determine the reciprocal of the maximal serum dilution reaching this value. For IgG subisotypes, an endpoint OD titer was calculated by dividing the experimental OD values with that achieved by the positive control on the same plate.

PNAG expression by clinical isolates of R. equi

Clinical isolates of *R. equi* were obtained from the culture collection at the Equine Infectious Disease Laboratory, Texas A&M University College of Veterinary Medicine & Biomedical Sciences. All strains were originally isolated from foals diagnosed with *R. equi* pneumonia and were obtained from geographically distinct locations. *R. equi* strains were grown overnight on BHI agar then swabbed directly onto glass slides, air dried and fixed by exposure for 1 minute to methanol at 4ºC. Samples were labeled with either 5 µl of a 5.2 µg/ml concentration of MAb F598 to PNAG directly conjugated to Alexa Fluor 488 or control MAb F429 to alginate, also directly

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conjugated to Alexa Fluor 488, for 4 hours at room temperature. During the last 5 minutes of this incubation, 500 nM of Syto83 in 0.5% BSA/PBS pH 7.4 was added to stain nucleic acids (red fluorophore). Samples were washed and mounted for immunofluorescent microscopic examination as described (176).

Analysis of PNAG expression in infected horse tissues and human monocyte-derived macrophage cultures

The Texas A&M College of Veterinary Medicine & Biomedical Sciences histopathology laboratory provided paraffinized sections of lungs obtained at necropsy from foals with *R. equi* pneumonia. Slides were deparaffinized using EzDewax and blocked overnight at 4°C with 0.5% BSA in PBS. Samples were washed then incubated with the fluorophore-conjugated MAb F598 to PNAG or control MAb F429 to alginate described above for 4 hours at room temperature. Simultaneously added was a 1:500 dilution (in BSA/PBS) of a mouse antibody to the virulence associated Protein A (VapA) of *R. equi*. Binding of the mouse antibody to *R. equi* was detected with a donkey antibody to mouse IgG conjugated to Alexa Fluor 555 at a dilution of 1:250 in BSA/PBS. Samples were washed and mounted for immunofluorescence microscopic examination. To detect PNAG expression in cultured human monocyte-derived macrophages (MDM), prepared as described below in opsonic killing assays, the infected MDM were washed and fixed with 4% paraformaldehyde in PBS for 1 hour at room temperature. To visualize PNAG on the surface of infected cells, MDM cultures were incubated with the fluorophore-conjugated MAb F598 to PNAG or control MAb F429 to alginate for 4-6 hours at room temperature. Samples were then imaged by

confocal microscopy to visualize extracellular PNAG expression. Next, these same samples were treated with 100% methanol at 4ºC for 5 min at room temperature to permeabilize the plasma membrane. Samples were washed with PBS then incubated with either 5.2 μg/ml of MAb F598 to PNAG or MAb F429 to alginate for 1-2 hours at room temperature, washed in PBS then a 1:300 dilution in PBS of donkey antibody to human IgG labeled with Alexa Fluor 555 added for 4-6 hours at room temperature. Samples were washed and mounted for immunofluorescence microscopic examination.

C1q deposition assays

An ELISA was used to determine the serum endpoint titers for deposition of equine complement component C1q onto purified PNAG. ELISA plates were sensitized with 0.6 μg PNAG/ ml and blocked with skim milk as described above, dilutions of different horse sera added in 50 μl-volumes after which 50 μl of 10% intact, normal horse serum was added as a source of C1q. After 60 minutes incubation at 37ºC, plates were washed and 100 μl of goat anti-human C1q, which also binds to equine C1q, diluted 1:1,000 in incubation buffer, added and plates incubated at room temperature for 60 minutes. After washing, 100 μl of rabbit anti-goat IgG whole molecule conjugated to alkaline phosphatase and diluted 1:1,000 in incubation buffer was added and a 1-hour incubation at room temperature carried out. Washing and developing of the color indicator was then carried out as described above, and endpoint titers determined as described above for IgG titers by ELISA.

Opsonic killing assays

To determine opsonic killing of *R. equi*, bacterial cultures were routinely grown overnight at 37°C on chocolate-agar plates, and then killing assessed using modifications of previously described protocols (203). Modifications included use of EasySep Human Neutrophil Isolation Kits (Stem Cell Technologies Inc., Cambridge, Massachusetts, USA) to purify PMN from blood, and use of gelatin-veronal buffer supplemented with Mg^{2+} and Ca^{2+} (Boston Bioproducts, Ashland, Massachusetts, USA) as the diluent for all assay components. Final assay tubes contained, in a 400-µl volume, 2×10^5 human PMN, 10% (final concentration) *R. equi* absorbed horse serum as a complement source, 2×10^5 *R. equi* cells and the serum dilutions. Tubes were incubated with end-over-end rotation for 90 minutes then diluted in BHI with 0.05% Tween and plated for bacterial enumeration. For intracellular opsonic killing assays, human monocytes were isolated from peripheral blood using the EasySep Direct Human Monocyte Isolation Kit (Stem Cell Technologies) and 2×10^4 cells placed in a 150 µl volume of RPMI and 10% heat-inactivated autologous human serum in flat-bottom 96-well tissue culture plates for 5-6 days with incubation at 37°C in 5% CO_2. Differentiated cells were washed and 5×10^5 cfu of either *R. equi*, *M. avium*, *S. aureus*, *N. gonorrhoeae*, *L. monocytogenes* or *B. pertussis*, initially grown on blood or chocolate agar plates at 37°C overnight in 5% CO_2, suspended in RPMI and 10% heat-inactivated autologous human serum added to the human cells for 30 minutes. Next, these cells were washed and 150 µl of RPMI plus 10% autologous serum with 50 µg gentamicin sulfate/ml added and cells incubated for 24 hours at 37°C in 5% CO_2. For some experiments, 50 µl of 400 µg/ml of either chitinase (Sigma-Aldrich) or dispersin B (Kane Biotech, Winnipeg, Manitoba), a PNAG-

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degrading enzyme (192, 204), dissolved in Tris-buffered saline, pH 6.5, were added directly to gentamicin containing wells and plates incubated for 2 hours at 37°C in 5% CO_2. Cell cultures were washed then combinations of 50 μl of MAb or foal serum, 50 μl of 30% human serum absorbed with the target bacterial strain as a complement source, or heat-inactivated complement as a control, and 50 μl containing 1.5×10^5 human PMN, isolated as described above, added. Controls lacked PMN or had heat-inactivated complement used in place of active complement, and final volumes were made up with 50 μl of RPMI 1640 medium. After a 90-minute incubation at 37°C in 5% CO_2, 10 μl samples were taken from selected wells for analysis of lysis by lactate dehydrogenase release, and 100 μl of trypsin/EDTA with 0.1% Triton X100 added to all wells lyse the cells via a 10-minute incubation at 37°C. Supernatants were diluted and plated on chocolate or blood agar for bacterial enumeration as described above.

Cell-mediated immunity

The cell-mediated immune response to vaccination was assessed by measuring IFNγ production from isolated horse PBMCs that were stimulated with an *R. equi* antigen lysate of strain EIDL 5-331, or the same lysate digested for 24 hours at 37°C with 100 μg/ml of dispersin B. The PBMCs were isolated using a Ficoll-Paque gradient separation (GE Healthcare, Piscataway, NJ, USA) and resuspended in 1X RPMI-1640 media with L-glutamine (Gibco, Life Technologies, Grand Island, NY, USA), 15% fetal bovine serum (Gibco), and 1.5% penicillinstreptomycin (Gibco). The PBMCs were cultured for 48 hours at 37°C in 5% CO_2 with either media only, the mitogen Concanavalin A (positive control; 2.5 μg/ml, Sigma-Aldrich), or *R. equi* lysate

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representing a multiplicity of infection of 10. After 48 hours, supernatants from each group were harvested and frozen at -80°C until examined for IFNγ production using an equine IFNγ ELISA kit (Mabtech AB, Nacka Strand, Stockholm, Sweden) according to the manufacturer's instructions. Optical densities were determined using a microplate reader and standard curves generated to determine IFNγ concentrations in each sample using the Gen 5 software (Biotek, Winooski, VT, USA).

Statistical methods

Categorical variables with independent observations were compared using chi-squared or, when values for expected cells were ≤5, Fisher's exact tests. For estimation of the 95% C.I. of the relative risk, the Koopman asymptotic score (188) was determined. Continuous, independent variables were compared between 2 groups using either paired t-tests or Mann-Whitney tests and between > 2 groups using the Kruskal-Wallis test with pairwise *post hoc* comparisons made using Dunn's procedure. Continuous variables with non-independent observations (*i.e*., repeated measures) were compared using linear mixed-effects modeling with an exchangeable correlation structure and individual mare or foal as a random effect. Survival times were compared non-parametrically using the log-rank test. All analyses were performed using S-PLUS statistical software (Version 8.2, TIBCO, Inc., Seattle, Wash, USA) or the PRISM 7 statistical program. Mixed-effect model fits were assessed using diagnostic residual plots and data were transformed (log10) when necessary to meet distributional assumptions of modeling; post hoc pairwise comparisons among levels of a variable (*e.g*., age) were

made using the method of Sidak (205). Significance was set at $P \leq 0.05$ and adjustment

for multiple comparisons made.

CHAPTER III

PNAG-SPECIFIC EQUINE IGG$_1$ MEDIATES SIGNIFICANTLY GREATER

OPSONIZATION AND KILLING OF *RHODOCOCCUS EQUI* THAN DOES IGG$_{4/7}$

Synopsis

Rhodococcus equi is a facultative intracellular bacterial pathogen that causes

severe pneumonia in foals 1 to 6 months of age, whereas adult horses are highly resistant

to infection. We have shown that vaccinating pregnant mares against the conserved

surface polysaccharide capsule, β-1→6-linked poly-*N*-acetyl glucosamine (PNAG)

elicits opsonic killing antibody that transfers via colostrum to foals and protects them

against experimental infection with virulent *R. equi*. We hypothesized that equine IgG$_1$

might be more important than IgG$_{4/7}$ for mediating protection against *R. equi* infection in

foals. To test this hypothesis, we compared complement component 1 (C1) deposition

and polymorphonuclear cell-mediated opsonophagocytic killing (OPK) mediated by

IgG$_1$ or IgG$_{4/7}$ enriched from either PNAG hyperimmune plasma (HIP) or standard

plasma. Sub-isotype IgG$_1$ and IgG$_{4/7}$ from PNAG HIP and standard plasma were

precipitated onto a diethylaminoethyl ion exchange column, then further enriched using

a protein G Sepharose column. We determined C1 deposition by ELISA and estimated

OPK by quantitative microbiologic culture. Anti-PNAG IgG$_1$ deposited significantly (P

< 0.05) more C1 onto PNAG than did IgG$_{4/7}$ from PNAG HIP or Sub-isotype IgG$_1$ and

IgG$_{4/7}$ from standard plasma. In addition, IgG$_1$ from PNAG HIP mediated significantly

(P < 0.05) greater OPK than IgG$_{4/7}$ from PNAG HIP or IgG$_1$ and IgG$_{4/7}$ from standard

plasma. Our findings indicate that anti-PNAG IgG$_1$ is a correlate of protection against

R. equi in foals, which has important implications for understanding the immunopathogenesis of *R. equi* pneumonia, and as a tool for assessing vaccine efficacy and effectiveness when challenge is not feasible.

Introduction

Rhodococcus equi is a ubiquitous facultative intracellular pathogen that causes severe pyogranulomatous pneumonia in foals 3 weeks to 6 months of age, and is among the leading causes of morbidity and mortality for foals (1, 2). Although effective treatments exist (82, 206-208), no vaccine to prevent *R. equi* pneumonia in foals is commercially available. Recently, our laboratory has demonstrated that vaccination of pregnant mares targeting the highly conserved microbial surface polysaccharide, β-1→6-linked poly-*N*-acetyl glucosamine (PNAG) protects their foals against subsequent intrabronchial infection with virulent *R. equi* (70). Antibodies do not cross the equine epitheliochorial placenta during gestation (209) and thus foals are born agammaglobulinemic. Consequently, vaccination of mares during late gestation was presumed to protect foals by antibodies transferred postnatally from mares to foals via colostrum. Foals born to PNAG-vaccinated mares acquired antibody to PNAG from colostrum of the immunoglobulin (IgG) Sub-isotype 1 (IgG_1; a.k.a., IgGa) and Sub-isotype $IgG_{4/7}$ (a.k.a., IgGb), with differences in titers between vaccinates and controls of the former being generally higher than those of the latter. This finding was considered potentially important clinically because different equine IgG Sub-isotype are known to mediate different effector functions in immune responses (37, 46, 67, 165, 166). Direct

comparison of the function of these Sub-isotype, however, was not determined in our prior study.

Conflicting data exist regarding the role of IgG Sub-isotype in immunity to *R. equi* (37, 46, 67) and other equine pathogens (165, 166). It has been proposed that the predominant Sub-isotype response against *R. equi* determines whether a foal develops a protective Th1-type response (mediated by IgG_1) or a non-protective Th2-type response (mediated by $IgG_{3/5}$ and $IgG_{4/7}$) (37, 46, 67). Other studies, however, indicate that the $IgG_{4/7}$ Sub-isotype provides protective immunity against *R. equi* and other intracellular pathogens (46, 67, 165, 166). Immunoglobulin G can opsonize *R. equi* for efficient phagocytosis by equine polymorphonuclear cells (62, 71, 167), and both IgG_1 and $IgG_{4/7}$ contribute to protection against bacterial infection by their ability to: 1) elicit a strong respiratory burst from equine peripheral blood mononuclear cells (67); 2) bind to Fc receptors (FcRs) on effector cells (67, 164); and, 3) bind complement (C') component 1 (C1) and activate C' via the classical pathway (67). Although $IgG_{3/5}$ also elicits a respiratory burst and interacts with FcRs (67), there is contradictory evidence as to whether it fixes C', or inhibits C' fixation by IgG_1 or $IgG_{4/7}$ (37, 164, 210). The presence of $IgG_{3/5}$ antibody to *R. equi* is indicative of exposure to infection, but not of a protective immune response (211). The finding that anti-PNAG IgG_1 titers were generally higher relative to foals of unvaccinated mares than those of $IgG_{4/7}$ in vaccinated and protected foals suggested that anti-PNAG IgG_1 could represent a correlate of protective immunity in foals. Thus, we sought to directly compare functional responses (*viz.*, deposition of C1 onto PNAG and opsonophagocytic killing [OPK] of virulent *R. equi* by PMNs) of IgG_1 isolated from plasma hyperimmune to

PNAG (PNAG HIP) with those of $IgG_{4/7}$ from PNAG HIP, and with those of IgG_1 or $IgG_{4/7}$ from standard plasma.

Results

Enrichment of IgG sub-isotypes from equine plasma

To compare functional responses of IgG Sub-isotype IgG_1 and $IgG_{4/7}$ from PNAG HIP and standard plasma, we first needed to enrich these Sub-isotype from plasma. We used a diethylaminoethyl (DEAE) ion exchange column to enrich whole IgG molecules from standard commercial equine plasma and PNAG HIP, provided by the same manufacturer (Mg Biologics, Ames, IA) (Fig. 7). This resulted in the release of IgG into chromatography fractions collected from the flow-through represented in the first peak in Fig. 7. We confirmed the presence of IgG_1 and $IgG_{4/7}$ in this flow-through by enzyme-linked immunosorbent assay (ELISA) (Table 4). We stripped the remaining proteins bound to the DEAE column from the column with a salt wash-out (second peak in Fig 7). We monitored the quality of the IgG enrichment from equine plasma by using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on saved samples of the enrichment process: the original whole plasma, the supernatant from dextran sulphate precipitation, the dialysate from desalination, chromatography fractions with whole IgG (first peak of DEAE ion exchange column in Fig. 7), and chromatography fractions from the column salt wash-out (second peak of DEAE ion exchange column in Fig. 8).
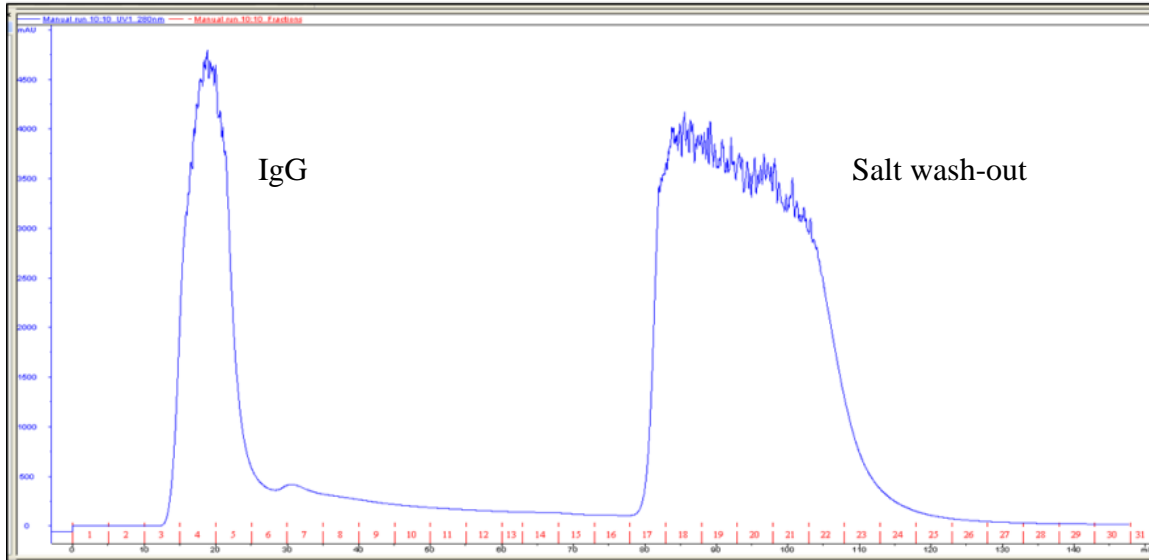
Fig 7. Protein profile of DEAE ion exchange column.

First peak to the left depicts flow-through of IgG (includes IgG subclasses IgG_1 and $IgG_{4/7}$), which does not bind to the DEAE ion exchange column. The second peak to the right represents other proteins which were retained in the DEAE ion exchange column and were removed with a salt wash-out.

Table 4. Concentrations (ng/ml) of IgG$_1$ and IgG$_{4/7}$ from the combined fractions of the flow-through of the DEAE ion exchange column (first peak in Fig 7).
An ELISA plate coated with antibodies specific for either equine IgG$_1$ or IgG$_{4/7}$ immunoglobulins detected these IgG Sub-isotype in the flow-through from PNAG hyper immune plasma (PNAG HIP) and standard plasma (Standard) (shown in two-fold dilutions). Dilutions of flow-through highlighted in orange were tested in wells coated with IgG$_1$ and dilutions of flow-through highlighted in blue were tested in wells coated with IgG$_{4/7}$. The concentration of IgG$_1$ in the PNAG HIP and standard plasmas was detected within the range of concentrations set by the standard IgG$_1$ (approx. 150 to 7 ng/ml). The Sub-isotype IgG$_{4/7}$ was highly concentrated in the flow-through of PNAG HIP and standard plasma, as every dilution surpassed the concentration intervals set by the IgG$_{4/7}$ standard (>209 ng/ml). Concentrations of each IgG Sub-isotype dilution represent the mean of the dilution in duplicate.

ELISA of DEAE flow-through					
		Plates coated with			
		Anti-IgG$_1$ (ng/ml)		Anti-IgG$_{4/7}$ (ng/ml)	
Standard IgG$_1$ & IgG$_{4/7}$	Flow-through dilution	PNAG HIP	Standard	PNAG HIP	Standard
200	Neat	139.35	149.05	>209	>209
100	1:2	108.05	74.73	>209	>209
50	1:4	67.22	50.38	>209	>209
25	1:8	48.65	32.10	>209	>209
12.5	1:16	30.98	20.11	>209	>209
6.25	1:32	21.73	12.31	>209	>209
3.125	1:64	14.32	7.49	>209	>209
Blank	-	3.24	4.47	9.517	6.11

Verification of IgG enrichment using sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

To detect potential protein loss during the enrichment process, we performed SDS-PAGE on samples of the different fractions obtained from the plasma enrichment of IgG (Fig. 8). We found substantial amounts of albumin present in the whole (untouched) plasma, supernatant (after precipitation), and dialysate (after desalination), suggesting that there was no major protein loss. We showed fractions from the first peak in Fig. 7 to be depleted of albumin, and to instead have strongly enriched bands that corresponded to heavy and light IgG chains, confirming presence of IgG (see Fig. 8). The strong band of albumin reappeared in the wash-out (second peak in Fig. 7), that had

only trace bands of heavy and light chains of IgG. It should be noted that this gel was heavily loaded with proteins such that small amounts of protein can be detected. This figure demonstrates the successful enrichment of the fractions containing predominately IgG that was substantiated by the results of ELISA testing (Table 4).
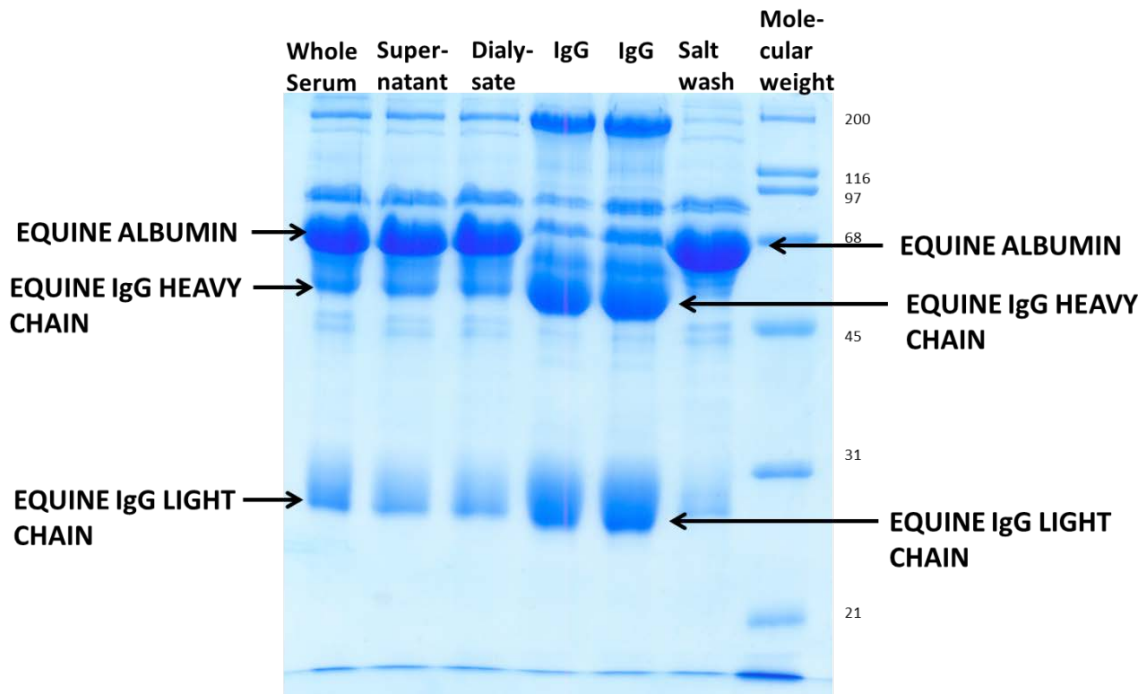
Fig 8. SDS PAGE of samples from different phases of plasma enrichment.
Columns left to right: whole serum; supernatant from dextran sulphate; dialysate after desalination; IgG from flow-through (from first peak to the left of DEAE in Fig 1); salt wash-out used to clean column of other retained proteins (from second peak of DEAE in Fig 1); and, molecular weight standard. Whole serum, supernatant, dialysate, and the wash-out all had robust bands that correspond to albumin protein (see arrows). The flow-through, containing IgG molecules, was clear of the albumin band and showed prominent bands that correspond to equine IgG heavy and light chains (see arrows).

Isolation of IgG₁ and IgG₄/₇ using protein G Sepharose columns

For the isolation of IgG_1 and $IgG_{4/7}$ from the flow-through in the DEAE ion exchange columns (first peak to the left, Fig. 7) we used a Protein G Sepharose column (Fig. 9). A continual decrease in pH (represented by the green line in Fig. 9) allowed for the capture of IgG_1 in the fractions represented in the smaller peak beneath the pH gradient in Fig. 9. Further decrease in the pH allowed for the dissociation of $IgG_{4/7}$ from the Protein G Sepharose column and its collection into the fractions represented by the taller peak beneath the pH gradient in Fig 3. We confirmed the presence or absence of IgG_1 and $IgG_{4/7}$ in the eluted fractions by ELISA (Table 5).
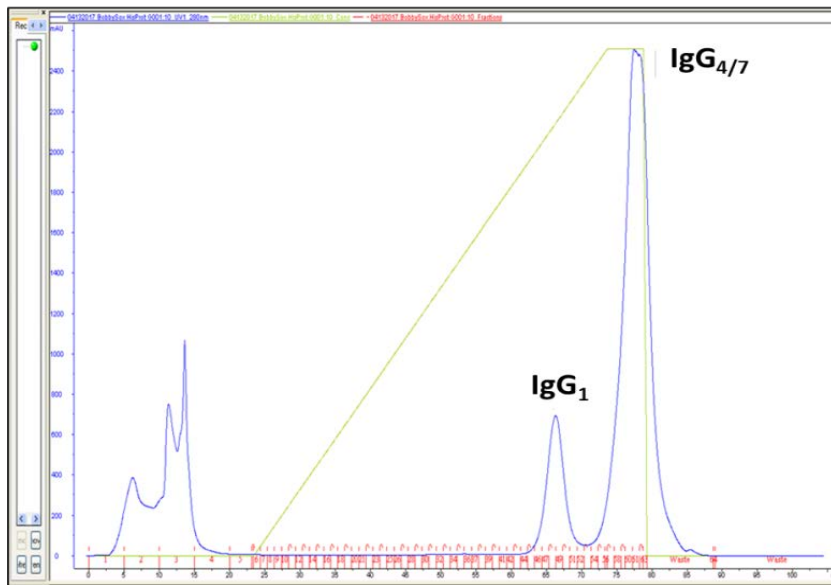
Fig 9. Protein profile of the isolation of IgG subisotypes using a pH gradient of 8.0 to 2.0 in a protein G sepharose column.
The subisotype IgG_1 was eluted into fractions (comprising of the smaller peak to the left) by a pH gradient (green line) while other proteins remained bound to the protein G column. Further decrease in pH (rise in the green line) eluted $IgG_{4/7}$ into another set of fractions (represented by the taller peak to the right). The elution of the 2 IgG Sub-isotype at different pH values allowed for their isolation. Presence of these Sub-isotype in these fractions was confirmed by ELISA (see Table 5).

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Table 5. Concentrations (ng/ml) of IgG$_1$ and IgG$_{4/7}$ sub-isotypes were simultaneously determined by ELISA from the chromatography fractions collected from the protein G column from Fig 9.

Fractions 47 to 51 (highlighted in orange) corresponded to the smaller peak in Fig 9. In these fractions, the presence of IgG$_1$ was detected at a peak concentration of 63.25 ng/ml. There was no IgG$_{4/7}$ detected in these fractions. Fractions 57 to 65 (highlighted in blue) corresponded to the second (taller) peak in Fig 9 where the presence of IgG$_{4/7}$ was detected by ELISA at a peak concentration of 93.9 ng/ml. There was no IgG$_1$ detected in these fractions. Neither IgG Sub-isotype were detected by ELISA in the remaining fractions before and after the pH gradient.

Fx	IgG$_1$ ELISA Conc (ng/ml)	IgG$_{4/7}$ ELISA Conc (ng/ml)	Dilution factor	ng/ml	ug/ml
1	0	0	5000	0	0
2	0	0	5000	0	0
3	0	0	5000	0	0
4	0	0	5000	0	0
45	0	0	5000	0	0
47	4.041	0	5000	20205	20.205
49	12.650	0	5000	63250	63.250
51	6.985	0	5000	34925	34.925
55	0	0	5000	0	0
57	0	2.893	5000	14465	14.465
59	0	14.430	5000	72150	72.150
61	0	18.790	5000	93950	93.950
63	0	11.750	5000	58750	58.750
65	0	2.850	5000	14250	14.250
67	0	0	5000	0	0

Complement component C1q deposition of standard plasma, PNAG HIP and their IgG sub-isotypes

To test for differences in classical C' pathway activation between IgG$_1$ and IgG$_{4/7}$ from PNAG HIP and standard plasma, we compared their ability to deposit C1 onto PNAG. We found that antibodies from whole PNAG HIP (positive control) deposited significantly (P < 0.05; linear mixed-effects modeling, using the method of Sidak for pair-wise comparisons) more C1 onto PNAG than did those from whole standard plasma (Fig. 10), Sub-isotype IgG$_1$ or IgG$_{4/7}$ isolated from PNAG HIP, and Sub-isotype IgG$_1$ or

IgG$_{4/7}$ isolated from standard plasma. IgG$_1$ isolated from PNAG HIP deposited significantly (P < 0.05) more C1 than did whole standard plasma, IgG$_{4/7}$ derived from PNAG HIP, and IgG$_1$ or IgG$_{4/7}$ from standard plasma. No other differences were significant. For the PNAG HIP and Sub-isotype IgG$_1$ and IgG$_{4/7}$ derived from it, however, there were significant (P < 0.05; linear mixed-effects modeling using the method of Sidak for pair-wise comparisons) decreases of optical densities (ODs) with dilution. Collectively, these results indicate that IgG$_1$ derived from PNAG HIP deposited significantly (P < 0.05) more C1 onto PNAG than did IgG$_{4/7}$ from PNAG HIP, or IgG$_1$ or IgG$_{4/7}$ isolated from standard plasma.
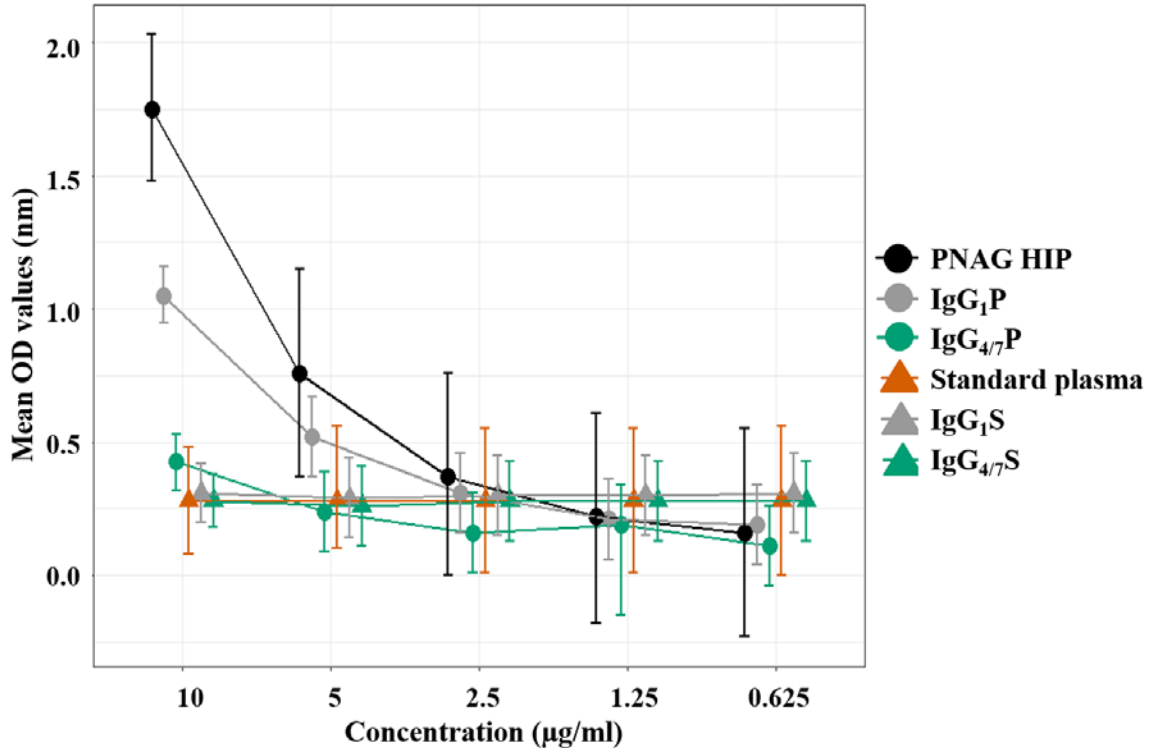
Fig 10. Impact of IgG-subisotype and source on PNAG C1q deposition.
Model-estimated mean OD values for deposition of C1q onto PNAG for standard plasma, PNAG HIP, IgG$_1$ from PNAG HIP (IgG$_1$P), IgG$_{4/7}$ from PNAG HIP (IgG$_{4/7}$P), IgG$_1$ from standard plasma (IgG$_1$S), and IgG$_{4/7}$ from standard plasma (IgG$_{4/7}$S). Plasmas and IgG Sub-isotype were serially diluted by 1:1 from 10 to 0.625 μg/ml. It should be noted that the concentration for plasma is reflective of IgG$_1$ in plasma (but not IgG$_{4/7}$). The OD values are derived from the amount of complement deposited onto plates coated with PNAG as determined by ELISA. Sidak method for mean comparison for concentrations with different symbols differed significantly (P < 0.05) from the other conditions (*PNAG HIP; #IgG$_1$P).

Determination of OPK activity using IgG sub-isotypes

To further assess the functional capacity of each Sub-isotype, we determined

opsonic killing of *R. equi* by PMNs in the presence of C'. We used 2 negative controls:

a bacterial control (Contrl Bact, Fig. 11) comprised of only media and *R. equi*, and a

second control omitting antibodies (Contrl C'PMN, Fig. 5; presence of media, *R. equi*,

equine C', and equine PMNs). We incubated tubes with end-over-end rotation for 90

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minutes, and then serially diluted and plated for bacterial enumeration. Each of the IgG Sub-isotype resulted in significant ($P < 0.05$; linear mixed-effects modeling) killing relative to the Contrl Bact, but the Contrl C'PMN did not differ significantly from the Contrl Bact ($P=0.2106$; linear mixed-effects modeling). The proportion of bacteria surviving that were opsonized by IgG_1 from PNAG HIP (mean proportion surviving, 69%; 95% CI 61% to 78%) was significantly ($P < 0.05$; linear mixed-effects modeling using the method of Sidak for pair-wise comparisons) less than those opsonized with IgG_1 from standard plasma (mean proportion surviving 79%; 95% CI, 70% to 87%), $IgG_{4/7}$ from PNAG HIP (mean proportion surviving, 81%; 95% CI, 73% to 89%), and $IgG_{4/7}$ from standard plasma (mean proportion surviving, 80%; 95% CI, 72% to 88%) (Fig. 11). The proportion of bacteria surviving that were opsonized by IgG_1 from PNAG HIP was also significantly ($P < 0.05$; linear mixed effects modeling using the method of Sidak for pair-wise comparisons) less than that of bacteria treated without antibodies (Cntrl C'PMNs = 90%; [95% CI, 79% to 100%]). No other pairwise comparisons among treatments differed significantly (Fig. 11).
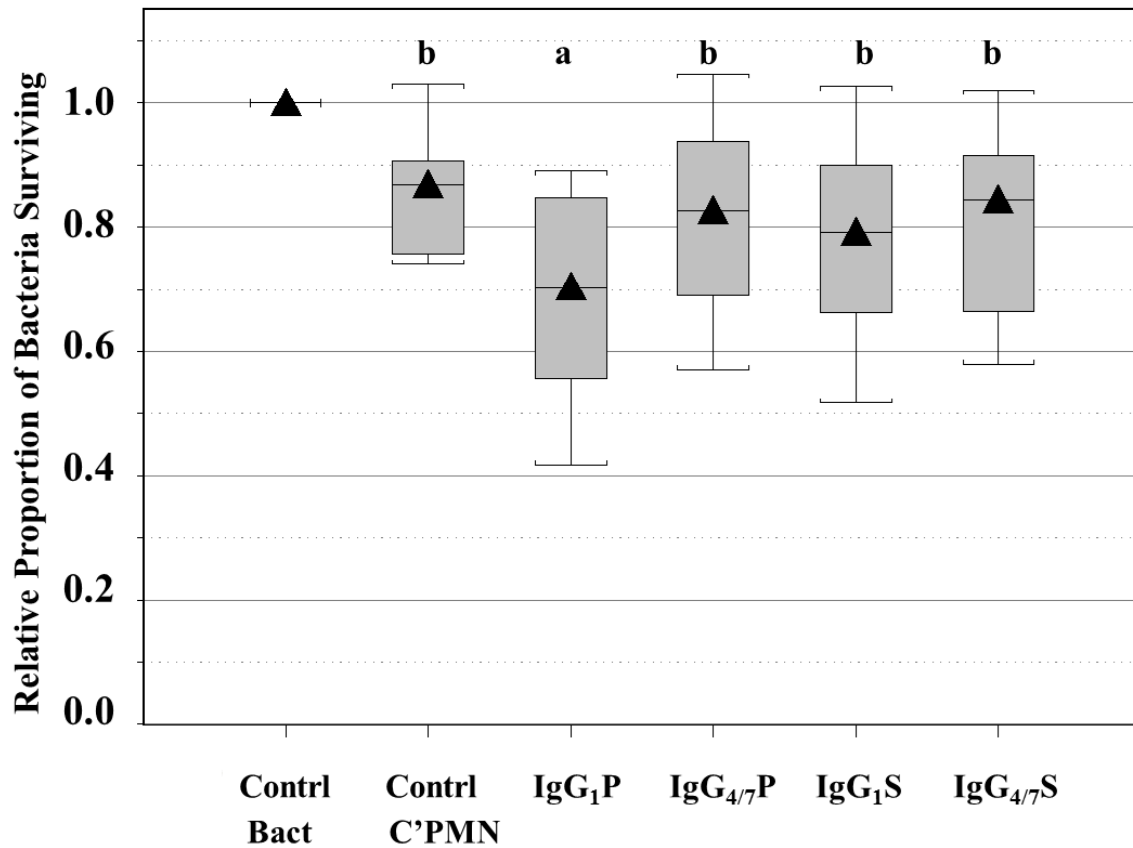
Fig 11. **Effect of IgG-subtype and source on PMN killing of opsonized *R. equi*.**
Boxplot of proportional equine PMN killing of virulent *R. equi* when opsonized with IgG_1
derived from either PNAG HIP or standard plasma (IgG_1P and IgG_1S, respectively) or $IgG_{4/7}$
derived from PNAG HIP or standard plasma ($IgG_{4/7}P$ and $IgG_{4/7}S$, respectively). Controls had
bacteria and media only (Contrl Bact; reference category) or bacteria, media, complement (C'),
and polymorphonuclear (PMN) cells (no antibodies; Contrl C'PMN). Triangles represent median
values; bottoms and tops of boxes represent the 25th and 75th percentiles, respectively. Whiskers
extend to a multiple (1.75) of the inter-quartile distance. Sidak method for mean comparison:
boxes labelled with different letters differ significantly ($P < 0.05$) relative to control.

Discussion

We have previously shown that maternal vaccination with PNAG protects foals against experimental infection with live, virulent *R. equi*, and that this protection is likely mediated following colostral transfer to foals of maternal antibodies raised to PNAG (70). Moreover, relative differences between titers of vaccinated foals and control foals of anti-PNAG IgG_1 were greater than those for $IgG_{4/7}$, suggesting anti-PNAG IgG_1 was more important for immunity to *R. equi*. To further investigate this possibility, we compared the *in vitro* capacity of these 2 IgG Sub-isotype enriched from standard plasma and PNAG HIP to deposit C1on to PNAG) which leads to efficient OPK. Our results indicate a functional basis for the observed association between relatively greater concentrations of IgG_1 against PNAG than $IgG_{4/7}$ in protected foals (70) supporting the importance of anti-PNAG IgG_1 as a correlate of immunity against *R. equi* infection in foals.

There is compelling evidence that antibodies can protect against intracellular infections, although conflicting results exist (26, 212-214). Toxin neutralizing antibodies can contribute to protection against progression of *R. equi* pneumonia (75), though the protection they mediate is incomplete (1, 75, 93). Beyond neutralizing activity, antigen-specific antibodies can alter inflammatory responses against certain intracellular pathogens through FcR-mediated signaling (27, 96, 138, 139), altering microbial physiology (55, 146), stimulating respiratory burst (92), opsonizing and activating C' (92, 167, 215), enhancing phagocytosis (71), and via antibody-dependent cellular cytotoxicity of OPK (92, 162). Activation of FcR could play an important role in *R. equi* protection, as the interaction of antibodies with $Fc\epsilon RII$-CD23 or $Fc\gamma IIIa$ leads

to control of proliferation of ingested of *Mycobacterium tuberculosis* (Mtb) (139, 213, 216), an organism similar to *R. equi*. Antibody binding to the surface of the facultative intracellular pathogens *C. neoformans* (145) or Mtb (55) triggers transcriptional responses intracellularly that can interfere with microbial physiology. Antibodies may enter cells via pinocytosis (144, 145) and mediate protection against intracellular pathogens by activity within infected cells. For example, antibodies mediate protection against *Listeria monocytogenes,* by neutralizing the toxin listeriolysin <u>*within*</u> infected phagocytic cells; the toxin is delivered to the bacterial phagosome by intracellular transport (141).

The ability of antibodies to mediate killing of intracellular pathogens can vary among IgG Sub-isotype. In this study, we report that anti-PNAG IgG_1 – but not anti-PNAG $IgG_{4/7}$ – mediated protective responses against *R. equi*. In other studies of *R. equi* infections*,* foals that remained healthy after experimental infection (41, 46, 84) with *R. equi* or during natural exposure (37, 38) to *R. equi* had high IgG_1 titers against *R. equi*, which were indicative of either immunity or humoral response to *R. equi.* There is also a link between IgG Sub-isotype and Th1 and Th2 cytokine responses (41, 217, 218). It is possible that IgG_1 directs a Th1-type response resulting in enhanced cell-mediated immunity (CMI) against *R. equi* (219-221), whereas $IgG_{4/7}$ and $IgG_{3/5}$ are associated with greater Th2 responses that do not enhance CMI (37). A Th1 response has been linked to protection against *R. equi* in mice (30, 35, 222). Varying protection among different Sub-isotype also has been observed for other intracellular pathogens such as Mtb, in which murine IgG Sub-isotype IgG_1 and IgG_3 prolonged survival of mice infected with Mtb that were associated with CMI (212, 222). Arabinomannan-

specific IgG_3 elicited by a polysaccharide conjugate vaccine targeting Mtb conferred

protection in infected mice (27, 212), possibly by altering expression of genes regulating

bacterial metabolism (55). Human IgG_2 is responsible for immunity to certain bacterial

infections by binding to bacterial capsular polysaccharide antigens on which it forms

hexamers that increase its avidity for C1 (223). Studies with *C. neoformans* show IgG_1 -

but not IgG_3 - against a capsular polysaccharide protects against infection in mice (224).

Opsonization of *C. neoformans* with IgG_1 increases phagocytosis by macrophages and

arrests intracellular fungal growth (225).

The mechanisms by which anti-PNAG IgG_1 mediates protection against *R. equi*

are not fully explored, but a number of mechanistic insights have been experimentally

derived. It has recently been shown that PNAG derived from the surface of intracellular

R. equi appears to be transported to the cytoplasmic membrane of infected macrophages

(70). The PNAG on the surface of the infected macrophages was detected by an anti-

PNAG monoclonal antibody, which could also facilitate deposition of C1 and

chemotaxis of neutrophils to the infected cells, with subsequent lysis of the macrophages

and release of the intracellular microbes for further opsonic killing. Furthermore,

antibodies to PNAG increased the release of IFNγ from PBMCs isolated from foals born

to vaccinated mares in response to PNAG (70). Equine Sub-isotype IgG_1 could

orchestrate interactions with other cell types by activating FcR on effector cells, and

consequently mediating phago-lysosomal fusion within alveolar macrophages infected

with virulent *R. equi* (96, 163). The enhanced ability of equine IgG_1 from PNAG HIP to

mediate killing of *R. equi* could be due to higher specificity (epitope location and

accessibility) than that of $IgG_{4/7}$ to *R. equi*, as occurs for specific Sub-isotype in

mediating protection against other intracellular pathogens (26, 28, 163). These mechanisms likely change as the foal's immune system matures and requirements change, but more studies are needed.

In summary, our results provide compelling evidence that equine IgG_1 antibody to PNAG is a mediator of protective immunity to *R. equi* in foals from pregnant mares vaccinated against PNAG. It remains to be demonstrated, however, whether anti-PNAG IgG_1 administered to foals specifically mediates protection *in vivo* against either experimental or natural infection with *R. equi*. Intracellular clearance of *R. equi* likely relies on the cooperation between innate, humoral, and CMI (27, 71) factors, as components of these systems are all involved in mediating protection against *R. equi* challenge. The possibility to protect against intracellular pathogens by vaccines that elicit humoral immunity requires further understanding of how antibodies alter host cell-pathogen interactions, and how antibodies interact with leukocytes to control and eliminate intracellular infections such as *R. equi*. In horses, differences in functional activities of IgG Sub-isotype appear to impact such protective immunity, indicating that more molecular insights into protective immunity against *R. equi* and other similar pathogens can be garnered from additional studies of horse IgG Sub-isotype functionality.

Materials and methods

List of reagents and media

1X RPMI medium 1640 [+] L-glutamine (ThermoFisher Scientific) with 5% heat-inactivated fetal bovine serum (ThermoFisher Scientific) as the diluent for all

components in polymorphonuclear (PMN) cell isolation, C' acquisition from depleting

R. equi specific antibodies from serum, and OPK assays.

Standard sandwich ELISA kits (Horse IgG$_1$ ELISA Quantitation Set and Horse

IgG$_{4/7}$ ELISA Quantitation Set, Bethyl Laboratories, Inc, Montgomery, TX, USA) to

detect and quantify IgG sub-isotypes during the enrichemnet and isolation process.

ELISA buffers and solutions for:

- Coating: 0.05 M Carbonate-Bicarbonate, pH 9.6,

- Washing: 50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0

- Blocking: 50 mM Tris, 0.14 M NaCl, 1% BSA, 0.05% Tween 20, pH 8.0

- Diluent: 50 mM Tris, 0.14 M NaCl, 1% BSA, 0.05% Tween 20, pH 8.0

- Stopping enzymatic reaction: 0.18 M H$_2$SO$_4$

Enrichment and isolation of IgG sub-isotypes IgG$_1$ and IgG$_{4/7}$

We performed the IgG Sub-isotype isolation from plasma at the Texas A&M

University Protein and Chemistry Laboratory, based on the method of Sugiura *et al.*

(226). The plasma source used for the isolation of IgG Sub-isotype was standard, non-

immune, horse plasma with low titers to *R. equi* (Immunoglo Serial 1700, Mg Biologics,

Ames, IA, USA) and PNAG HIP produced using previously described methods by Mg

Biologics (70). We removed the contaminants from 50 ml of plasma (either PNAG HIP

or non-immune) using dextran sulphate to precipitate lipoproteins in the presence of

cations such as Ca^{2+}, and the salt (Ca^{2+}) in the resulting protein solution (supernatant)

using dialysis with a 5M Tris pH 8.0 buffer. Then we passed the desalted protein

solution through a DEAE Sepharose ion exchange column (GE Healthcare, Chicago, IL,

USA) to which IgG_1 and $IgG_{4/7}$ do not bind. We collected the flow-through material into different fractions and detected presence of IgG_1 and $IgG_{4/7}$ by standard sandwich ELISA. The flow-through fractions positive for IgG_1 and $IgG_{4/7}$ were bound to a Protein G Sepharose column (GE Healthcare) and eluted using a pH gradient ranging from 8.0 to 2.0 to separate IgG_1 from $IgG_{4/7}$ on an AKTA pure chromatography system controlled by Unicorn (v7.0.3, GE Healthcare). We collected the fractions in Tris buffer (1M, pH 7.5), combined, and concentrated using spin columns with molecular weight cutoff (MWCO) of 10,000 (VIVASPIN 15R, Sartorius Stedim Biotech, Goettingen, Germany). Isolation of IgG steps were monitored using SDS-PAGE) (60).

ELISA for detection of IgG sub-isotypes

We detected the concentration (in ng/ml) of IgG_1 and $IgG_{4/7}$ Sub-isotype collected from the chromatography fractions by standard sandwich ELISA. We tested samples in duplicates in a 96-well plate that was coated with total horse IgG_1 or $IgG_{4/7}$. After 1 hour of incubation at room temperature ($22^{o}C$), we washed the plate 5 times and blocked for 30 minutes at room temperature. We washed the plate again before adding diluted standard and IgG Sub-isotype samples from chromatography fractions and incubated at room temperature for 1 hour. Afterwards we added diluted horseradish peroxidase conjugated antibodies against IgG_1 and $IgG_{4/7}$ to the corresponding wells, and incubated for another hour at room temperature. After a final wash, SureBlue Reserve One Component TMB Microwell Peroxidase Substrate (SeraCare, Gaithersburg, MD, USA) we added to the wells and incubated for 15 minutes at room temperature in the

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dark. We stopped the reaction before determining the optical densities at 450 nm by using microplate reader Synergy 2 (Biotek, Winooski, VT, USA).

Polymorphonuclear (PMN) cell isolation

For all procedures requiring blood in this study, blood was collected from university-owned donor horses using a protocol approved by the Texas A&M University IACUC (AUP# 2017-0440). To isolate equine PMNs, we collected whole blood in plastic sodium heparin vacutainer tubes (ThermoFisher Scientific, Grand Island, NY, USA) by layering over Histopaque 1191 (Sigma-Aldrich Co, St. Louis, MO, USA) and Histopaque 1077 (Sigma-Aldrich Co), and centrifuging at 700 x g for 30 minutes at 22°C yielded 2 opaque rings or layers. We collected PMNs from the middle layer, washed once in 1X Hanks' Balanced Salt Solution (ThermoFisher Scientific), and resuspended in 1X RPMI medium.

Complement acquisition from depleting <u>R. equi</u>-specific antibodies from serum

We used the C' source from commercial equine serum (Sigma-Aldrich Co) diluted to a 40% solution containing *R. equi* which was grown overnight in brain-heart infusion broth and suspended to a concentration of an OD_{600} nm of 1. To allow time for *R. equi*-specific antibodies in the commercial serum to bind to the *R. equi* cells, we incubated this serum/*R. equi* solution on ice for 30 minutes. After this incubation time, we centrifuged the serum solution for 3 minutes at 6,000 x g at 4°C, discarded the pellet, incubated the supernatant again with *R. equi* at an OD_{600} nm of 1 for 30 minutes on ice, and centrifuged for 3 minutes at 6,000 x g at 4°C. To remove residual bacterial cells we

filter-sterilized the supernatant through a 0.2-µm filter, and then we aliquoted, and stored at -80°C.

Complement protein C1q deposition

This assay was performed as previously described (70). Briefly, we sensitized ELISA plates with PNAG and added dilutions of equine standard plasma, PNAG HIP, or the IgG Sub-isotype in 50-µl volumes with 50 µl of 10% intact horse serum as the source of C1 (Sigma-Aldrich Co). After 60 minutes incubation at 37°C, we washed the plates and added 100 µl of goat anti-human C1 (Cedarlane, Burlington, NC, USA), which also binds to equine C1, diluted 1:1,000, and incubated the plates at room temperature for 60 minutes. After washing, we added 100 µl of rabbit anti-goat IgG whole molecule (Sigma-Aldrich Co) conjugated to alkaline phosphatase diluted to 1:2,000 and incubated for 1 hour at room temperature. We finished by washing the plate and developing the color indicator before reading the optical densities.

Opsonophagocytic killing (OPK) assay

We determined the OPK of *R. equi* in the presence of PMNs, C', and antibodies in 2-ml round-bottom tubes. The total volume of each tube was 400 µl and comprised of 4×10^6 equine PMN (1×10^7 cells/ml), 40% *R. equi*-absorbed horse serum as a C' source, 4×10^6 cfu of *R. equi* (1×10^7 cfu/ml), and 10 µg/ml antibodies of either Sub-isotype (IgG_1 or $IgG_{4/7}$) isolated from standard plasma or PNAG HIP. We used two negative controls: a bacterial control (Contrl Bact), comprised only of media and *R. equi*; and a control omitting antibodies (Contrl C'PMN; presence of media, *R. equi*, equine C'

and PMNs). We incubated the tubes with end-over-end rotation for 90 minutes then serially diluted and plated for bacterial enumeration.

Statistical analysis

We analyzed the C1 deposition data (OD_{405} nm) using linear mixed-effects regression. The outcome variable was the OD value and the dependent fixed variables were source of antibody (standard plasma, PNAG HIP, and IgG_1 and $IgG_{4/7}$ from either PNAG HIP or standard plasmas a categorical variable), concentration (as an ordered categorical variable), and their bivariate interaction terms, with individual experiment as a random effect. The 95% confidence intervals were estimated using maximum likelihood methods. For post hoc comparisons among dilutions and treatments, we used the method of Sidak (227), a significance level of $P < 0.05$.

We analyzed the OPK data from 16 experiments conducted using PMNs from 16 horses to compare the killing capacity of PMNs infected with either IgG_1 or $IgG_{4/7}$ derived from PNAG HIP or standard plasma. We determined the proportional killing by dividing the number of bacteria recovered from PMNs by the number from control samples. We analyzed data using linear mixed-effects regression with the proportional killing as the dependent variable (standard plasma, PNAG HIP, IgG_1P, IgG_1S, $IgG_{4/7}P$, and $IgG_{4/7}S$), and estimated the 95% confidence intervals using maximum likelihood methods. For *post hoc* comparisons among dilutions and treatments we used the method of Sidak (227), a significance level of $P < 0.05$. For all linear mixed-effects regression models, the goodness of model fit was assessed by inspecting diagnostic residual plots and examining AIC and BIC values.

CHAPTER IV

SUMMARY AND FUTURE DIRECTIONS

Summary of our findings

The studies presented in this dissertation indicate that interaction of antibodies against the highly conserved microbial surface polysaccharide, β-1→6-linked poly-*N*-acetyl glucosamine (PNAG) with different leukocytes mediated protection against intra-bronchial infection of 1-month-old foals with virulent *R. equi*. Below is a summary of our findings.

Antibodies can provide immunity to intracellular pathogens

Our results have shown that a vaccine targeting PNAG protects foals against experimental intra-bronchial infection with live, virulent *R. equi* and suggests that this protection is primarily antibody-mediated (please see Chapter II). Further, our evidence indicates that this protection is attributable principally to anti-PNAG immunoglobulin G (IgG) sub-isotype 1 (IgG_1) (please see Chapter III). We theorize, based on preliminary results (summarized in Chapter II) that PNAG antibodies , protect against *R. equi* infections by identifying *R. equi*-derived PNAG embedded on the surface of infected macrophages causing lysis of the antibody-targeted macrophages by neutrophils and complement, and further killing of released intracellular *R. equi* by standard opsonophagocytic mechanisms.

Antibodies may indirectly prime peripheral blood mononuclear cells (PBMCs) to produce interferon-γ (IFNγ)

In vitro stimulation of PBMCs from vaccinated and naturally protected foals with *R. equi* resulted in PNAG-specific release of IFNγ compared to controls. This indicates PNAG vaccination also induced an antibody-dependent cellular release of this critical immune cytokine. Overall, antibody-mediated opsonic killing and IFNγ release in response to PNAG seems to play a role in protection against diseases caused by *R. equi* and possibly other intracellular bacterial pathogens.

Future Directions

To expand our knowledge of the functional properties of these protective antibodies, we need to further investigate our results. Relevant questions include the following.

1) Is anti-PNAG IgG_1 more effective than anti-PNAG $IgG_{4/7}$ at protecting foals against experimental infection with live, virulent *R. equi*?

2) Is PNAG identified in bacterial membranes embedded on the surface of alveolar macrophages?

3) Does anti-PNAG IgG_1 induce opsonic killing of *R. equi*-infected foal alveolar macrophages by neutrophils better than $IgG_{4/7}$?

4) Can alveolar macrophages be activated with anti-PNAG antibodies to kill intracellular *R. equi*?

5) Can IFNγ produced by protected foals activate macrophages to kill intracellular *R. equi*?

6) By what mechanism do anti-PNAG antibodies mediate protection against *R. equi* infections?

Is anti-PNAG IgG$_1$ more effective than anti-PNAG IgG$_{4/7}$ at protecting foals against experimental infection with live, virulent R. equi?

The IgG sub-isotype IgG$_1$ from PNAG hyperimmune plasma (HIP) seems to be more effective than IgG$_{4/7}$ (from PNAG HIP) at mediating opsonophagocytic killing (OPK) of *R. equi* by neutrophils and depositing complement component 1q (C1q) on to PNAG. These data suggest that anti-PNAG IgG$_1$ is a correlate of protective immunity against *R. equi*. To test this *in vivo*, we would need to show that transfusion of anti-PNAG IgG$_1$ but not anti-PNAG IgG$_{4/7}$ can protect against *R. equi* pneumonia. As there is no small animal model of *R. equi* pneumonia, we would have to do challenge experiments in foals. We know that transfusion of 2 L of PNAG HIP protects foals against experimental infection with *R. equi* so we would need to isolate IgG$_1$ and IgG$_{4/7}$ from the equivalent 2 L of PNAG HIP for the experiment. Two groups of foals would be transfused with the equivalent of 2 L of either IgG$_1$ or IgG$_{4/7}$ from PNAG HIP within 24 hours of birth and experimentally infected at approximately 1 month of age. Anti-PNAG IgG$_1$ would be associated with protection against *R. equi* pneumonia if the group of foals transfused with this sub-isotype has significantly (P < 0.05) fewer pneumonic foals or if their clinical signs are significantly (P < 0.05) mitigated when compared to foals transfused with anti-PNAG IgG$_{4/7}$.

Is PNAG identified in bacterial membranes embedded on the surface of alveolar macrophages?

This question can be answered *in vitro* using fluorescently-labeled monoclonal antibodies and membrane dyes to microscopically identify PNAG on *R. equi* vesicles embedded in the surface of macrophages infected with live virulent *R. equi* but not on uninfected macrophages. First, bronchoalveolar lavages (BAL) would be performed using foals to harvest alveolar macrophages to infect with *R. equi*. Bacterial and macrophage membranes would be stained separately with fluorescent dyes (Deep Red and Green CMFDA dyes respectively) prior to *in vitro* infection. Co-localization of PNAG detected with (orange Rhodamine Red-X) fluorescently labeled human anti-PNAG monoclonal antibody F598 onto the deep red-stained *R. equi* membrane embedded in the green alveolar membrane of infected macrophages, and its absence in uninfected alveolar macrophages, will confirm that this surface PNAG came from bacterial vesicles within infected alveolar macrophages.

Does anti PNAG IgG$_1$ induce opsonic killing of <u>R. equi</u>-infected foal alveolar macrophages by neutrophils better than IgG$_{4/7}$?

This question can be answered by infecting macrophages *in vitro* as well as *in vivo* to see the effect of antibody-mediated OPK by neutrophils in the presence of complement and the different IgG sub-isotyes (IgG$_1$ or IgG$_{4/7}$) from PNAG hyperimmune or standard plasmas. The efficacy of the different anti-PNAG IgG sub-isotypes would be compared in their ability to recognize PNAG on the surface of infected macrophages to target them for opsonic killing by neutrophils. Based on our

previous results, we would expect anti-PNAG IgG$_1$ to mediate the most killing of infected macrophages and their intracellular *R. equi*.

In the *in vitro* experiments, foal alveolar macrophages obtained from a BAL will be incubated with *R. equi* at a multiplicity of infection (MOI) of 10 for 45 min at 37 °C. These macrophages will serve as the target cells for opsonic killing by equine neutrophils in the presence of equine complement and the different IgG sub-isotyes. After incubating for 4h at 37 °C, the efficacy of the PNAG-specific antibodies mediating killing of *R. equi* can be evaluated by lysing the macrophages and enumerating bacteria on brain heart infusion (BHI) plates after 48h of incubation at 37 °C. The lower the bacterial count will correlate with the higher efficacy of the PNAG-specific antibody used in the assay. An alternative method is to use flow cytometry for bacterial enumeration. Here, *R. equi* could be labeled with FITC or modified to produce GFP before infection. After the opsonic assay, macrophages would be stained with Alexa Fluor 647 to detect viability. Cells could then be analyzed by flow cytometry and the total number of macrophage-associated bacteria could be estimated by total fluorescence and the intracellular bacteria could be calculated after quenching with trypan blue and subtracting from the total fluorescence. In addition, viability of intact macrophages and the number of intracellular and extracellular *R. equi* could be quantified.

In the *ex vivo* experiments, macrophages recovered from BAL from experimentally infected foals would act as the *R. equi*-infected target cells for recognition by anti-PNAG antibodies in the OPK assay. Bacterial enumeration would be determined through lysis of macrophages and plating of *R. equi* onto BHI plates. If the results from the *in vitro* experiments correspond with the *ex vivo* experiments, then we

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could correlate *R. equi* protection with anti-PNAG IgG$_1$ antibodies *in vivo* through the

new mechanism of PNAG recognition on the surface of macrophages by antibodies and

their destruction through opsonic killing by neutrophils.

Can alveolar macrophages be activated with anti-PNAG antibodies to kill intracellular

R. equi?

Above, we theorize that the *R. equi*-infected macrophage acts as a target cell for

opsonic killing by neutrophils. In this case, neutrophils seem to be the effector cells

responsible for directly killing *R. equi* and clearing infections. It would make sense that

macrophages would be the effector cell responsible for the control and elimination of

virulent *R. equi* infections, but to what extent are they actually responsible for active

elimination of *R. equi*? We have tried activating macrophages to kill intracellular

R. equi by pre-incubating macrophages before *R. equi* infection with PNAG

hyperimmune, *R. equi* hyperimmune, or nonimmune plasmas, and by opsonizing *R. equi*

with each of those plasmas as well as each of the anti-PNAG IgG sub-isotypes and non-

immune IgG sub-isotypes before infecting macrophages. These experiments failed to

indicate any influence of antibodies on enhancing the killing of *R. equi*. Although we

have been unsuccessful in showing antibodies to enhance killing of *R. equi* by

macrophages *in vitro*, we may be studying the wrong kind of macrophage. The initial

interaction of macrophages with soluble mediators, such as cytokines and antibodies,

determines the functional phenotype of the cells (228). Macrophages can be

differentiated to become type 1 macrophages (M1), which are linked with helper T

lymphocytes (Th) type 1 (Th1) responses or type 2 (M2) macrophages which are linked

with Th2 responses (228). While M1 cells rely on IFNγ to mediate killing of intracellular pathogens and production of reactive oxygen species, it is the M2 cells that rely on ligation of their receptors for the constant region of antibodies (Fc receptors, or FcRs) with IgG from immune complexes to produce a Th2 response which subsequently decreases cellular responsiveness to IFNγ and inhibits the synthesis of reactive oxygen species (228). Therefore, if *R. equi*-immunity in foals is attributable to deficient Th1-type responses and relies on an antibody-mediated responses, then the macrophage is probably not the major effector cell responsible for clearance of *R. equi* infections in foals (1). However, M1 cells may be indirectly activated by antibodies, because of the antibody-mediated IFNγ released by PBMCs of PNAG-protected foals we saw in our results. Furthermore, equine IgG$_1$ sub-isotype is associated with activation of Th1 CD4+ cells and IFNγ production, both of which play pivotal roles in stimulation of infected target cells and in activation of intracellular effector killing mechanisms (44, 46). It would be interesting to repeat our experiments of infecting macrophages with antibody-opsonized *R. equi* but this time, stimulating the macrophages with IFNγ and determining whether the macrophages in our assays are M1 or M2. This will likely benefit our understanding of the role(s) of macrophages in mediating protection against *R. equi* infections.

Can IFNγ produced by protected foals activate macrophages to kill intracellular
<u>*R. equi*</u>*?*

Maternal vaccination with PNAG and transfer of maternal antibodies to foals induced an antibody-dependent cellular release of IFNγ in response to *R. equi*

stimulation from PBMCs obtained from protected foals just prior to challenge. To show whether the IFNγ released by PBMCs of immune foals activates macrophages to kill intracellular *R. equi,* we could stimulate PBMCs of vaccinated foals with *R. equi* to release IFNγ into the supernatant, and incubate that supernatant with *R. equi*-infected macrophages and see if there is any increase in bacterial killing. It would also be important to study what other cell types PNAG antibodies could interact with other than lymphocytes, neutrophils, and macrophages as well as the mechanisms behind these interactions for a bigger picture of how the immune system orchestrates immunity against *R. equi.* To provide cellular immunity T-cell effectors must be activated by recognizing microbial antigens presented to them by cell surface proteins called major histocompatibility complex (MHC) found, for example, on antigen presenting cells (APCs) such as dendritic cells (DC), B-cells, or macrophages. This response can potentially be achieved with an antibodies by internalization of antigen-antibody immune complexes by FcR on APCs, enabling them to prime CD4+ T cells to produce IFNγ. In this way, vaccine-primed CD4+ Th1 cells (or CD8+ cells) can lead to vaccine-specific circulating T cells producing INFγ.

By what mechanism do anti-PNAG antibodies mediate protection against R. equi infections?

The mechanism by which IgG$_1$ mediates protection against *R. equi* is unknown. For example, we don't know if IgG$_1$ protects by altering host cell-pathogen interactions or by interacting with leukocyte receptors to control and eliminate intracellular *R. equi* infections. The specificity of antibodies will define its protection against intracellular

bacteria. In particular, the different sub-isotypes may vary in efficacy against intracellular infections. For example, 2 different IgG sub-isotypes can bind to a bacterial surface antigen with similar affinities but one may have higher affinity to FcγR and therefore be more protective than the other (109, 229). Furthermore, some IgG sub-isotypes can be more effective in complement activation than others or can even be associated with Th1 CD4+ T cells and IFNγ production (37, 38, 40, 67, 229). The interaction of IgG_1 with the FcR of a leukocyte, such as a macrophage infected with intracellular *R. equi*, could enable apoptosis and the destruction of intracellular virulent *R. equi*, by containing intracellular growth of *R. equi* and depriving the pathogen of its niche cell (230). Activation of FcR by antibodies through the uptake of IgG-opsonized pathogens has shown to be important in antibody-mediated elimination of intracellular pathogens by inducing a respiratory burst, which produces reactive oxygen intermediates, enables fusion with lysosomes, and increases production of pro-inflammatory cytokines (229, 230). The nature of the macrophage receptor recognition, signaling, inflammation, and antigen presentation pathways differ during different stages of infection and disease, and the virulence status of the infecting *R. equi* strain also contributes to this diversity of responses (230). Furthermore, formation of immune complexes and their binding to FcγR can trigger intracellular signaling pathways in macrophages, which are associated with microbicidal activity (27, 96, 138, 139). The interaction of the expression of different cell surface receptors and pathogen initiates a cascade of signaling events that result in the release of soluble (cytokines) and cell-associated antimicrobial and innate immune mediators. These signaling pathways

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related to the host's response to infection are unknown and depends on the site and stage of infection.

Final Remarks

Ultimately, understanding how antibodies influence the assortment of effector cells responsible for killing of *R. equi* and how they interact with each other will help us better understand how the PNAG vaccine mediates protection in foals. Furthermore, understanding the mechanisms by which these antibodies make the difference between resistance and susceptibility to *R. equi* will allow us to uncover the mysteries behind the virulence of *R. equi* and implement these discoveries on other intracellular pathogens.

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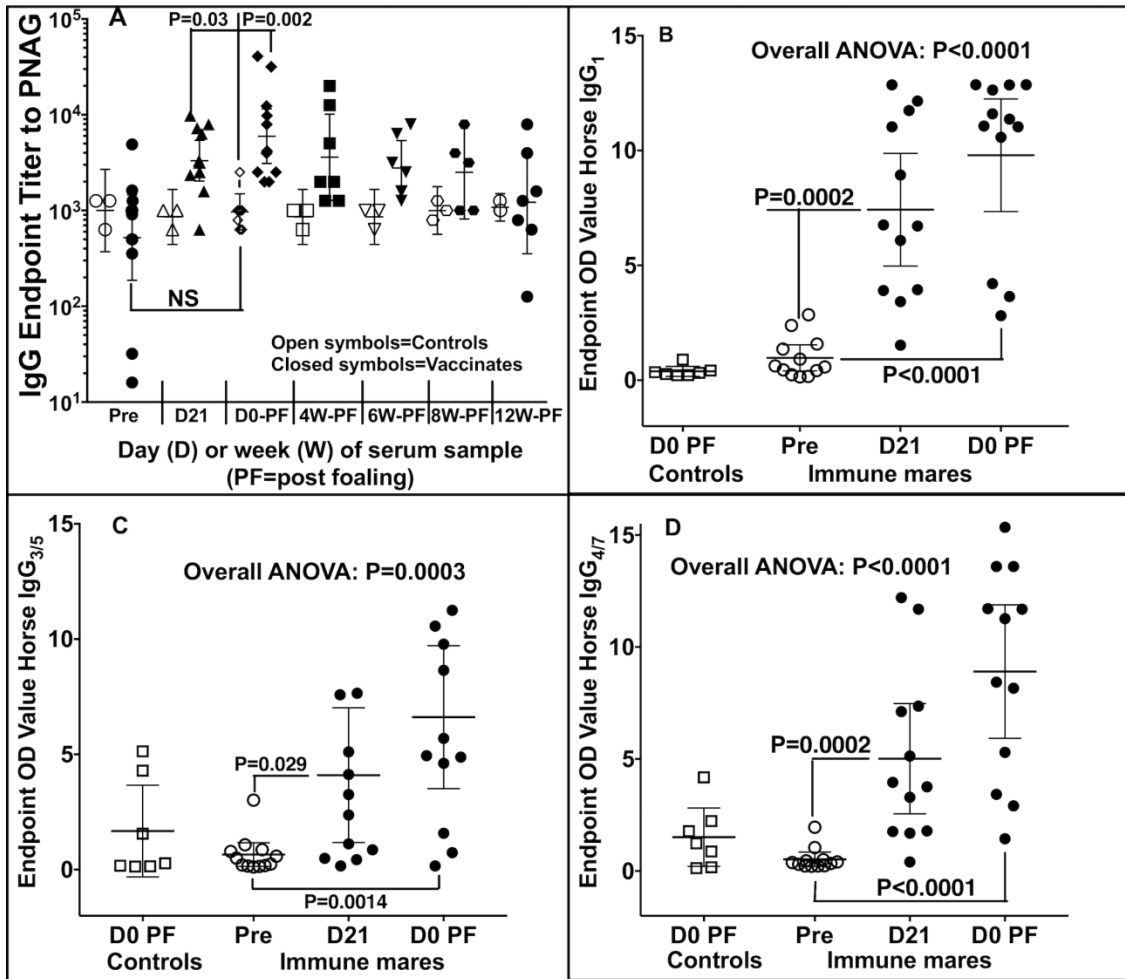
S1 Fig. Serum IgG and IgG subisotype titers to PNAG in immunized mare sera.
Serum end-point titers of IgG or IgG subisotypes are plotted by vaccine group as a function of
age in days. A: Total IgG antibody end-point titers to PNAG were significantly higher in sera of
immunized mares at D21 and D0 PF compared with titers in sera of control mares at D0 PF.
There was no significant (NS) difference in the IgG titers of the vaccinated mares at pre-
immunization and controls at D0 PF. B-D: Concentrations of IgG1, $IgG_{4/7}$, and $IgG_{3/5}$ were
significantly higher in mares in the vaccinated group at D21 and D0 PF as indicated on the
figure. Statistical comparisons were made by linear mixed-effects regression with
exchangeable correlation structure, using the mare as random effect (to account for
repeated measures) and multiple comparisons determined by the method of Sidak.
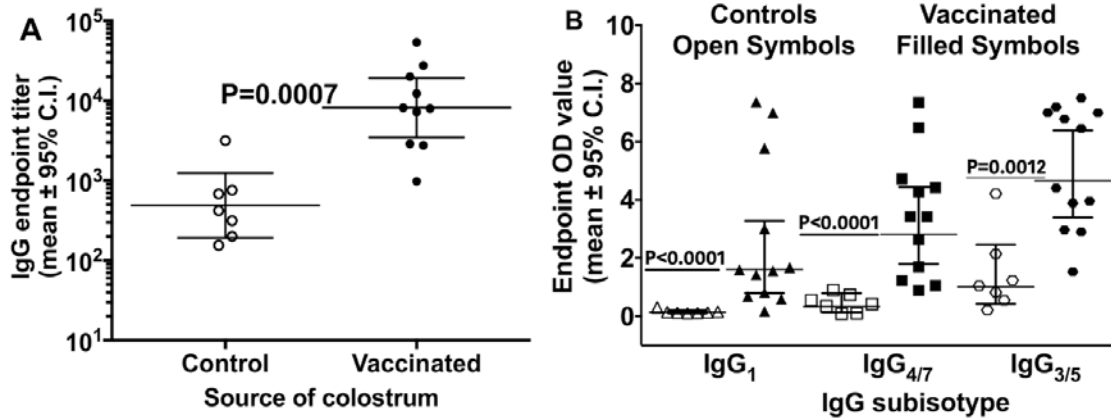
S2 Fig. **IgG and IgG subisotype titers in mare colostra on day of foaling.**
End-point colostral titers of IgG or IgG subisotype. End-point values are plotted by vaccine group. A: Total IgG antibody end-point titers to PNAG were significantly higher in colostra of vaccinated mares (N = 10, 2 samples not tested due to limited quantities) compared with colostra of control mares N = 7). B: Concentrations of IgG1, $IgG_{4/7}$, and $IgG_{3/5}$ were significantly higher in colostra of mares in the vaccinated group (N = 12). Statistical comparisons were made by the Wilcoxon rank-sum test.
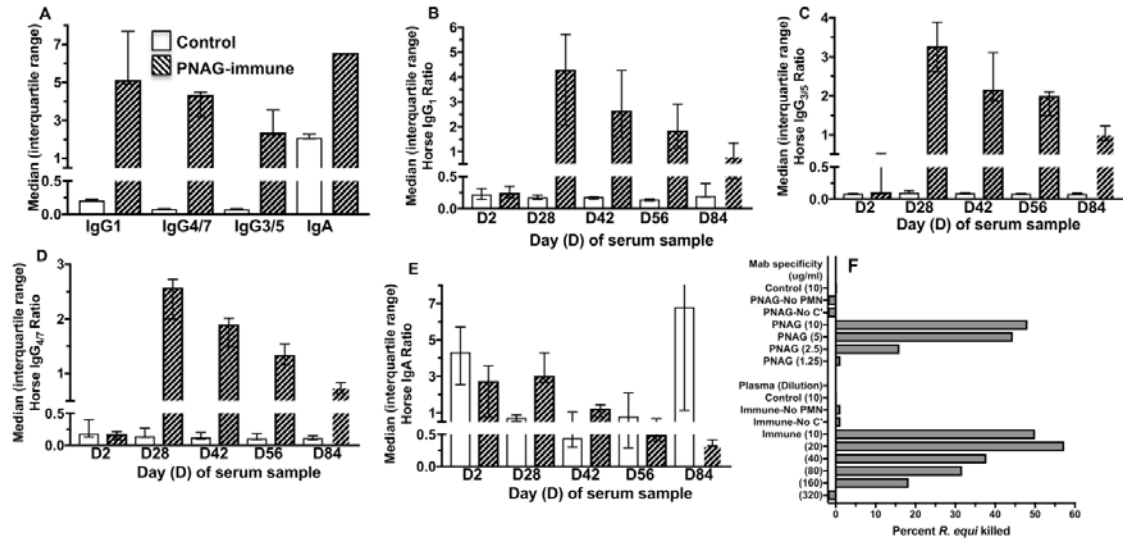
S3 Fig. Antibody titers to PNAG in control and hyperimmune horse plasma infused into newborn foals on day 1 of life.

A: Titers of IgG subisotypes and IgA in control (open bars) and PNAG-immune (hatched bars) plasmas. **B**: horse IgG_1, **C**: horse, $IgG_{3/5}$, **D**: horse $IgG_{4/7}$, or **E:** horse IgA in sera at day indicated on X-axis. Bars represent medians, error bars the interquartile ranges. OD ratios of IgG_1, $IgG_{4/7}$, and $IgG_{3/5}$ did not differ significantly over time in controls but were significantly ($P < 0.05$) greater than age 2 days in foals transfused with anti-PNAG plasma at ages 28, 42, and 56 days (IgG_1), or ages 28, 42, 56, and 84 for $IgG_{4/7}$ and $IgG_{3/5}$. OD ratio of IgA did not differ significantly ($P > 0.05$) among the different days for anti-PNAG-transfused foals, but controls had significantly ($P < 0.05$) higher IgA titers at age 2 days compared to control titers on days 28, 42, and 56. The OD ratio values for control foals' IgA on day 84 was significantly ($P < 0.05$) greater than those of control foals on days 28, 42, and 56. IgA titers between controls and anti-PNAG-transfused foals differed significantly ($P < 0.05$) at day 84 only. All P values were determined by linear mixed-effects regression. **F**: Opsonic killing of *R. equi* EIDL 990 by antibody in control or immune plasma. Monoclonal antibodies (MAb) were used as controls, as were tubes lacking PMN or complement (C') as indicated. Bars represent means of technical replicates.

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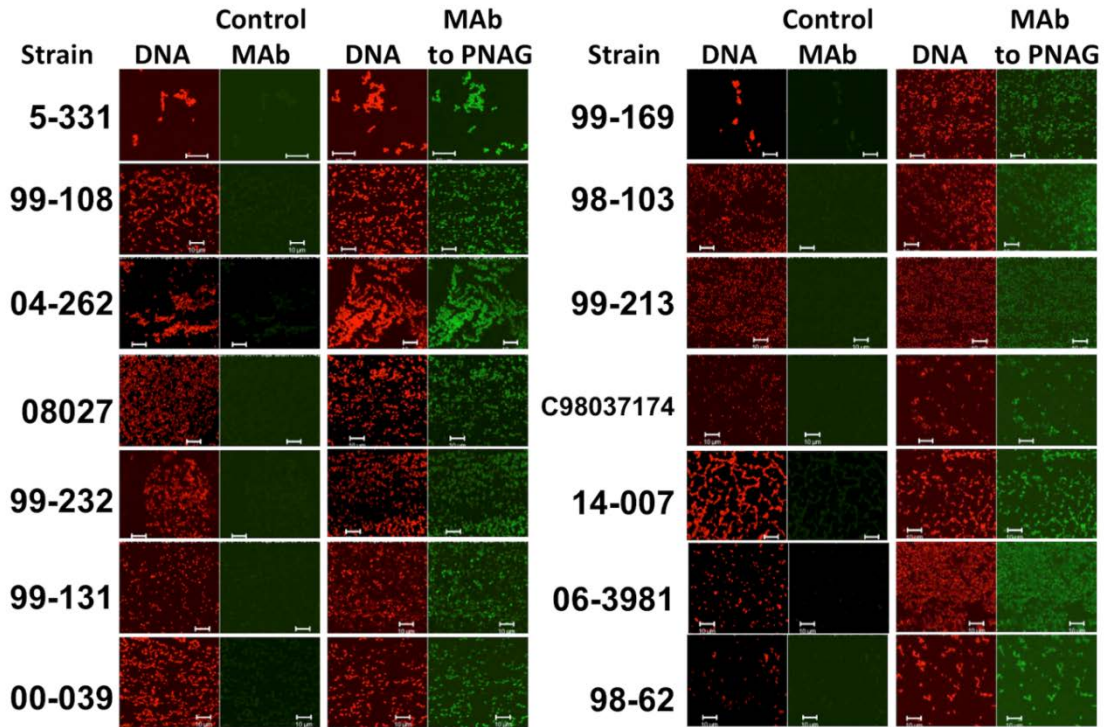
S4 Fig. PNAG expression by *R. equi* clinical isolates.
Designated individual clinical isolates of *R. equi* were reacted with either control MAb F429 to
P. aeruginosa alginate or MAb F598 to PNAG, both directly conjugated to Alexa Fluor 488.
Binding to PNAG on bacterial cells was visualized by immunofluorescence microscopy. Left-
hand panel in each pair shows DNA visualized by red-fluorophore Syto 83. Right-hand panel in
each pair is green if PNAG detected by MAb F598.
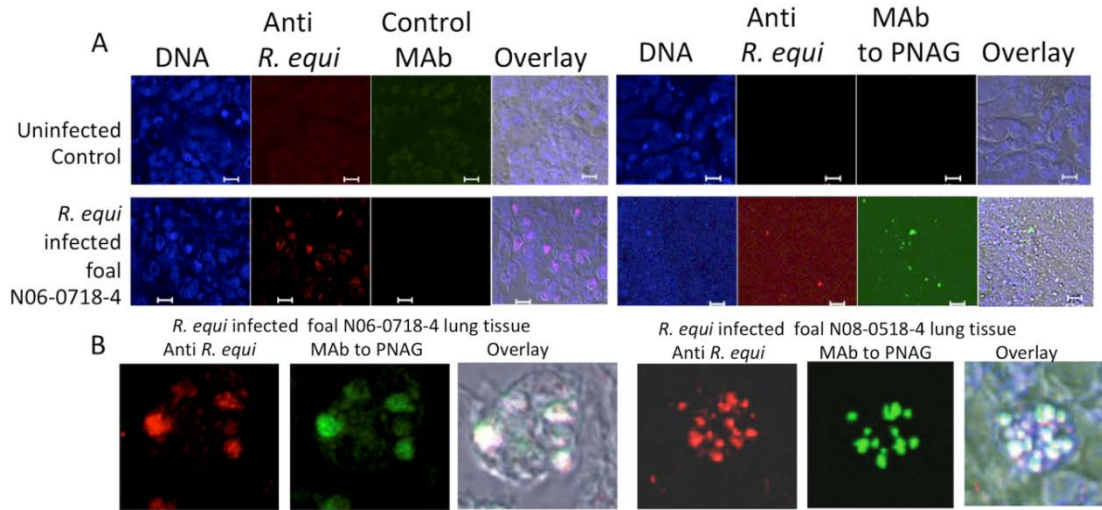
S5 Fig. Expression of PNAG in lungs of *R. equi* infected foals.
Either an uninfected control lung or lungs from foals with *R. equi* pneumonia were reacted with
the indicated antibody to detect the presence of *R. equi* (red, antibody to VapA protein), PNAG
(Green, MAb F598) or control MAb F429 to alginate. A: Low power (40X) sections indicating
presence of *R. equi* and closely associated PNAG in infected lung. Bars = 10 μm. B: Higher
magnification (60 X) shows individual infected cells in 2 different foal lung sections with
PNAG-expressing *R. equi* contained in apparent intracellular vesicles.
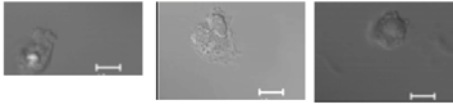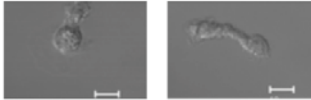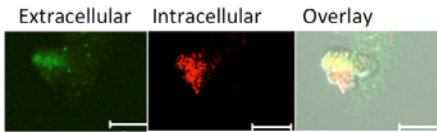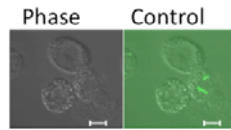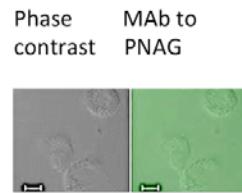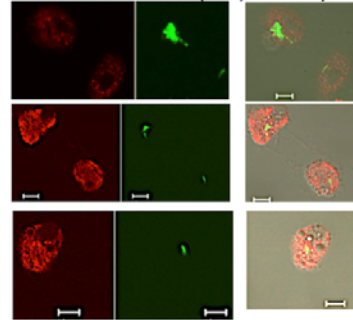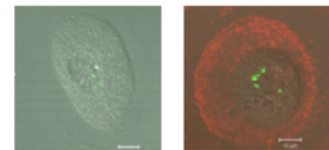
S6 Fig. Surface and intracellular expression of PNAG in infected human MDM.
Detection of PNAG either on the surface or within the infected cell was determined by first
reacting cultures with MAb F598 to PNAG or control MAb F429, both directly conjugated to
Alexa Fluor 488 (green fluorophore), on paraformaldehyde-fixed cells then washing and
permeabilizing the cells with ice-cold methanol followed by reaction with the MAbs and
secondary antibody to human IgG conjugated to Alexa Fluor 555 (red/orange). A-G: Cells and
treatments indicated in figure. White bars = 10 μm.
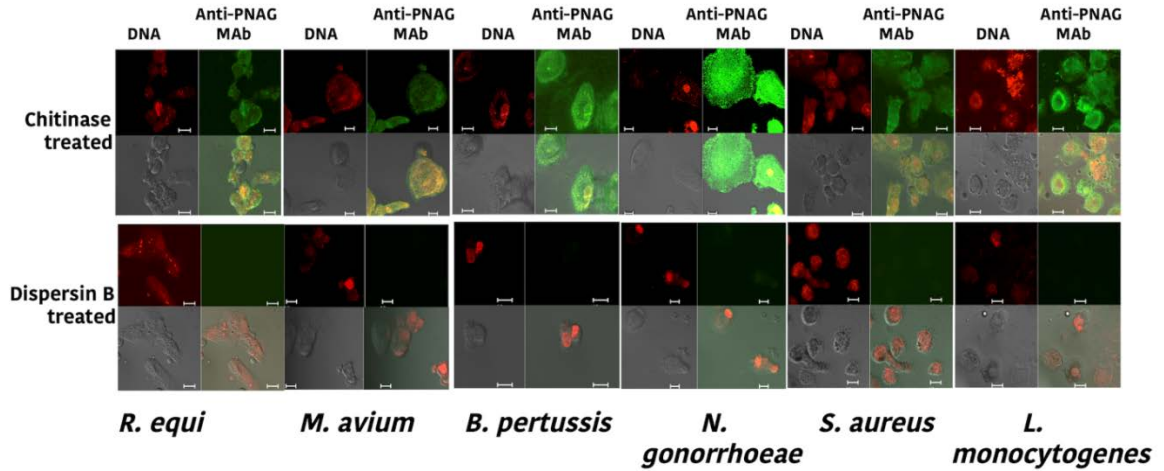
S7 Fig. Human MDM cells infected with PNAG-expressing intracellular pathogens have high levels of the PNAG antigen on their surface that is removed by treatment with Dispersin B.

PNAG on infected cell surfaces was detected by reacting cultures with MAb F598 to PNAG or control MAb F429, both directly conjugated to Alexa Fluor 488 (green fluorophore), on paraformaldehyde-fixed cells. Infected bacterial strains and treatments indicated in figure. For each figure, upper left quadrant shows nuclear DNA (red), upper right quadrant shows PNAG (green if present), lower left quadrant shows phase contrast, lower right quadrant shows co-localization of DNA and PNAG (yellow-green to yellow to orange if present). White bars = 10 μm.
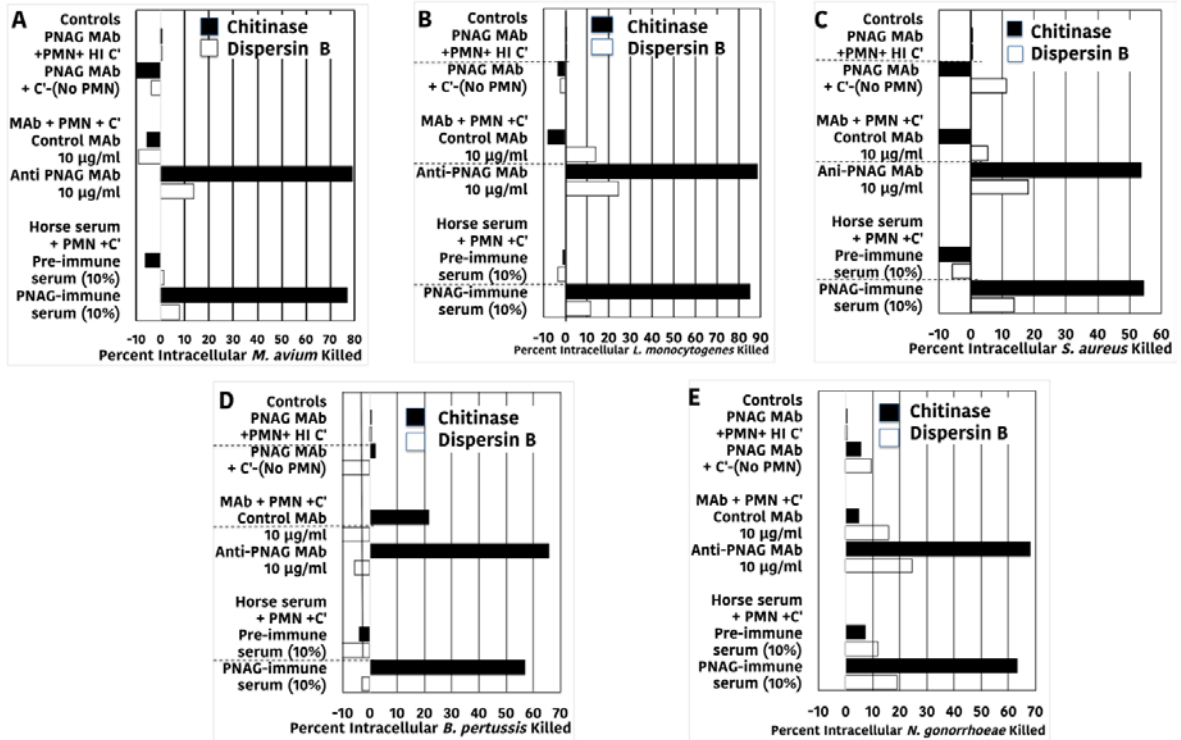
**S8 Fig. Opsonic killing of intracellular pathogens by antibody to PNAG, complement (C')
and PMN depends on infected-cell surface expression of PNAG**

A-E: Killing of intracellular bacteria by antibody, PMN and C' was markedly reduced following
treatment of infected cells with Dispersin B (open bars) to digest surface PNAG compared with
treatment with the control enzyme, Chitinase (black bars). Depicted data are representative of 2-
3 independent experiments. Bars represent means of 6 technical replicates. Bars showing <0%
kill represent data wherein the cfu counts were greater than the control of PNAG MAb + PMN +
HI C'.
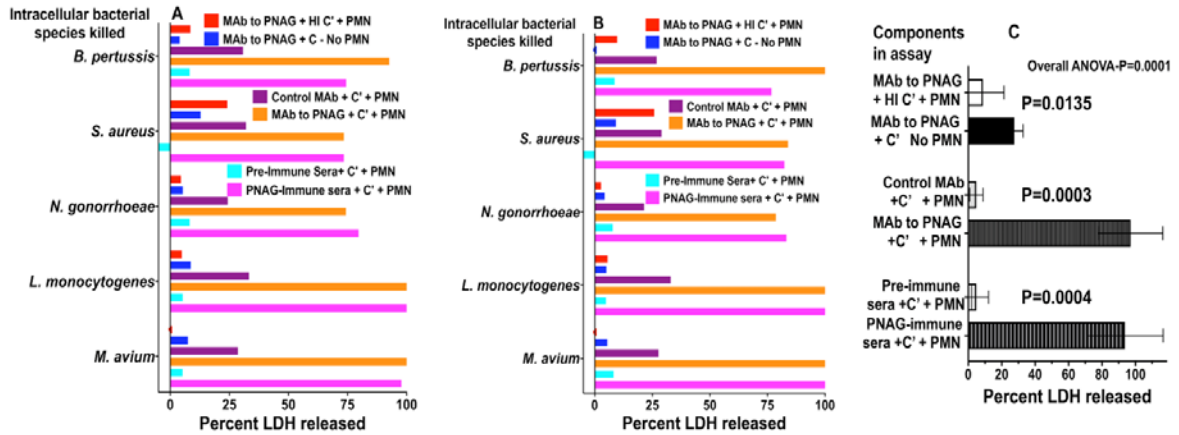
S9 Fig. Release of LDH from cells infected with intracellular pathogens following antibody plus immune effector treatment.
A and **B**: Replicate experiments measuring release of LDH from cells infected with indicated pathogen treated with 10 μg/ml control or anti-PNAG monoclonal or 10% polyclonal antibody plus indicated immune effector. Bars indicate means of quadruplicates. C: Summary of LDH release for experiment in figure S9B. Mean (bars) and 95% C.I. (error bars) indicate release of LDH from cells infected with all five intracellular pathogens. Overall ANOVA P value by one-way repeated measures ANOVA, pair wise comparisons determined by two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli.