Antibody-based vaccine for TB: pre-clinical validation in horse foals challenged with the TB-related pathogen *Rhodococcus equi*

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Abstract:

Immune correlates for protection against *Mycobacterium tuberculosi*s (Mtb) infection and other intracellular pathogens are largely undetermined. Whether there is a role for antibody-mediated immunity is controversial. *Rhodococcus equi* is an intracellular pathogen causing severe pneumonia in young horse foals, eliciting a disease with many similarities to TB including intracellular residence, formation of granulomas and induction of severe respiratory distress. No purified vaccine antigens exist for *R. equi* or Mtb infections. Both express the microbial surface polysaccharide antigen poly-*N*-acetyl glucosamine (PNAG). Vaccination of pregnant mares with a synthetic PNAG oligosaccharide conjugated to tetanus toxoid elicited antibody that transferred to foals via colostrum and provided nearly complete protection against *R. equi* pneumonia in a randomized, controlled, blinded challenge trial. Infusion of PNAG-hyperimmune plasma protected 100% of foals against *R. equi* pneumonia. Vaccination induced opsonic antibodies that killed extracellular and intracellular *R. equi* and other intracellular pathogens. Killing of intracellular organisms was dependent on antibody recognition of surface expression of PNAG on infected macrophages, complement deposition and PMN-assisted lysis of infected macrophages. Protection also correlated with PBMC release of interferon-γ in response to PNAG. Antibody-mediated opsonic killing and interferon-γ release in response to PNAG may protect against disease caused by intracellular bacterial pathogens.

40 **INTRODUCTION**

41 *Mycobacterium tuberculosis* (Mtb) infection is now considered the greatest cause of
42 serious worldwide infection and associated death from a single microbe, with a clear
43 major need for new prophylactic and therapeutic interventions (*1*). Correlates of human
44 cellular and humoral immunity to Mtb capable of informing vaccine development are
45 unknown, and protection studies to date, primarily in laboratory rodents and non-human
46 primates, have not led to an effective human vaccine (*2, 3*) outside of the limited
47 efficacy of the Bacillus Calmette-Guerin whole-cell vaccine (*3-5*). *Rhodococcus equi* is a
48 Gram-positive, facultative intracellular pathogen that primarily infects alveolar
49 macrophages of foals following inhalation, resulting in a granulomatous pneumonia that
50 is pathologically similar to that caused by Mtb in humans (*6*). Like Mtb, *R. equi* also
51 causes extrapulmonary disorders including osseous and intra-abdominal lymphadenitis
52 (*6-8*). The disease is of considerable importance to the equine industry (*6, 8*), and has
53 relevance to human health both as a cause of granulomatous pneumonia (*9*), and as a
54 platform for studying Mtb infections, due to the similar pathological and clinical findings
55 (*6-8, 10*). Additionally, both Mtb and *R. equi* synthesize the conserved surface capsule-
56 like polysaccharide, poly-*N*-acetyl glucosamine (PNAG), a target for the development of
57 a broadly-protective vaccine against many pathogens (*11, 12*). These findings indicate
58 that a study evaluating vaccination against PNAG for induction of protective immunity to
59 *R. equi* challenge in foals could provide critical information and support for testing a
60 PNAG vaccine or PNAG-specific monoclonal antibody against human Mtb infections.

61 To justify the premises underlying a *R. equi* vaccine study, multiple considerations
62 regarding immunity to intracellular pathogens, pathogenesis of Mtb and *R. equi*

63 infections, and properties of natural and vaccine-induced immunity to PNAG had to be
64 taken into account. Many investigators consider effective immunologic control of these
65 intracellular pathogens to be primarily based on cell-mediated immune (CMI) responses
66 (*13*), a finding clearly in evidence for *R. equi*. Disease occurs almost exclusively in foals
67 less than 6 months of age, but by ~ 9 months of age most young horses become highly
68 resistant to this pathogen (*6, 8*). This acquired natural resistance is obviously not
69 antibody-mediated inasmuch as the solid immunity to infection in healthy horses > 9
70 months of age, which obviously includes pregnant mares, is not transferred to
71 susceptible foals via antibody in the colostrum. Colostrum is the only source of
72 maternal antibody in foals and the offspring of other animals producing an
73 epitheliochorial placenta. Human development of TB often correlates with defective
74 CMI (*1, 14*), particularly in those infected with the human immunodeficiency virus (*14,*
75 *15*). Nonetheless, considerable evidence has emerged to indicate that antibodies to Mtb
76 have the potential to be major mediators of protective immunity (*16-20*). Both Mtb and
77 *R. equi* survive within alveolar macrophages and induce granulomas, supporting the
78 use of *R. equi* as a relevant model for TB pathogenesis, immunity, and vaccine
79 development.
80 In regards to targeting the conserved PNAG surface polysaccharide, the production
81 of this antigen by many microbes induces natural IgG antibody in most humans and
82 animals (*21, 22*), but natural antibody is generally ineffective at eliciting protection
83 against infection. These antibodies do not activate the complement pathway and cannot
84 mediate microbial killing (*21-23*). By removing most of the acetate substituents from the
85 *N*-acetyl glucosamine sugars comprising PNAG (*24, 25*), or using synthetic

86 oligosaccharides composed of only β-1→6-linked glucosamine conjugated to a carrier
87 protein such as tetanus toxoid (TT), (*11, 23, 26, 27*) complement-fixing, microbial-killing,
88 and protective antibody to PNAG can be induced. A final premise justifying evaluation
89 of vaccine-induced immunity to *R. equi* by immunizing pregnant mares is that foals are
90 considered to be infected soon after birth (*28*) when they are more susceptible to
91 infection (*29*) and when their immune system is less effective in responding to vaccines,
92 (*30-33*) which precludes active immunization of very young foals as a strategy for
93 vaccine evaluation against *R. equi*.
94 Therefore, in order to ascertain if *R. equi* pneumonia could be prevented by
95 antibody to PNAG, pregnant mares were vaccinated with the 5GlcNH$_2$-TT vaccine, the
96 transfer of functional opsonic antibodies via colostrum to foals verified, and foals were
97 then challenged at 25-28 days of life with virulent *R. equi.* This approach protected 11 of
98 12 (92%) foals born to immunized mares against essentially all signs of clinical
99 pneumonia, whereas 6 of 7 (86%) foals born to non-immune, control mares developed
100 *R. equi* pneumonia. A follow-up study in which 2 liters (approximately 40 ml/kg) of
101 hyperimmune plasma from adult horses immunized with the 5GlcNH$_2$-TT vaccine were
102 infused into newborn foals on day 1 of life similarly protected 5 of 5 (100%) of the
103 recipients from *R. equi* pneumonia following challenge at 4 weeks of life whereas
104 recipients of control plasma all developed clinical signs of pneumonia (4/4). *In vitro*
105 correlates of immunity were further investigated, and a novel mechanism of killing of
106 intracellular *R. equi* and other intracellular bacterial pathogens was identified, wherein
107 intracellular bacterial growth resulted in high levels of surface-expressed PNAG
108 intercalated into the plasma membrane of infected host cells, which served as a target

109 for the complement-fixing antibody to PNAG, which, along with added
110 polymorphonuclear neutrophils (PMNs), lysed the infected cell and released the
111 intracellular organisms that were further opsonized and killed in this setting. The results
112 establish a solid basis for evaluating vaccination or MAb therapy against PNAG for
113 prevention or treatment of human TB and provide a clear immunologic rationale for
114 antibody-mediated protection against intracellular pathogens.

115 **RESULTS**

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

118 Mares were immunized twice approximately 6 and 3 weeks prior to their estimated date
119 of parturition (based on last known breeding date) with 125 or 200 µg of the 5GlcNH$_2$
120 vaccine conjugated to TT (AV0328 from Alopexx Vaccine, LLC) adjuvanted with 100 µl
121 of Specol. Immunization of mares resulted in no detectable local or systemic reaction
122 following either 1 or 2 vaccine doses except for a slightly swollen muscle 24 h after the
123 first vaccination followed at day 2 by a small dependent edema that resolved by day 3 in
124 a single mare. Serum samples from mares immunized in 2015 were only collected on
125 the day of foaling, so statistical comparisons with immunized mare titers were only
126 made between all 7 control samples collected on the day of foaling (D0 post-foaling
127 (PF)) with 12 vaccinated samples collected pre-immunization, on day 21 prior to the
128 booster dose, and on D0 PF (Fig. S1). When compared with IgG titers to PNAG in non-
129 immune controls obtained on D0 PF, immunization of mares gave rise to significant ($P <$
130 0.05) increases in total IgG to PNAG as well as the equine IgG subisotypes IgG$_1$, IgG$_{3/5}$,
131 and IgG$_{4/7}$ in serum (Fig. S1) on day 21 after a single immunization, as well as on D0

132 PF, representing the serum sample obtained after the booster immunization. Similarly,
133 total IgG and IgG subisotype titers were significantly higher in the colostrum obtained on
134 the day of foaling from vaccinated mares compared with controls (Fig. S2). Notably,
135 non-immunized mares had antibody titers to PNAG, representative of the natural
136 response to this antigen commonly seen in normal animal and human sera.

137 Successful oral delivery of antibody to the blood of foals born to vaccinated mares
138 (hereafter termed vaccinated foals) was shown by the significantly higher titers of serum
139 IgG to PNAG compared with foals from control mares at ages 2, 28, and 56 days, but
140 not 84 days (Fig. 1A). Foal serum concentrations of subisotypes IgG_1, $IgG_{3/5}$, and $IgG_{4/7}$
141 to PNAG were significantly higher at 2, 28, and 42 days of age (after colostral transfer)
142 in the vaccinated group compared with the control group, and subisotype IgG_1 titers
143 remained significantly higher through age 56 days (Figs. 1B-1D). The pattern in
144 vaccinated foals of decreasing titers to PNAG with increasing age was consistent with
145 the decay of maternally-transferred immunoglobulins.

146 **Orally obtained colostral antibody to PNAG protects foals against**
147 **intrabronchial infection with *R. equi***

148 Protection studies were undertaken using a randomized, controlled, blinded
149 experimental trial design. At days 25-28 of life, foals in the study were challenged with
150 1 X 10^6 CFU of live *R. equi* contained in 40 ml of vehicle, with half of the challenge
151 delivered to each lung by intrabronchial dosing with 20 ml. Foals were followed for
152 development of clinical *R. equi* pneumonia (Table S1) for 8 weeks. The proportion of
153 vaccinated foals that developed *R. equi* pneumonia (8%; 1/12) was significantly (P =
154 0.0017; Fisher's exact test) less than that of unvaccinated control foals (86%; 6/7),

155 representing a relative risk reduction or protected fraction of 84% (95% C.I. 42% to
156 97%, Koopman asymptotic score analysis (*34*)). The duration of clinical signs indicative
157 of *R. equi* pneumonia was significantly ($P \leq 0.027$, Wilcoxon rank-sum tests) longer for
158 foals from control than vaccinated mares (Table S2). Thoracic ultrasonographic
159 examination is the standard clinical technique for monitoring areas of pulmonary
160 abscessation or consolidation attributed to *R. equi* infection. The severity and duration
161 of ultrasonographic lesions were significantly greater in foals born to controls than
162 vaccinated mares (Fig. 2). Vaccinated foals that were protected against pneumonia had
163 less severe clinical signs and smaller and fewer ultrasonographic lesions compared with
164 control foals. Thus, maternal vaccination against PNAG demonstrated successful
165 protection against clinical *R. equi* pneumonia, a disease for which there is no current
166 vaccine, using a randomized, blinded experimental challenge model.

167 **Passive infusion with hyperimmune plasma to PNAG protects foals against *R.***
168 ***equi* pneumonia**

169 To substantiate that vaccination-mediated protection was attributable to antibody
170 to PNAG, hyperimmune plasma was prepared from the blood of 5GlcNH$_2$-TT-
171 immunized adult horses and 2 L (approximately 40 ml/kg) infused into 5 foals at 18-24
172 hours of age. Four controls were transfused at the same age with 2 L of standard
173 commercial horse plasma. Titers of control and hyperimmune plasma IgG subisotypes
174 and IgA antibody to PNAG and OPK activity against *R. equi* (Fig. S3) documented
175 significantly higher titers of functional antibody to PNAG in the plasma from vaccinated
176 donors and in foals transfused with the plasma from vaccinated donors compared to
177 foals transfused with standard plasma. After challenge with *R. equi* as described above,

178 there was a significant reduction in clinical signs in the foals receiving PNAG-
179 hyperimmune plasma, compared to controls, except for the duration of ultrasound
180 lesions (Table S3). None of the 5 foals receiving PNAG-hyperimmune plasma were
181 diagnosed with *R. equi* pneumonia, whereas 4 of 4 recipients of normal plasma had a
182 diagnosis of clinical pneumonia for at least 1 day (P=0.0079, Fisher's exact test; relative
183 risk reduction or protected fraction 100%, 95% C.I. 51%-100%, Koopman asymptotic
184 score (*34*)).

185 ***R. equi* expression of PNAG *in vitro* and *in vivo***

186 Using immunofluorescence microscopy, we demonstrated that 100% of 14 virulent
187 strains of *R. equi* tested express PNAG (Fig. S4). Moreover, we found that PNAG was
188 expressed in the lungs of foals naturally infected with *R. equi* (Fig. S5a), similar to our
189 prior demonstration of PNAG expression in the lung of a human infected with Mtb (*11*).
190 PNAG was detected within apparent vacuoles inside *R. equi*-infected horse
191 macrophages *in vivo* (Fig. S5b).

192 **PNAG vaccine-induced opsonic antibodies mediate killing of both extracellular**
193 **and intracellular *R. equi***

194 Testing of the functional activity of the antibodies induced in the pregnant mares and in
195 foal sera on the day of challenge demonstrated the antibodies could fix equine
196 complement component C1q onto the PNAG antigen (Fig. 3A). Notably, the natural
197 antibody to PNAG in sera of non-vaccinated, control mares and their foals did not
198 deposit C1q onto the PNAG antigen, consistent with prior findings that natural
199 antibodies are immunologically inert in these assays (*21, 22, 35*). Sera from vaccinated
200 foals on the day of *R. equi* infection mediated high levels of opsonic killing of

201 extracellular *R. equi* whereas control foals with only natural maternal antibody to PNAG
202 had no killing activity (Fig. 3B), again demonstrating the lack of functional activity of
203 these natural antibodies to PNAG.
204 As some of the vaccinated foals developed small subclinical lung lesions that
205 resolved rapidly (Table S1, Fig. 2) it appeared the bolus challenge did lead to some
206 uptake of *R. equi* by alveolar macrophages but without development of detectable
207 clinical signs of disease. This observation suggested that antibody to PNAG led to
208 resolution of these lesions and prevented the emergence of clinical disease. Based on
209 the finding that *R. equi*-infected foal lung cells expressed PNAG *in vivo* (Fig. S5), we
210 determined if macrophages infected with *R. equi in vitro* similarly expressed PNAG, and
211 also determined if this antigen was on the infected cell surface, intracellular, or both. We
212 infected cultured human monocyte-derived macrophages (MDM) for 30 min with live *R.*
213 *equi* then cultured them overnight in antibiotics to prevent extracellular bacterial
214 survival. To detect PNAG on the infected cell surface we used the human IgG1 MAb to
215 PNAG (MAb F598) conjugated to the green fluorophore Alexa Fluor 488. To detect
216 intracellular PNAG, we next permeabilized the cells with ice-cold methanol and added
217 either unlabeled MAb F598 or control MAb, F429 (*36*) followed by donkey anti-human
218 IgG conjugated to Alex Fluor 555 (red color). These experiments showed there was no
219 binding of the MAb to uninfected cells (Fig. S6A) nor binding of the control MAb to
220 infected cells (Fig. S6B). However, we found strong expression of PNAG both on the
221 infected MDM surface and within infected cells (Fig. S6C). Similarly, using a GFP-
222 labeled Mtb strain (Figs. S6 D-E) and a GFP-labeled strain of *Listeria monocytogenes*
223 (Fig. S6F) we also visualized intense surface expression of PNAG on infected human

224 MDMs in culture, even when the bacterial burden in the infected cell was apparently
225 low. Importantly, within infected cultures, only cells with internalized bacteria had PNAG
226 on their surface (Fig. S6G), indicating the antigen originated from the intracellular
227 bacteria. Thus, uninfected cells did not obtain PNAG from shed antigen or lysed
228 infected cells. This finding is consistent with published reports of intracellular bacterial
229 release of surface vesicles that are transported among different compartments of an
230 infected host cell (*37*).

231 Next, we examined if the surface PNAG on infected cells provided the antigenic
232 target needed by antibody to both identify infected cells and, along with complement
233 and PMN, lyse the cells, release the intracellular microbes, and kill them by classic
234 opsonic killing. Human MDM cultures were established *in vitro*, infected for 30 min with
235 live *R. equi*, and then cells were washed and incubated for 24 h in the presence of 100
236 µg gentamicin/ml to kill extracellular bacteria and allow for intracellular bacterial growth.
237 Then, various combinations of the human IgG_1 MAb to PNAG or the control MAb F429
238 along with human complement and human PMN were added to the cultures, and viable
239 *R. equi* determined after 90 min. While a low level of killing (≤30%) of intracellular *R.*
240 *equi* was obtained with PMN and complement in the presence of the control MAb, there
241 was a high level of killing of the intracellular *R. equi* when the full compendium of
242 immune effectors encompassing MAb to PNAG, complement, and PMN were present
243 (Fig. 4A). Similarly, testing of sera from vaccinated foals on the day of challenge,
244 representing animals with a low, medium, or high titers of IgG to PNAG, showed they
245 also mediated titer-dependent killing of intracellular *R. equi* (Fig. 4B). Measurement of
246 the release of lactate dehydrogenase as an indicator of lysis of the macrophages

247 showed that the combination of antibody to PNAG, complement, and PMN mediated
248 lysis of the infected human cells (Fig. 4C), presumably releasing the intracellular
249 bacteria for further opsonic killing.

250 PNAG can be digested with the enzyme dispersin B that specifically recognizes
251 the β-1→6-linked *N*-acetyl glucosamine residues (*38, 39*) but is unaffected by chitinase,
252 which degrades the β-1→4-linked *N*-acetyl glucosamines in chitin. Thus, we treated
253 human macrophages infected for 24 h with *R. equi* with either dispersin B or chitinase to
254 determine if the presence of surface PNAG was critical for killing of intracellular
255 bacteria. Dispersin B treatment markedly reduced the presence of PNAG on the
256 infected cell surface (fig. S7) as well as killing of intracellular *R. equi* by antibody,
257 complement, and PMN (Fig. 4D). Chitinase treatment had no effect on PNAG
258 expression (fig. S7) or killing, indicating a critical role for PNAG intercalated into the
259 macrophage membrane for antibody-mediated killing of intracellular *R. equi.*

260 **Antibody to PNAG mediates intracellular killing of other intracellular**
261 **pathogens**

262 To show that antibody to PNAG, complement, and PMN represent a general
263 mechanism for killing of disparate intracellular pathogenic bacteria that express PNAG,
264 we used the above-described system of infected human macrophages to test killing of
265 *M. avium, S. aureus, Neisseria gonorrhoeae*, *L. monocytogenes* and *Bordetella*
266 *pertussis* by the human MAb to PNAG or horse serum from a foal protected from *R.*
267 *equi* pneumonia. Human MDM infected with these organisms expressed PNAG on the
268 surface that was not detectable after treatment with dispersin B (fig. S7). When present
269 intracellularly, all of these organisms were killed in the presence of MAb to PNAG or

270 anti-PNAG immune horse serum, complement, and PMN following treatment of the
271 infected cells with the control enzyme, chitinase, but killing was markedly reduced in
272 infected cells treated with dispersin B (Figs. 5A and S8). As with *R. equi*, maximal lysis
273 of infected cells occurred when antibody to PNAG plus complement and PMN were
274 present (Fig. 5B and fig. S9), although when analyzing data from all 5 of these
275 experiments combined there was a modest but significant release of LDH release with
276 antibody to PNAG and complement alone (Fig. 5B and fig. S9).

277 **Maternal PNAG vaccination and antibody transfer to foals enhances *in vitro***
278 **cell-mediated immune responses against *R. equi***

279 Cell-mediated immune (CMI) responses in vaccinated and unvaccinated, control foals
280 were assessed by detecting production of IFN-γ from peripheral blood mononuclear
281 cells (PBMC) stimulated with a lysate of virulent *R. equi*. IFN-γ production at 2 days of
282 age was significantly ($P < 0.05$; linear mixed-effects modeling) lower than levels at all
283 other days for both the control and vaccinated groups (Fig. 6A, P value not on graph).
284 There was no difference in IFN-γ production between vaccinated and control foals at
285 day 2 of age. Vaccinated foals had significantly higher (~10-fold) production of IFN-γ in
286 response to *R. equi* stimulation (Fig. 6A) from cells obtained just prior to challenge on
287 days 25-28 of life compared to unvaccinated controls. Subsequent to infection, the
288 controls likely made a CMI response to *R equi*, removing differences days between
289 vaccinates and controls in PBMC IFN-γ production by the age of 56.

290 To substantiate the specificity of this CMI reaction, we demonstrated that stimulation
291 of horse PBMC from vaccinated foals with an *R. equi* lysate treated with the enzyme
292 dispersin B diminished IFN-γ responses by ~90% (Fig. 6B). We also made a *post hoc*

293 comparison of CMI responses between foals that remained healthy and foals that
294 developed pneumonia. In this analysis (Fig. 6C), foals that remained healthy (11
295 vaccinates and 1 control) had significantly ($P < 0.05$; linear mixed-effects modeling)
296 higher CMI responses at all ages, including age 2 days, than foals that became ill (1
297 vaccinate and 6 controls), suggesting that both innate and acquired cellular immunity
298 contribute to resistance to *R. equi* pneumonia. Overall, it appears the maternally
299 derived antibody to PNAG sensitizes foal PBMC to recognize the PNAG antigen and
300 release IFN-γ, which is a known effector of immunity to intracellular pathogens.
301

302 **Discussion**

303 In this study we showed maternal immunization against the deacetylated glycoform of
304 the conserved microbial surface polysaccharide PNAG induced antibodies that
305 protected 11 of 12 (91%) ~4-week-old foals from challenge with live, virulent *R. equi*.
306 The attack rate in the controls was 86% (6/7). A confirmatory passive transfer study
307 similarly showed antibody to PNAG infused into foal blood on the day of birth protected
308 5/5 (100%) of recipients against *R. equi* pneumonia following challenge at 4 weeks of
309 life whereas 100% of 4 controls were diagnosed with *R. equi* pneumonia after
310 challenge. Between the colostral and the infusion passive transfer experiments, a total
311 of 16 of 17 foals with vaccine-induced antibody to PNAG were protected against all
312 evidence of clinical pneumonia (Table S1) due to *R. equi* whereas 10 of 11 non-immune
313 controls had a clinical diagnosis of pneumonia ($P < 0.0001$, Fisher's exact test;
314 protected fraction 90%, (95% C.I. 59%-98%, Koopman asymptotic score (*34*)).
315 Vaccine-induced antibody to PNAG deposited complement component C1q onto the

316 purified PNAG antigen, mediated opsonic killing of both extracellular and intracellular *R.*
317 *equi*, and sensitized PBMC from vaccinated foals to release IFN-γ in response to PNAG.
318 The mechanism of killing of intracellular PNAG-expressing microbes was dependent
319 upon surface expression of this antigen, presumably intercalated into the plasma
320 membrane of the infected host cell following release and intracellular transport of
321 microbial surface vesicles (*37*). This mechanism of killing was shown to be applicable
322 to the PNAG-expressing intracellular pathogens *M. avium, L. monocytogenes, S.*
323 *aureus, B. pertussis*, and *N. gonorrhoeae*. Overall, these results imply a high potential
324 for efficacy of vaccination against PNAG in protecting against human intracellular
325 pathogens, and with the parallels in pathogenesis of *R. equi* in foals and Mtb in humans,
326 further imply a potential for protective efficacy against human TB.
327 While immunization-challenge studies such as those performed here are often
328 correlative with protective efficacy against infection and disease, such studies can have
329 limitations in their ability to predict efficacy in the natural setting. Bolus challenges
330 provide an acute insult and immunologic stimulus that mobilizes immune effectors and
331 clears infectious organisms, whereas in a field setting, such as natural acquisition of
332 *R. equi* by foals, infection likely occurs early in life with onset of disease signs taking
333 several weeks to months to develop (*6, 7*). Pulmonary acquisition of Mtb followed by a
334 latent period prior to the emergence of disease occurs in humans exposed to this
335 pathogen (*40*). Thus, it cannot be predicted with certainty that the protective efficacy of
336 antibody to PNAG manifest in the setting of acute, bolus challenge will also be effective
337 when a lower infectious inoculum and more insidious course of disease develops. In this
338 setting, activation of immune effectors may not be of sufficient intensity to take

339 advantage of the opsonic killing activity of antibody, thus allowing progression to
340 disease to occur. In the context of acute challenge, we noted that many of the
341 protected vaccinated foals developed small lung lesions after challenge that rapidly
342 resolved and no disease signs were seen. Finding such lesions by routine ultrasound
343 examination of foals that occurs on farms (*41*) might instigate treatment of subclinical
344 pneumonia if equine veterinarians are either unwilling to monitor foals until clinical signs
345 appear or unconvinced that disease would not ensue in vaccinated foals. This approach
346 would obviate the benefit of vaccination.

347 The use of animal models to predict vaccine efficacy in humans is fraught with
348 uncertainty, even when non-human primates are used (*42-44*). Thus, whether the
349 protective efficacy shown here for *R. equi* in foals is predictive of efficacy against human
350 infection with Mtb is unknown, even though these 2 organisms share significant
351 pathogenic properties. One difference between these microbes is their growth rates,
352 and this may impact the effectiveness of PNAG immunization of humans. *R. equi* grows
353 rapidly in laboratory culture but Mtb slowly. Also, while *R. equi,* Mtb, and other
354 pathogenic mycobacteria are reasonably closely related genetically, there are likely
355 some differences in surface composition and antigens that might impact the ability of
356 antibody to PNAG to kill mycobacteria, thus limiting the utility of this vaccine for human
357 Mtb. However, we did find that the antibody to PNAG could kill a variety of human
358 pathogens inside of infected macrophages, including a rapidly-growing mycobacterial
359 pathogen, dependent on antibody recognition of PNAG antigen intercalated into the
360 infected cell's plasma membrane. This finding not only provides an explanation for how
361 antibody to PNAG can mediate protective immunity to intracellular pathogens, but also

362 supports the potential of antibody to PNAG for providing broad-based protective
363 immunity to many intracellular pathogens.
364 The protection studies described here for *R. equi* disease in foals has led to the
365 implementation of a human trial evaluating the impact of infusion of the fully human IgG_1
366 MAb to PNAG (*21*) on latent and new onset TB. The MAb has been successfully tested
367 for safety, pharmacokinetic, and pharmacodynamic properties in a human phase I test
368 (*45*). The trial in TB patients began in September 2017 (South African Clinical Trials
369 Register). The MAb was chosen for initial evaluation to avoid issues of variable
370 immunogenicity that might arise if a vaccine were tried in a TB-infected population, and
371 to have a greater margin of safety in case of untoward effects of immunity to PNAG in
372 the human setting. It is expected the half-life of the MAb will lead to its reduction to pre-
373 infusion levels over 9 to 15 months whereas this might not be the case following
374 vaccination. A successful effect of the MAb on treatment or disease course in TB will
375 lead to an evaluation of immunogenicity and efficacy of a PNAG targeting vaccine in this
376 patient population. The vaccine used here in horse mares was part of a batch of
377 material produced for human phase 1 safety and immunogenicity testing
378 (ClinicalTrials.gov Identifier: NCT02853617), wherein early results indicate that among a
379 small number of vaccinates there were no serious adverse events and high titers of
380 functional antibody elicited in 7 of 8 volunteers given either 75 µg or 150 µg doses twice
381 28 days apart.
382 Numerous investigators have studied how antibodies can mediate protection
383 against intracellular bacterial pathogens like TB (*16, 19, 46*), although specific
384 mechanisms of immunity are not well defined. The *in vitro* results we derived indicated

385 that a cell infected with a PNAG-producing pathogen has prominent surface display of
386 this antigen that serves as a target for antibody, complement and PMN to lyse the
387 infected cell and release the intracellular organisms for subsequent opsonic killing.
388 Likely other bacterial antigens are displayed on the infected host cell as well, and thus
389 this system could be used to evaluate the protective efficacy and mechanism of killing of
390 antibodies to other antigens produced by intracellular organisms. Although we have not
391 investigated the basis for the appearance of PNAG in the plasma membrane of infected
392 host cells, we suspect that microbial extracellular vesicles, known to be released by
393 many microbes including Mtb (*47*), are a likely source of the plasma membrane antigen
394 due to trafficking from infected cellular compartments (*37*).
395 A notable component of the immune response in the foals was the association of
396 release of IFN-γ from PBMC in response to a *R. equi* cell lysate with the protective
397 efficacy of the maternally derived antibody. The response to the lysate significantly
398 dropped after treatment of the lysate with the PNAG-degrading enzyme dispersin B,
399 indicating that an antibody-dependent cellular response to PNAG underlay the IFN-γ
400 response. As this cytokine is well known to be an important component of resistance to
401 human TB (*13*), it was notable that the maternal immunization strategy led to an IFN-γ
402 response. It also appears that the reliance on traditional T-cell effectors recognizing
403 MHC-restricted microbial antigens to provide components of cellular immunity can
404 potentially be bypassed by an antibody-dependent mechanism of cellular responses,
405 further emphasizing how antibody can provide immunity to intracellular pathogens.
406 This study addressed many important issues related to vaccine development,
407 including the utility of maternal immunization to provide protection against an

408 intracellular pathogen via colostrum to immunologically immature offspring, the efficacy
409 and mechanism of action of antibody to PNAG in protective efficacy, and identification
410 of a role for antibody-dependent IFN-γ release in the response to immunization that
411 likely contributed to full immunity to challenge. The success of immunization in
412 protecting against *R. equi* challenge in foals targeting the broadly synthesized PNAG
413 antigen raises the possibility that this single vaccine could engender protection against
414 many microbial pathogens. While the potential to protect against multiple microbial
415 targets is encouraging, the findings do raise issues as to whether antibody to PNAG will
416 be protective against many microbes or potentially manifest some toxicities or
417 unanticipated enhancements of infection caused by some organisms. Thus, continued
418 monitoring and collection of safety data among animals and humans vaccinated against
419 PNAG is paramount until the safety profile of antibody to PNAG becomes firmly
420 established. Overall, the protective efficacy study in foals against *R. equi* has initiated
421 the pathway to development of PNAG as a vaccine for significant human and animal
422 pathogens, and barring unacceptable toxicity, the ability to raise protective antibodies to
423 PNAG with the 5GlcNH$_2$-TT conjugate vaccine portends effective vaccination against a
424 very broad range of microbial pathogens.

425 **Materials and Methods**

426 **Experimental** Design

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

442 **Samples size determination**

443 The sample size for the foal protection study was based on prior experience with
444 this model [6, 30, 48] indicating a dose of 10^6 CFU of *R. equi* delivered in half-portions to the
445 left and right lungs via intrabronchial instillation would cause disease in ~85% of foals.
446 Thus, a control group of 7 foals, anticipating 6 illnesses, and a vaccinated group of 12
447 foals, would have the ability to detect a significant effect at a P value of <0.05 if 75% of

448 vaccinated foals were disease-free using a 1-sided Fisher's exact test. A 2-sided test is
449 not feasible as one cannot realistically measure a disease rate in vaccinated foals
450 significantly greater than 85%, and any failure to reduce the disease rate would not be
451 indicative of vaccine efficacy. Thus, lack of reduction in disease would lead to rejection
452 of the hypothesis that vaccination against PNAG is effective in preventing *R. equi*
453 pneumonia. Similar criteria were applied to the passive infusion/protection study. All
454 clinical and immunological data to be collected were defined prior to the trial in mares
455 and foals, and no outliers were excluded from the analysis. The primary endpoint was
456 development of clinical *R. equi* pneumonia as defined under Clinical Monitoring below.
457 Experiments were performed over 3 foaling seasons: 2015 and 2016 for the active
458 immunization of pregnant mares, with results from the 2 years of study combined, and
459 2017 for the passive infusion study.

460 **Ethics statement**

461 All procedures for this study were reviewed and approved by the Texas A&M
462 Institutional Animal Care and Use Committee (protocol number AUP# IACUC 2014-
463 0374 and IACUC 2016-0233) and the University Institutional Biosafety Committee
464 (permit number IBC2014-112). The foals used in this study were university-owned, and
465 permission for their use was provided in compliance with the Institutional Animal Care
466 and Use Committee procedures. No foals died or were euthanized as a result of this
467 study.

468 **Vaccine**

469 Mares in the vaccine group received 125 μg (during 2015) or 200 μg (2016) of synthetic
470 pentamers of β 1→6-linked glucosamine conjugated to tetanus toxoid (ratio of

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

484 **Study populations and experimental infection**

485 Twenty healthy Quarter Horse mare/foal pairs were initially included in this study; 1
486 unvaccinated mare and her foal were excluded when the foal was stillborn. The
487 unvaccinated group consisted of 7 mare/foal pairs (n = 4 in 2015 and n = 3 in 2016) and
488 the vaccinated group consisted of 12 mare/foal pairs (n = 5 in 2015 and n = 7 in 2016).
489 For the passive infusion of hyperimmune plasma, 9 foals were used, 4 infused with 2 L
490 of commercial normal horse plasma (Immunoglo Serial 1700, Mg Biologics, Ames, IA,
491 USA) and 5 were infused with 2 L of PNAG-hyperimmune plasma produced using
492 standard methods by Mg Biologics. Group assignment was made using a randomized,
493 block design for each year. All foals were healthy at birth and had total serum IgG

494 concentrations >800 mg/dl at 48 h of life using the SNAP® Foal IgG test (IDEXX, Inc.,
495 Westbrook, Maine, USA), and remained healthy through the day of experimental
496 challenge. Immediately prior to experimental infection with *R. equi*, each foal's lungs
497 were evaluated by thoracic auscultation and thoracic ultrasonography to document
498 absence of evidence of pre-existing lung disease.

499 To study vaccine efficacy, foals were experimentally infected with 1 x 10^6 of live *R.*
500 *equi* strain EIDL 5–331 (a virulent, *vapA*-gene-positive isolate recovered from a
501 pneumonic foal). This strain was streaked onto a brain-heart infusion (BHI) agar plate
502 (Bacto Brain Heart Infusion, BD, Becton, Dickinson and Company, Sparks, MD, USA).
503 One CFU was incubated overnight at 37°C in 50 ml of BHI broth on an orbital shaker at
504 approximately 240 rpm. The bacterial cells were washed 3 times with 1 X phosphate-
505 buffered saline (PBS) by centrifugation for 10 min, 3000 x g at 4°C. The final washed
506 pellet was resuspended in 40 ml of sterile medical grade PBS to a final concentration of
507 2.5 x 10^4 CFU/ml, yielding a total CFU count of 1 x 10^6 in 40 ml. Half of this challenge
508 dose (20 ml with 5 x 10^5) was administered transendoscopically to the left mainstem
509 bronchus and the other half (20 ml with 5 x 10^5) was administered to the right mainstem
510 bronchus. Approximately 200 µl of challenge dose was saved to confirm the
511 concentration (dose) administered, and to verify virulence of the isolate using multiplex
512 PCR (23).

513 For transendoscopic infection, foals were sedated using intravenous (IV) injection of
514 romifidine (0.8 mg/kg; Sedivet, Boehringer-Ingelheim Vetmedica, Inc., St. Joseph, MO,
515 USA) and IV butorphanol (0.02 mg/kg; Zoetis, Florham Park, New Jersey, USA). An
516 aseptically-prepared video-endoscope with outer diameter of 9-mm was inserted via the

517 nares into the trachea and passed to the bifurcation of the main-stem bronchus. A 40-
518 mL suspension of virulent EIDL 5–331 *R. equi* containing approximately 1 x 10^6 viable
519 bacteria was administered transendoscopically, with 20 ml infused into the right
520 mainstem bronchus and 20 ml into the left mainstem bronchus via a sterilized silastic
521 tube inserted into the endoscope channel. The silastic tube was flushed twice with 20 ml
522 of air after each 20-ml bacterial infusion. Foals and their mares were housed
523 individually and separately from other mare and foal pairs following experimental
524 infection.

525 **Sample collections from mares and foals**

526 Colostrum was collected (approx. 15 ml) within 8 hours of foaling. Blood samples were
527 collected from immunized mares 6 weeks and 3 weeks before their predicted dates of
528 foaling, and on the day of foaling. Blood samples from 4 non-vaccinated mares in the
529 2015 study were only collected on the day of foaling, whereas blood was collected from
530 the 3 non-vaccinated mares in the 2016 study at the same time-points as those for
531 vaccinated mares. Blood for preparation of hyperimmune plasma was collected from
532 immunized adult horses 2 weeks after the second injection of 200 µg of the 5GlcNH$_2$-TT
533 vaccine in 0.1 ml of Specol.

534 Blood samples were drawn from foals on day 2 (the day after foaling), and at 4, 6, 8,
535 and 12 weeks of age. Samples at 4 weeks (25-28 days of life) were collected prior to
536 infection. Blood was collected in EDTA tubes for complete blood count (CBC) testing, in
537 lithium heparinized tubes for PBMC isolation, and in clot tubes for serum collection.
538 Transendoscopic tracheobronchial aspiration (T-TBA) was performed at the time of
539 onset of clinical signs for any foals developing pneumonia and at age 12 weeks for all

540 foals (end of study) by washing the tracheobronchial tree with sterile PBS solution
541 delivered through a triple-lumen, double-guarded sterile tubing system (MILA
542 International, Inc. Erlanger, KY, USA).
543
544 **Clinical monitoring**
545 From birth until the day prior to infection, foals were observed twice daily for signs of
546 disease. Beginning the day prior to infection, rectal temperature, heart rate, respiratory
547 rate, signs of increased respiratory effort (abdominal lift, flaring nostrils), presence of
548 abnormal lung sounds (crackles or wheezes, evaluated for both hemithoraces),
549 coughing, signs of depressed attitude (subjective evidence of increased recumbence,
550 lethargy, reluctance to rise), and nasal discharges were monitored and results recorded
551 twice daily through 12 weeks (end of study). Thoracic ultrasonography was performed
552 weekly to identify evidence of peripheral pulmonary consolidation or abscess formation
553 consistent with *R. equi* pneumonia. Foals were considered to have pneumonia if they
554 demonstrated ≥3 of the following clinical signs: coughing at rest; depressed attitude
555 (reluctance to rise, lethargic attitude, increased recumbency); rectal temperature
556 >39.4°C; respiratory rate ≥60 breaths/min; or, increased respiratory effort (manifested
557 by abdominal lift and nostril flaring). Foals were diagnosed with *R. equi* pneumonia if
558 they had ultrasonographic evidence of pulmonary abscessation or consolidation with a
559 maximal diameter of ≥2.0 cm, positive culture of *R. equi* from T-TBA fluid, and cytologic
560 evidence of septic pneumonia from T-TBA fluid. The primary outcome was the
561 proportion of foals diagnosed with *R. equi* pneumonia. Secondary outcomes included
562 the duration of days meeting the case definition, and the sum of the total maximum

563 diameter (TMD) of ultrasonography lesions over the study period. The TMD was
564 determined by summing the maximum diameters of each lesion recorded in the 4th to
565 the 17th intercostal spaces from each foal at every examination; the sum of the TMDs
566 incorporates both the duration and severity of lesions. Foals diagnosed with *R. equi*
567 pneumonia were treated with a combination of clarithromycin (7.5 mg/kg; PO; q 12
568 hour) and rifampin (7.5 mg/kg; PO; q 12 hour) until both clinical signs and thoracic
569 ultrasonography lesions had resolved. Foals also were treated as deemed necessary by
570 attending veterinarians (AIB; NDC) with flunixin meglumine (0.6 to 1.1 mg/kg; PO; q 12-
571 24 hour) for inflammation and fever.

572 **Immunoglobulin ELISAs**

573 Systemic humoral responses were assessed among foals by indirectly quantifying
574 concentrations in serum by ELISA from absorbance values of PNAG-specific total IgG
575 and by IgG subisotypes IgG_1, $IgG_{4/7}$, and $IgG_{3/5}$. ELISA plates (Maxisorp, Nalge Nunc
576 International, Rochester, NY, USA) were coated with 0.6 µg/ml of purified PNAG [49]
577 diluted in sensitization buffer (0.04M PO_4, pH 7.2) overnight at 4°C. Plates were washed
578 3 times with PBS with 0.05% Tween 20, blocked with 120 µl PBS containing 1% skim
579 milk for 1 hour at 37°C, and washed again. Mare and foal serum samples were added at
580 100 µl in duplicate to the ELISA plate and incubated for 1 hour at 37°C. Serum samples
581 were initially diluted in incubation buffer (PBS with 1% skim milk and 0.05% Tween 20)
582 to 1:100 for total IgG titers, 1:64 for IgG_1 and $IgG_{4/7}$ detection, and to 1:256 for $IgG_{3/5}$
583 detection. A positive control from a horse previously immunized with the 5GlcNH$_2$-TT
584 vaccine and known to have a high titer, along with normal horse serum known to have a
585 low titer, were included in each assay for total IgG titers. For the subisotype assays,

586 immune rabbit serum (rabbit anti-5GLcNH$_2$-TT) was diluted to a concentration of
587 1:102,400 as a positive control and used as the denominator to calculate the endpoint
588 OD ratio of the experimental OD values. The immune rabbit serum was used to account
589 for inter-plate variability and negative control of normal rabbit serum were included with
590 the equine serum samples. After 1 hour incubation at 37°C, the plates were washed 3
591 times as described above. For total IgG titers, rabbit anti-horse IgG whole molecule
592 conjugated to alkaline phosphatase (Sigma-Aldrich, St. Louis, MO, USA) was used to
593 detect binding. For IgG subisotype detection, 100 µl of goat-anti-horse IgG$_{4/7}$ (Lifespan
594 Biosciences, Seattle, WA, diluted at 1:90,000), or goat anti-horse IgG$_{3/5}$ (Bethyl
595 Laboratories, Montgomery, TX, USA, diluted at 1:30,000) conjugated to horseradish
596 peroxidase, or mouse anti-horse IgG$_1$ (AbD Serotec, Raleigh, NC, USA), diluted at
597 1:25,000) were added to the wells and incubated for 1 hour at room temperature. For
598 the IgG$_1$ subisotype, goat antibody to mouse IgG (Bio-Rad, Oxford, England, diluted at
599 1:1000) conjugated to peroxidase was used for detection. Plates were washed again,
600 and for the total IgG titers pNPP substrate (1 mg/ml) was added while for peroxidase-
601 conjugated antibody to mouse IgG, SureBlue Reserve One Component TMB Microwell
602 Peroxidase Substrate (SeraCare, Gaithersburg, MD, USA) was added to the wells.
603 Plates were incubated for 15 to 60 minutes at 22°C in the dark. The reaction was
604 stopped by adding sulfuric acid solution to the wells. Optical densities were determined
605 at 450 nm by using microplate readers. Equine subisotype concentrations of PNAG-
606 specific IgG$_1$, IgG$_{4/7}$, and IgG$_{3/5}$ were also quantified in colostrum of each mare using
607 the same protocol described above for serum. Colostral samples were diluted in
608 incubation buffer (PBS with 1% skim milk and 0.05% Tween 20) to 1:8,192 for IgG$_1$,

609 1:4096 for $IgG_{4/7}$ detection, and at 1:64 for $IgG_{3/5}$ detection. For total IgG endpoint titers
610 were calculated by linear regression using a final OD_{405}nm value of 0.5 to determine the
611 reciprocal of the maximal serum dilution reaching this value. For IgG subisotypes, an
612 endpoint OD titer was calculated by dividing the experimental OD values with that
613 achieved by the positive control on the same plate.

614 **PNAG expression by clinical isolates of *R. equi***

615 Clinical isolates of *R. equi* were obtained from the culture collection at the Equine
616 Infectious Disease Laboratory, Texas A&M University College of Veterinary Medicine &
617 Biomedical Sciences. All strains were originally isolated from foals diagnosed with
618 *R. equi* pneumonia and were obtained from geographically distinct locations. *R. equi*
619 strains were grown overnight on BHI agar then swabbed directly onto glass slides, air
620 dried and fixed by exposure for 1 min to methanol at 4°C. Samples were labeled with
621 either 5 µl of a 5.2 µg/ml concentration of MAb F598 to PNAG directly conjugated to
622 Alexa Fluor 488 or control MAb F429 to alginate, also directly conjugated to Alexa Fluor
623 488, for 4 hours at room temperature. During the last 5 min of this incubation, 500 nM
624 of Syto83 in 0.5% BSA/PBS pH 7.4 was added to stain nucleic acids (red fluorophore).
625 Samples were washed and mounted for immunofluorescent microscopic examination as
626 described [11].

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

629 Paraffinized sections of lungs obtained at necropsy from foals with *R. equi* pneumonia
630 were provided by the Texas A&M College of Veterinary Medicine & Biomedical
631 Sciences histopathology laboratory. Slides were deparaffinized using EzDewax and

632 blocked overnight at 4C with 0.5% BSA in PBS. Samples were washed then incubated
633 with the fluorophore-conjugated MAb F598 to PNAG or control MAb F429 to alginate
634 described above for 4 hours at room temperature. Simultaneously added was a 1:500
635 dilution (in BSA/PBS) of a mouse antibody to the virulence associated Protein A of *R.*
636 *equi*. Binding of the mouse antibody to *R. equi* was detected with a donkey antibody to
637 mouse IgG conjugated to Alexa Fluor 555 at a dilution of 1:250 in BSA/PBS. Samples
638 were washed and mounted for immunofluorescence microscopic examination.
639 To detect PNAG expression in cultured human monocyte-derived macrophages
640 (MDM), prepared as described below in opsonic killing assays, the infected MDM were
641 washed and fixed with 4% paraformaldehyde in PBS for 1 hour at room temperature. To
642 visualize PNAG on the surface of infected cells, MDM cultures were incubated with the
643 fluorophore-conjugated MAb F598 to PNAG or control MAb F429 to alginate for 4-6
644 hours at room temperature. Samples were then imaged by confocal microscopy to
645 visualize extracellular PNAG expression. Next, these same samples were treated with
646 100% methanol at 4°C for 5 min at room temperature to permeabilize the plasma
647 membrane. Samples were washed with PBS then incubated with either 5.2 µg/ml of
648 MAb F598 to PNAG or MAb F429 to alginate for 1-2 hours at room temperature,
649 washed in PBS then a 1:300 dilution in PBS of donkey antibody to human IgG labeled
650 with Alexa Fluor 555 added for 4-6 hours at room temperature. Samples were washed
651 and mounted for immunofluorescence microscopic examination.
652 **C1q deposition assays**
653 Serum endpoint titers for deposition of equine complement component C1q onto
654 purified PNAG were determined by ELISA. ELISA plates were sensitized with PNAG

655 and blocked with skim milk as described above, dilutions of different horse sera added
656 in 50 µl-volumes after which 50 µl of 10% intact, normal horse serum was added. After
657 60 minutes incubation at 37°C, plates were washed and 100 µl of goat anti-human C1q,
658 which also binds to equine C1q, diluted 1:1,000 in incubation buffer added and plates
659 incubated at room temperature for 60 minutes. After washing, 100 µl of rabbit anti-goat
660 IgG whole molecule conjugated to alkaline phosphatase and diluted 1:1,000 in
661 incubation buffer was added and a 1-hour incubation at room temperature carried out.
662 Washing and developing of the color indicator was then carried out as described above,
663 and endpoint titers determined as described above for IgG titers by ELISA.

664 **Opsonic killing assays**

665 To determine opsonic killing of *R. equi,* bacterial cultures were routinely grown overnight
666 at 37°C on chocolate-agar plates, then killing assessed using modifications of
667 previously described protocols [49]. Modifications included use of EasySep™ Human
668 *Neutrophil Isolation* Kits (Stem Cell Technologies Inc., Cambridge, Massachusetts,
669 USA) to purify PMN from blood, and use of gelatin-veronal buffer supplemented with
670 Mg^{++} and Ca^{++} (Boston Bioproducts, Ashland, Massachusetts, USA) as the diluent for
671 all assay components. Final assay tubes contained, in a 400-µl volume, 2 X 10^5 human
672 PMN, 10% (final concentration) *R. equi*-absorbed horse serum as a complement
673 source, 2 X 10^5 *R equi* cells and the serum dilutions. Tubes were incubated with end-
674 over-end rotation for 90 minutes then diluted in BHI with 0.05% Tween and plated for
675 bacterial enumeration.

676 For intracellular opsonic killing assays, human monocytes were isolated from
677 peripheral blood using the EasySep™ Direct Human Monocyte Isolation Kit (Stem Cell

678 Technologies) and 2 x 10^4 cells placed in a 150 µl volume of RPMI and 10% heat-
679 inactivated autologous human serum in flat-bottom 96-well tissue culture plates for 5-6
680 days with incubation at 37°C in 5% CO_2. Differentiated cells were washed and 5 X 10^5
681 CFU of *R. equi* in RPMI and 10% heat-inactivated autologous human serum added for
682 30 minutes. Next, cells were washed and 150 µl of RPMI plus10% autologous serum
683 with 50 µg gentamicin sulfate/ml added and cells incubated for 24 hours at 37°C in 5%
684 CO_2. For some experiments, 50 µl of 400 µg/ml of either chitinase (Sigma-Aldrich) or
685 dispersin B (Kane Biotech, Winnipeg, Manitoba), a PNAG-degrading enzyme [37, 50],
686 dissolved in Tris-buffered saline, pH 6.5, were added directly to gentamicin containing
687 wells and plates incubated for 2 hours at 37°C in 5% CO_2. Cell cultures were washed
688 then combinations of 50 µl of MAb or foal serum, 50 µl of 30% *R. equi*-absorbed human
689 serum as a complement source, or heat-inactivated complement as a control, and 50 µl
690 containing 1.5 X 10^5 human PMN, isolated as described above, added. Controls lacked
691 PMN or had heat-inactivated complement used in place of active complement, and final
692 volumes made up with 50 µl of RPMI 1640 medium. After a 90-minute incubation at
693 37°C in 5% CO_2, 10 µl samples were taken from selected wells for analysis of lysis by
694 lactate dehydrogenase release, and 100 µl of trypsin/EDTA with 0.1% Triton X100
695 added to all wells lyse the cells via a 10-minute incubation at 37°C. Supernatants were
696 diluted and plated on chocolate agar for bacterial enumeration as described above.

697 **Cell-mediated immunity**

698 The cell-mediated immune response to vaccination was assessed by measuring IFN-γ
699 production from isolated horse PBMCs that were stimulated with an *R. equi* antigen
700 lysate of strain EIDL 5–331, or the same lysate digested for 24 hours at 37°C with 100

701 μg/ml of dispersin B. The PBMCs were isolated using a Ficoll-Paque gradient
702 separation (GE Healthcare, Piscataway, NJ, USA) and resuspended in 1X RPMI-1640
703 media with L-glutamine (Gibco, Life Technologies, Grand Island, NY, USA), 15% fetal
704 bovine serum (Gibco), and 1.5% penicillin-streptomycin (Gibco). The PBMCs were
705 cultured for 48 hours at 37°C in 5% CO_2 with either media only, the mitogen
706 Concanavalin A (positive control; 2.5 μg/ml, Sigma-Aldrich), or *R. equi* lysate
707 representing a multiplicity of infection of 10. After 48 hours, supernatants from each
708 group were harvested and frozen at -80°C until examined for IFN-γ production using an
709 equine IFNγ ELISA kit (Mabtech AB, Nacka Strand, Stockholm, Sweden) according to
710 the manufacturer's instructions. Optical densities were determined using a microplate
711 reader and standard curves generated to determine IFN-γ concentrations in each
712 sample using the Gen 5 software (Biotek, Winooski, VT, USA).

713 **Statistical methods**

714 Categorical variables with independent observations were compared using chi-squared
715 or, when values for expected cells were ≤5, Fisher's exact tests. For estimation of the
716 95% C.I. of the relative risk, the Koopman asymptotic score (*34*) was determined.
717 Continuous, independent variables were compared between 2 groups using either
718 paired t-tests or Mann-Whitney tests and between > 2 groups using the Kruskal-Wallis
719 test with pairwise *post hoc* comparisons made using Dunn's procedure. Continuous
720 variables with non-independent observations (*i.e.*, repeated measures) were compared
721 using linear mixed-effects modeling with an exchangeable correlation structure and
722 individual mare or foal as a random effect. Survival times were compared non-
723 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 ($\log_{10}$) 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 (*48*). Significance was set at $P \leq 0.05$ and adjustment for multiple comparisons made.

Acknowledgments:

Funding: This study was funded by a grant from the Morris Animal Foundation (MAF) and Alopexx Vaccine LLC. The results have not been reviewed or endorsed by MAF, and the views expressed do not necessarily reflect the views of the MAF, its officers, directors, affiliates or agents. Additional support was provided by the Link Equine Research Endowment, Texas A&M University. We thank Dr. Deborah Hung from the Broad Institute of MIT and Harvard and the Massachusetts General Hospital for provision of GFP-expressing *M. tuberculosis* cells for analysis. **Author contributions**: C.C-B., J.N.R., A.I.B., M.V., S.R.,T.Z., S.G. G.B.P. and N.D.C. designed and executed experiments. C.C-B., J.N.R., A.I.B., D.V., N.D.C., and G.B.P. analyzed data. J.N.R., A.I.B., and N.D.C. conducted the animal immunization and challenge studies. N.D.C. and G.P. conducted the statistical analysis. C.C-B., J.N.R., A.I.B., D.V., N.D.C. and G.B.P. wrote and edited the manuscript.

746 **Conflict of interest:**

747 Gerald B. Pier is an inventor of intellectual properties [human monoclonal antibody to
748 PNAG and PNAG vaccines] that are licensed by Brigham and Women's Hospital to
749 Alopexx Vaccine, LLC, and Alopexx Pharmaceuticals, LLC, entities in which GBP also
750 holds equity. As an inventor of intellectual properties, GBP also has the right to receive
751 a share of licensing-related income (royalties, fees) through Brigham and Women's
752 Hospital from Alopexx Pharmaceuticals, LLC, and Alopexx Vaccine, LLC. GBP's
753 interests were reviewed and are managed by the Brigham and Women's Hospital and
754 Partners Healthcare in accordance with their conflict of interest policies.

755 Colette Cywes-Bentley is an inventor of intellectual properties [use of human
756 monoclonal antibody to PNAG and use of PNAG vaccines] that are licensed by Brigham
757 and Women's Hospital to Alopexx Pharmaceuticals, LLC. As an inventor of intellectual
758 properties, CC-B also has the right to receive a share of licensing-related income
759 (royalties, fees) through Brigham and Women's Hospital from Alopexx Pharmaceuticals,
760 LLC.

761 Noah D. Cohen has received an unrestricted gift to the EIDL from Alopexx
762 Vaccines, LLC.

763 Daniel Vlock, holds an equity share and potential royalty income from Alopexx
764 Vaccines, LLC for vaccines to PNAG and monoclonal antibody to PNAG from Alopexx
765 Pharmaceuticals, LLC.

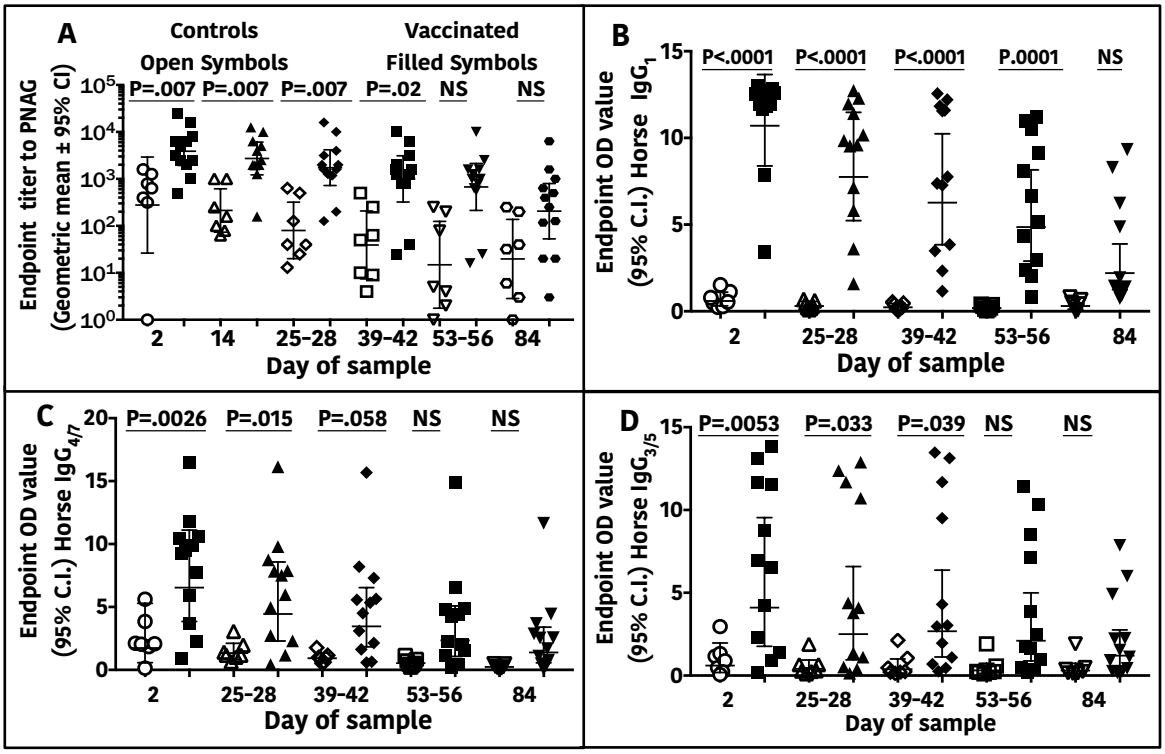
766

767 **Data and materials availability:**

768 Materials used in this study including vaccines, serum samples, and microbial strains

769 may be obtained through an MTA with either Brigham and Women's Hospital or Texas

770 A&M College of Veterinary Medicine & Biomedical Sciences.



Figures

Fig 1. Total IgG and IgG subisotype antibody titers to PNAG in sera of horse foals.

Endpoint serum titers (N=7 Non-vaccinated, 12 vaccinated) 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 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 D39-42 of life. (**B-D**) Concentrations of $IgG_{1,}$ $IgG_{4/7}$, and $IgG_{3/5}$ 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.

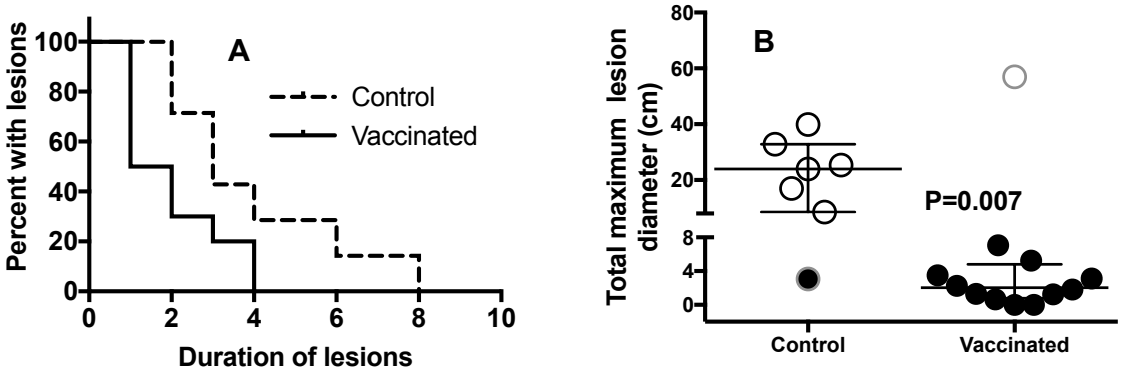


Fig. 2. Comparison of induction and regression of ultrasonographic lesions in foals from vaccinated or unvaccinated mares following *R. equi* challenge.

(**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 Unvaccinated, 12 Vaccinated). The sums of the cumulative maximum diameters were significantly (P = 0.007; Wilcoxon rank-sum test) shorter 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.

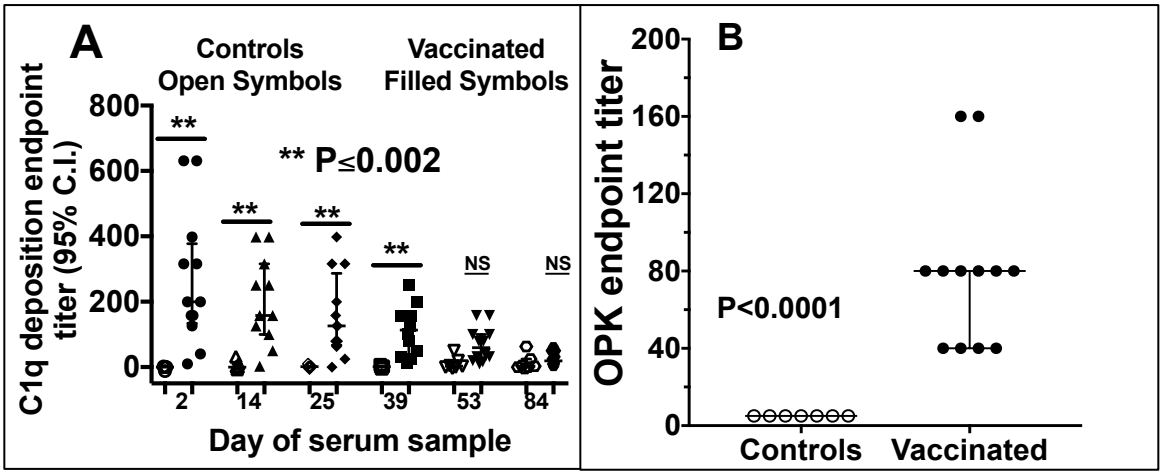


Fig. 3. Functional activity of antibody in foal sera on day of challenge with *R. equi*.

(**A**) Serum endpoint titer (N=7 unvaccinated controls, 12 vaccinated) of deposition of equine C1 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 ≥ 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.

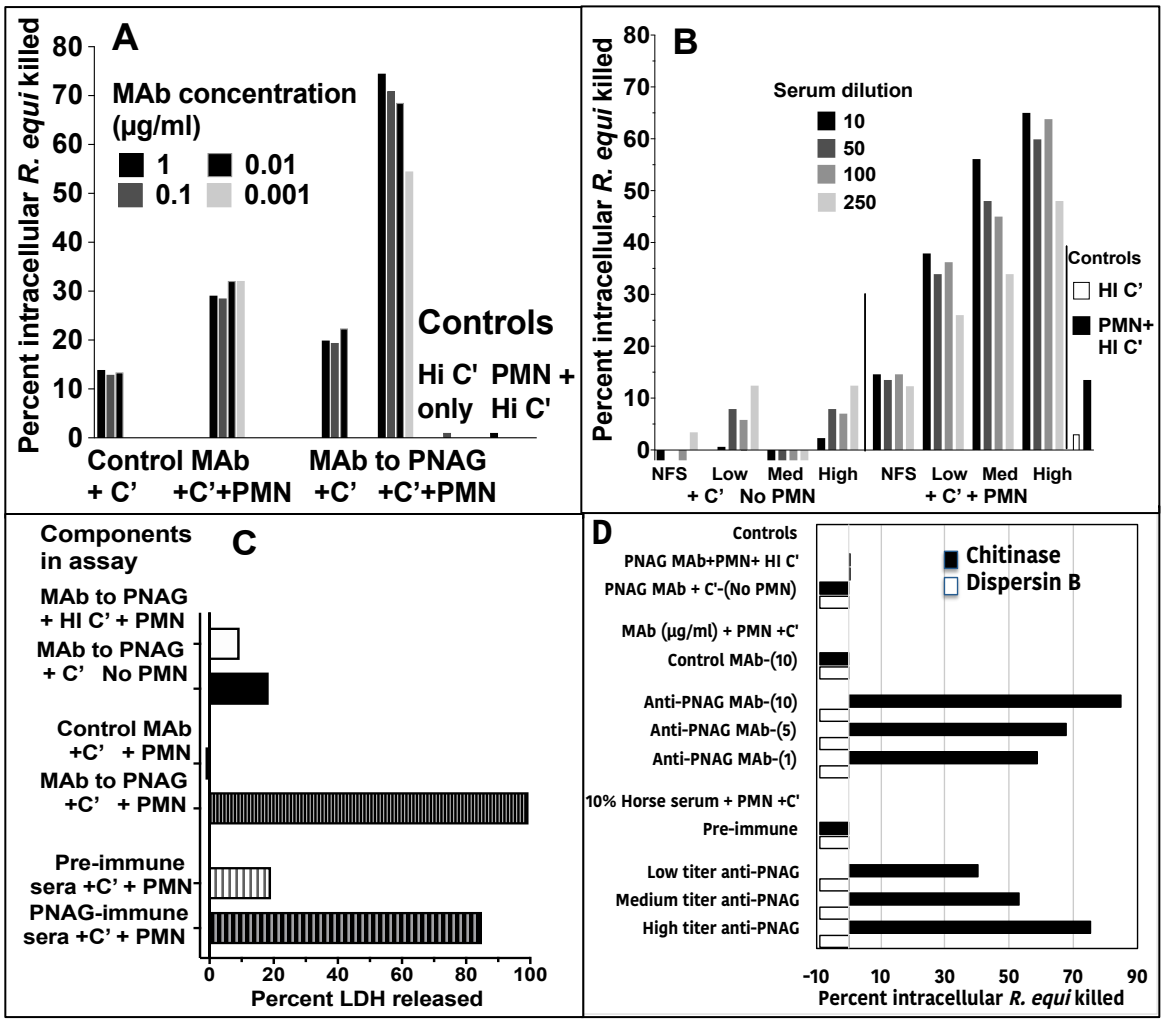


Fig. 4. Opsonic killing of intracellular *R. equi*.

(**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 (NFS) or immune foal sera obtained on the day of challenge with *R. equi* representing animals with low, medium (Med) or high titers to PNAG 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'.

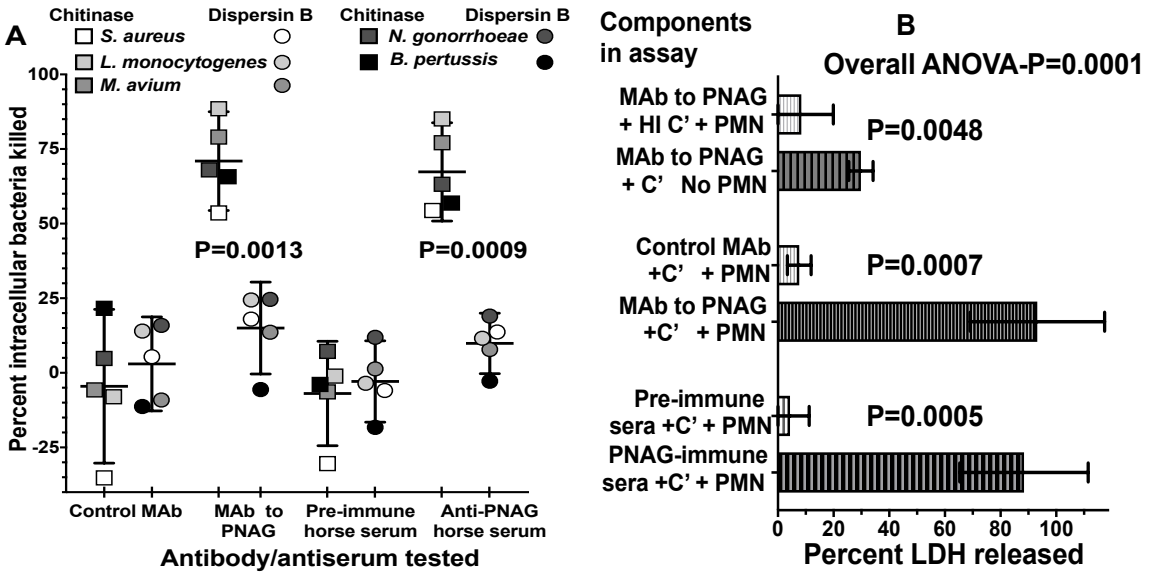


Fig. 5. Opsonic killing of multiple intracellular pathogens by antibody to PNAG, complement (C') and PMN depends on infected-cell surface expression of PNAG and is associated with release of LDH

(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 with treatment with the control enzyme, Chitinase (squares). Symbols represent indicated bacterial target strain. Horizontal bars represent means, error bars 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.

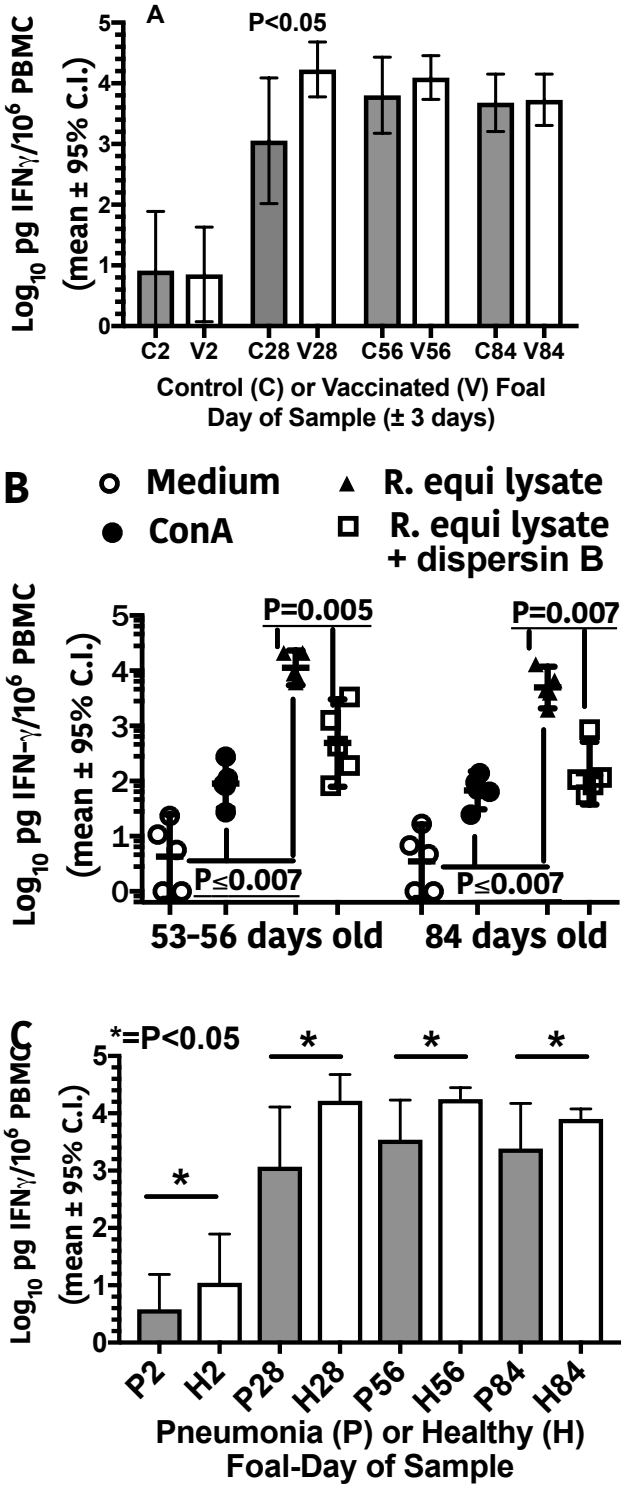


Fig. 6. Cell-mediated immune responses of foal PBMCs. (**A**) Foals (N=7 controls, 12 vaccinated) from vaccinated mares (V) had significantly (P <0.05; linear-mixed effects modeling) higher concentrations of IFN-γ produced at 28 days of age (prior to challenge) than control (C) foals in response to stimulation by a lysate of *R. equi*. IFN-γ 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**) IFN-γ production from PBMC from 5 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

control at both day 56 and 84 (Overall ANOVA for repeated measures ($P < 0.0001$); $P \leq 0.0070$ 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 IFN-γ expression at each day relative to foals that remained healthy (H).

Table S1. 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 > 103.5oF

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^{o}F

Tachypnea (respiratory rate $\geq$ 60 breaths/min)

Increased respiratory effort (abdominal lift, flaring nostrils)

Table S2. 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^{o}F (range)	8 (1 to 32)	2 (0 to 12)	0.0263
Days temperature > 103.0^{o}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

Table S3. 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^{o}F (range)	11.5 (1 to 15)	2 (0 to 4)	0.0811
Days temperature > 103.0^{o}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 Wilcoxon rank-rum test.

[b]Median

[c]TMD = total (sum) of maximum diameters of lesions observed on a given day

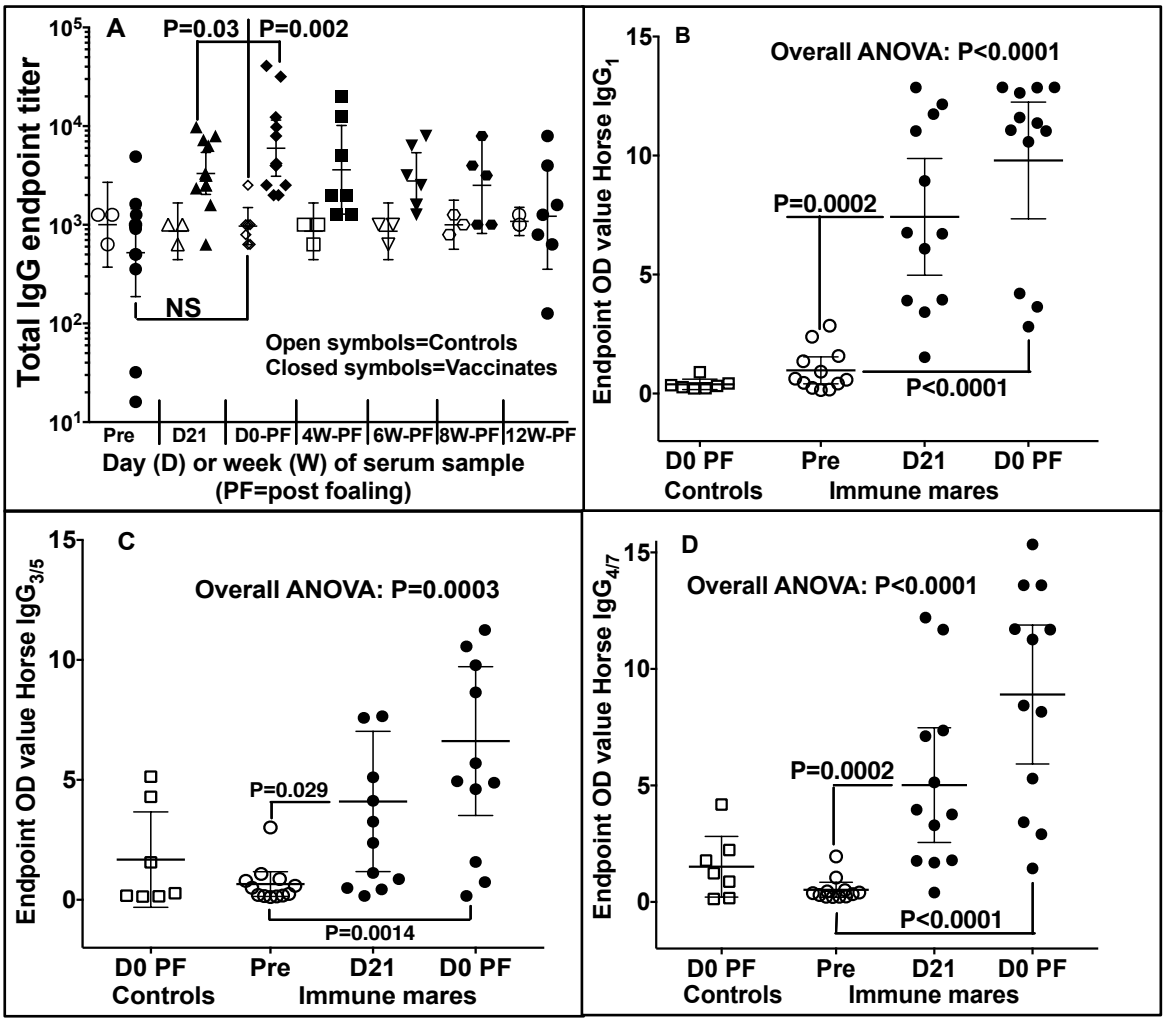


Fig. S1. Serum IgG and IgG subisotype titers to PNAG in immunized mare sera.

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 IgG_1, $IgG_{4/7}$, 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.

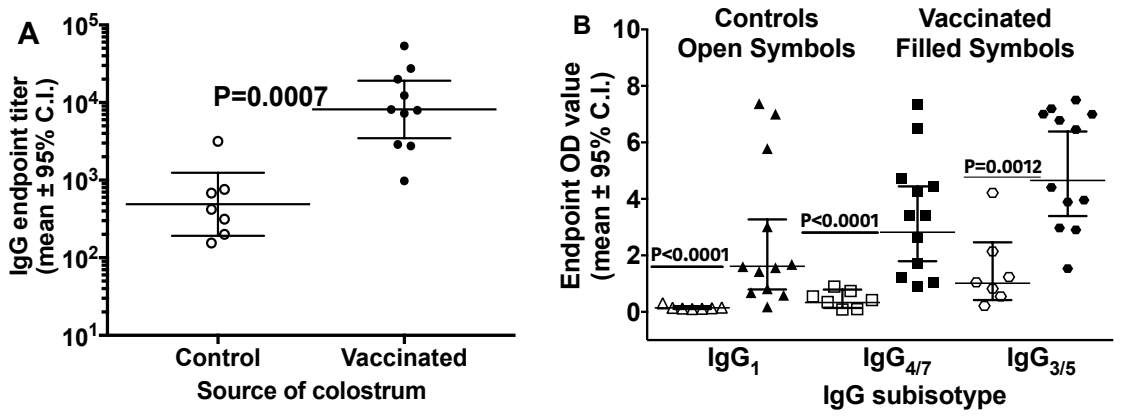


Fig. S2. IgG and IgG subisotype titers in mare colostra on day of foaling. End-point colostral titers of IgG or IgG subisotype. End-point values are plotted by vaccine group. (**A**) Total IgG antibody end-point titers to PNAG were significantly higher in colostra of vaccinated mares (N=10, 2 samples not tested due to limited quantities) compared with colostra of control mares N=7). (**B**) Concentrations of IgG_1, $IgG_{4/7}$, 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.

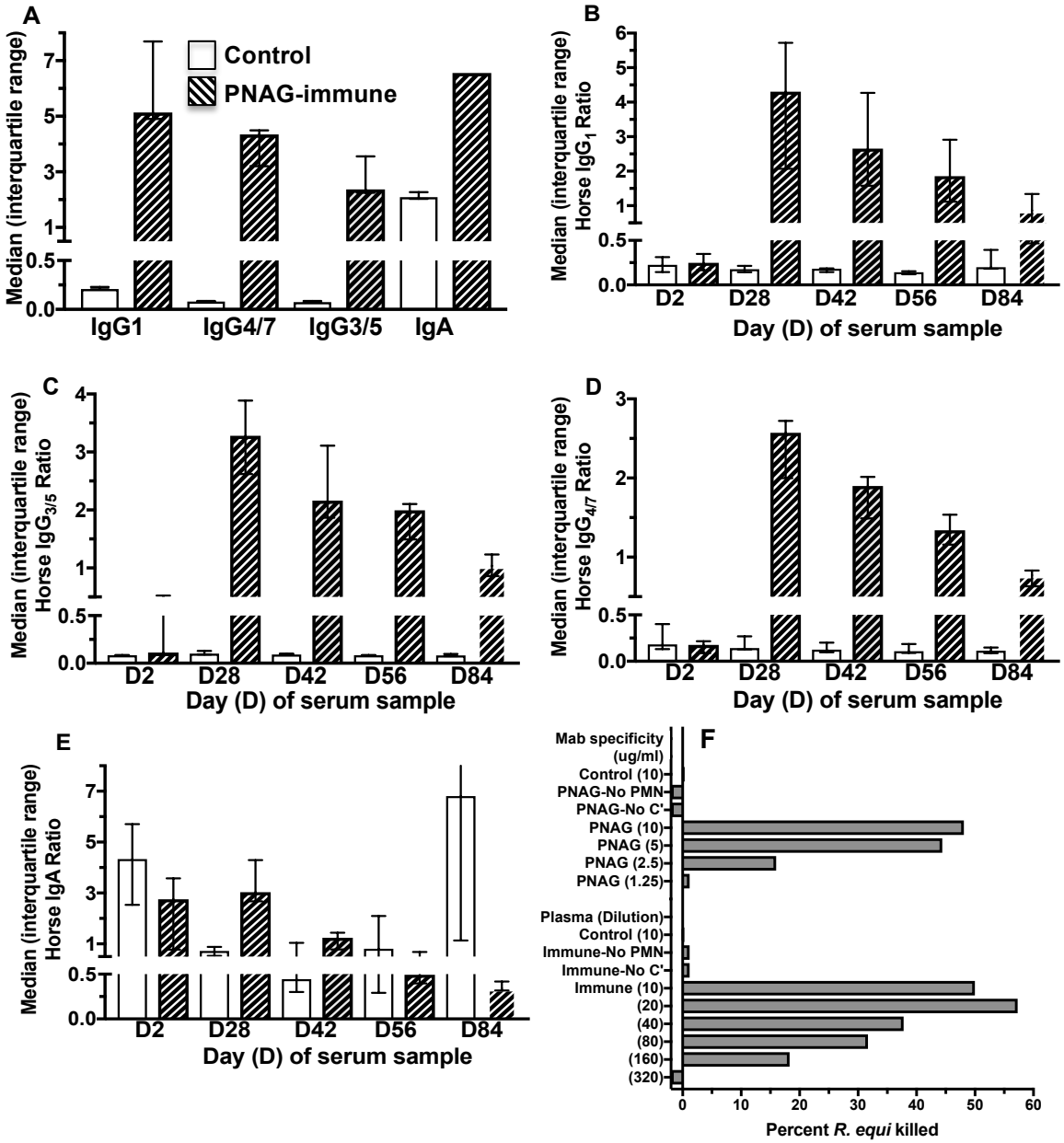


Fig S3. Antibody titers to PNAG in control and hyperimmune horse plasma infused into newborn foals on day 1 of life.

(**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 mixed-effects linear 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.

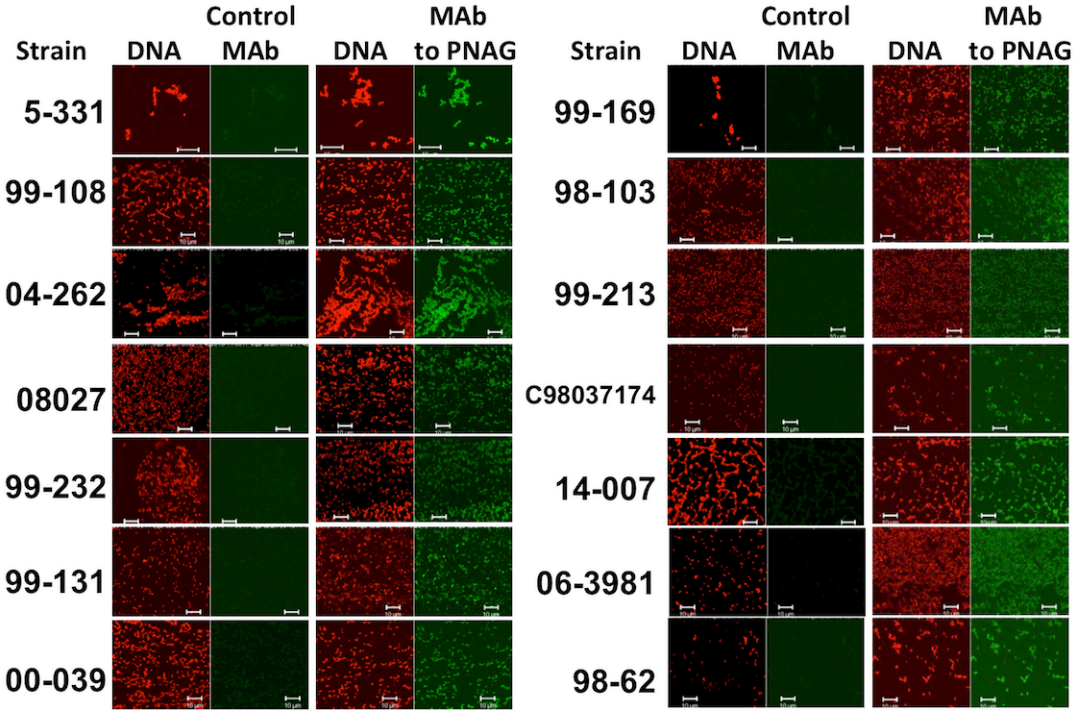


Fig. S4. PNAG expression by *R. equi* clinical isolates.

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.

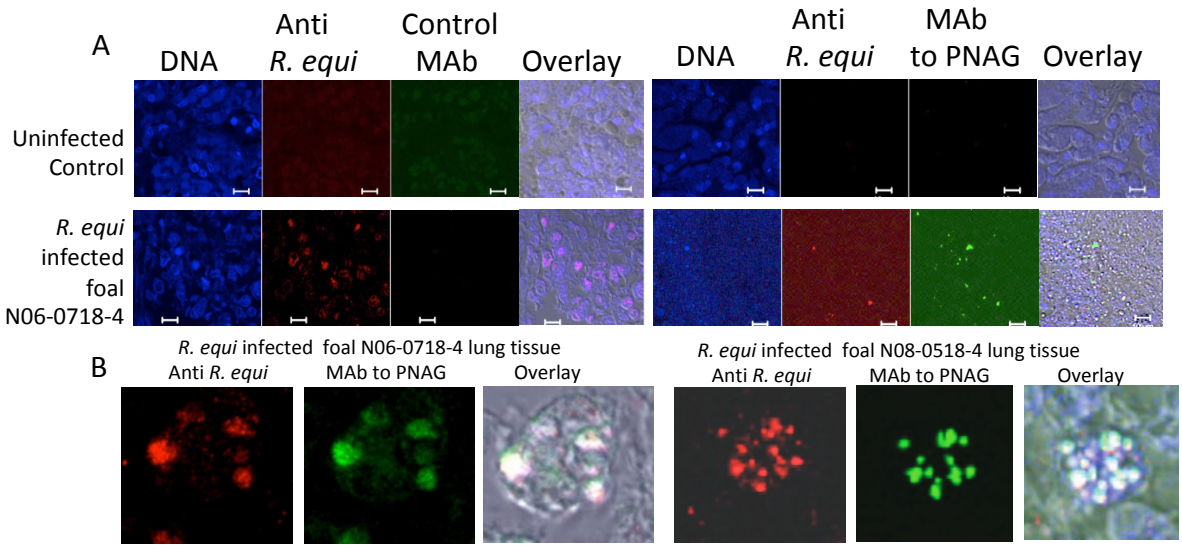


Fig. S5. Expression of PNAG in lungs of *R. equi* infected foals

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.

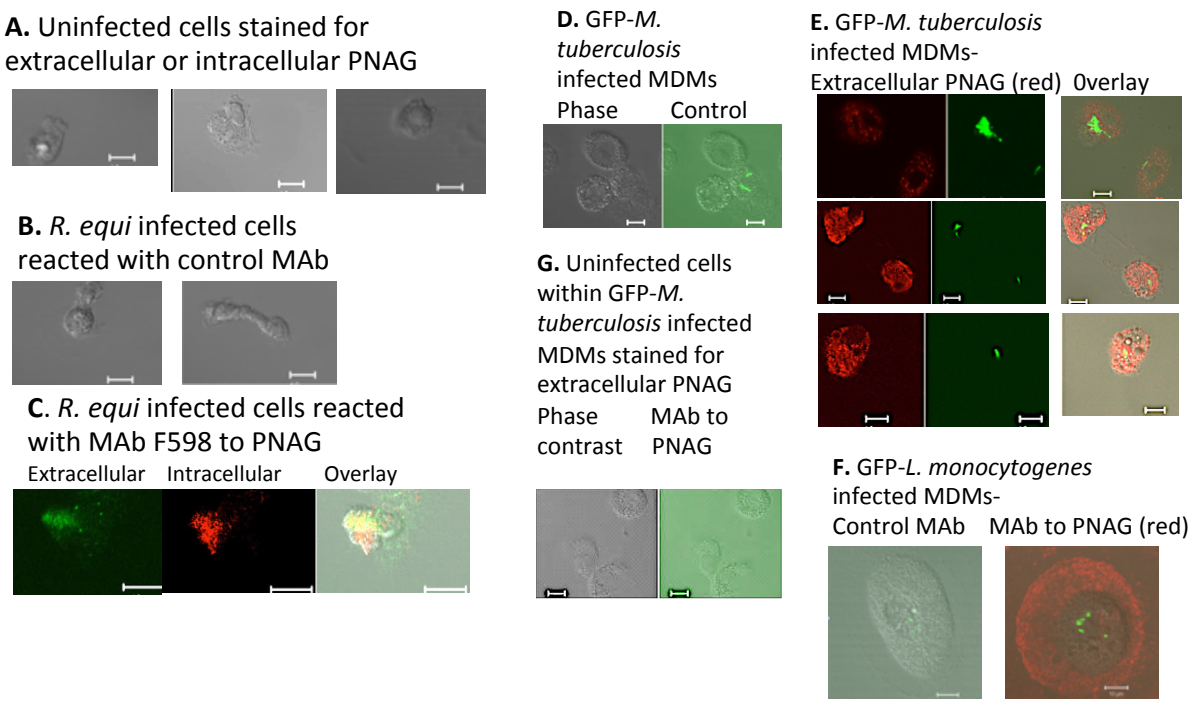


Fig. S6. Surface and intracellular expression of PNAG in infected human MDM.

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.

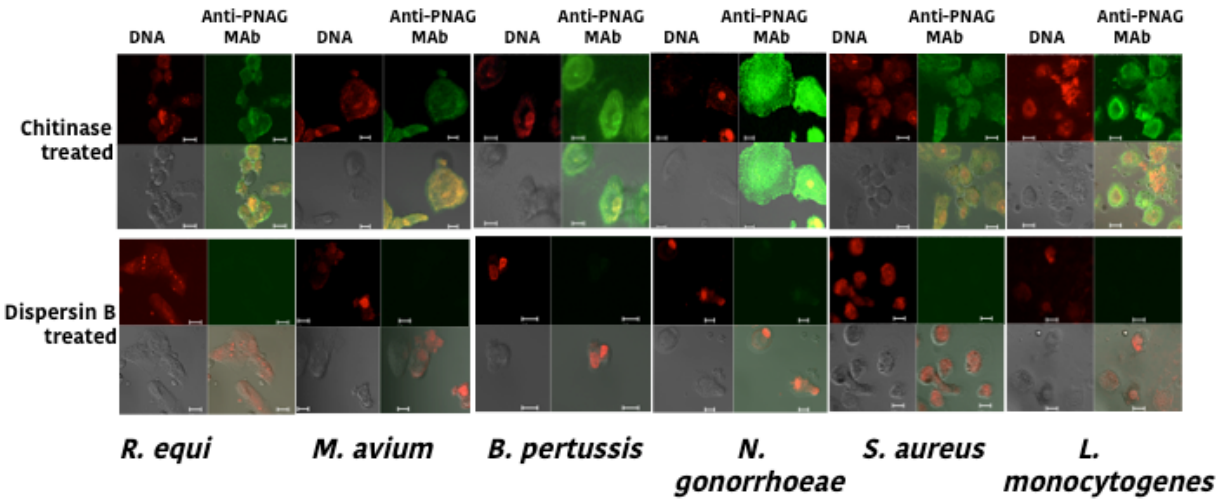


Fig. S7. Human MDM cells infected with PNAG-expressing intracellular pathogens have high levels of the PNAG antigen on their surface that is removed by treatment with dispersin B.

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.

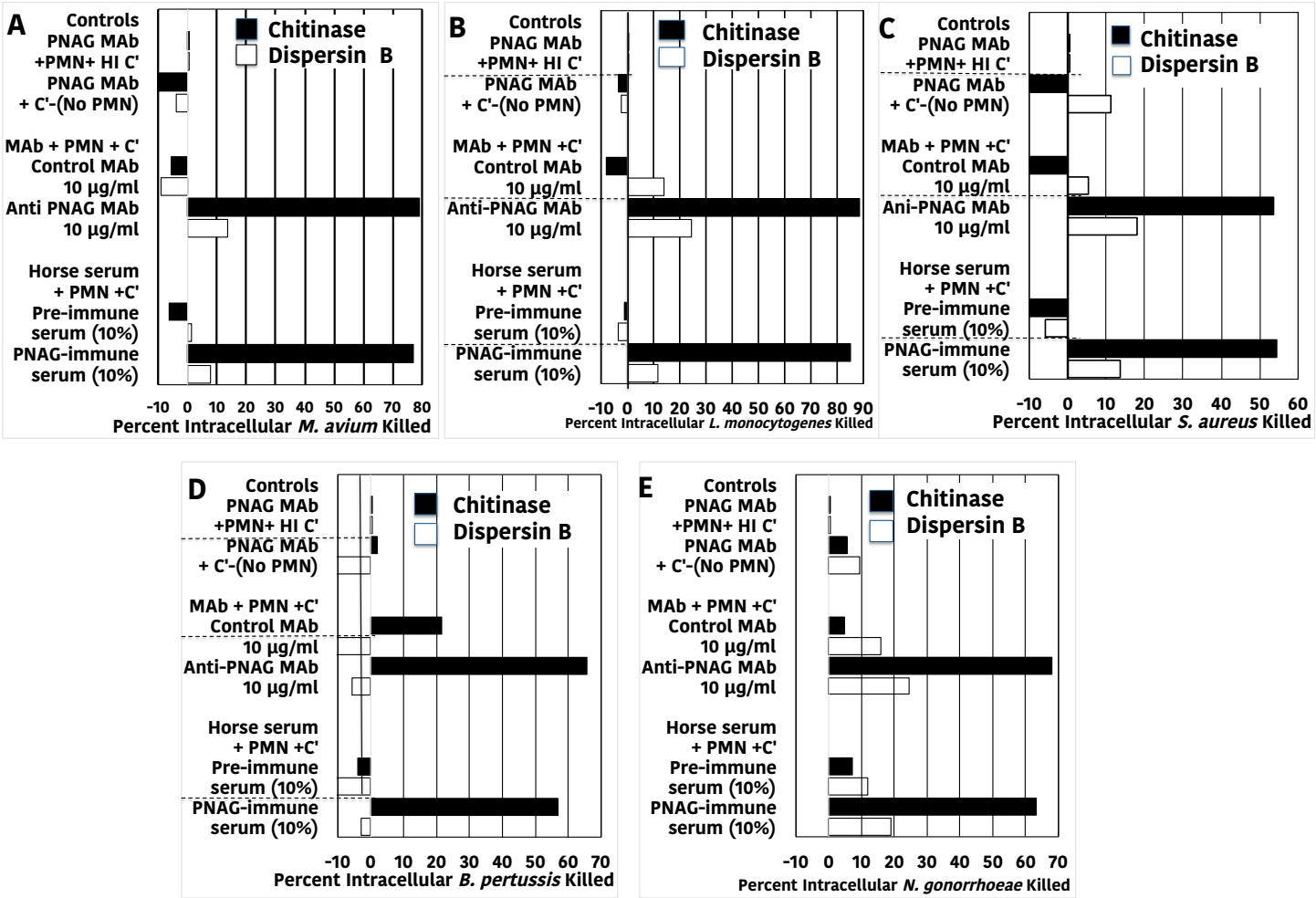


Fig. S8. Opsonic killing of intracellular pathogens by antibody to PNAG, complement (C') and PMN depends on infected-cell surface expression of PNAG. (**A-F**) Killing of intracellular bacteria by antibody, PMN and C' was markedly reduced following treatment of infected cells with Dispersin B (open bars) 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'.

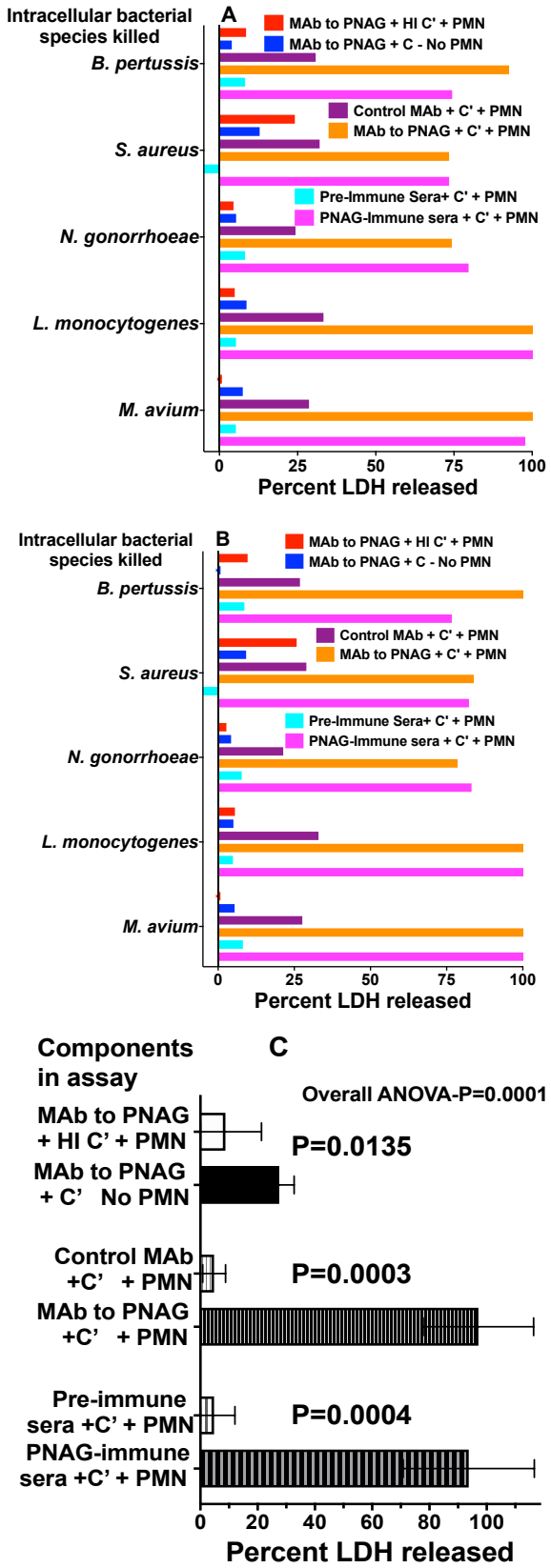


Fig. S9. Release of LDH from cells infected with intracellular pathogens following antibody plus immune effector treatment. (**A and B**) Replicate experiments measuring release of LDH from cells infected with indicated pathogen treated with 10 µg/ml control or anti-PNAG monoclonal or 10% polyclonal 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.

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