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Research paper

Cytokine expression by neutrophils of adult horses stimulated with virulent and avirulent *Rhodococcus equi in vitro*

Jessica R. Nerren [a,1], Susan Payne [b], Natalie D. Halbert [b], Ronald J. Martens [a], Noah D. Cohen [a,*]

[a] Department of Large Animal Clinical Sciences, College of Veterinary Medicine, Texas A&M University, College Station, TX 77843-4475, United States
[b] Department of Veterinary Pathobiology, College of Veterinary Medicine, Texas A&M University, College Station, TX 77843-4467, United States

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ABSTRACT

Rhodococcus equi is an intracellular pathogen of macrophages that causes rhodococcal pneumonia in foals and immunocompromised people. Evidence exists that neutrophils play a vital role in resistance to infection with *R. equi*; however, the means by which neutrophils exert their effects have not been clearly defined. In addition to directly killing bacteria, neutrophils also may exert a protective effect by linking innate and adaptive immune responses. In the present study we evaluated the cytokine expression profiles of adult equine neutrophils in response to stimulation with isogenic strains of virulent and avirulent *R. equi in vitro*. After 2 and 4 h incubation with virulent or avirulent *R. equi*, adult equine neutrophils expressed significantly ($P < 0.05$) greater tumor necrosis factor alpha (TNFα), interleukin (IL)-12p40, IL-6, IL-8 and IL-23p19 mRNA, but not interferon gamma (IFNγ) or IL-12p35 mRNA than unstimulated neutrophils. Furthermore, virulent *R. equi* induced significantly greater IL-23p19 mRNA than avirulent *R. equi*. These results demonstrate that *R. equi*-stimulated neutrophils are a source of many proinflammatory cytokines. Furthermore, these results suggest that IL-23 may be preferentially expressed over IL-12 in response to exposure with *R. equi*, and that this response may be more strongly induced by virulent *R. equi* than avirulent *R. equi*. Collectively, the data presented herein suggest a non-phagocytic role for neutrophils that may influence the type of adaptive immune response to *R. equi*.

1. Introduction

Rhodococcus equi is a gram-positive facultative intracellular pathogen of macrophages that causes severe and often fatal pneumonia in young foals and immunocompromised people (Giguere and Prescott, 1997). Infection in both horses and humans is characterized by severe pyogranulomatous pneumonia, similar to pneumonia caused by the closely related bacterium *Mycobacterium tuberculosis*. The impact of this disease is large because the associated morbidity and mortality rates are high, and treatment is generally prolonged, expensive, and associated with adverse effects (Giguere and Prescott, 1997). Furthermore, affected foals that survive are less likely than age-matched cohorts to race (Ainsworth et al., 1998). Currently, there are no vaccines available to prevent disease caused by *R. equi*. The only method shown to be effective for preventing *R. equi* pneumonia is transfusion of hyperimmune plasma (Martens et al., 1989), a procedure which is expensive, labor-intensive, and not universally effective (Hurley and Begg, 1995; Giguere and Prescott, 1997).

Many aspects of the epidemiology of *R. equi* remain unknown, despite the opportunities for prevention and

* Corresponding author. Tel.: +1 979 845 0741; fax: +1 979 847 8863.
E-mail address: ncohen@cvm.tamu.edu (N.D. Cohen).
[1] Present address: USDA-ARS-SPARC-FFSRU; 2881 F&B Rd., College Station, TX 77845, United States.

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control that would arise from such knowledge. In particular, it is unclear why some foals at endemic farms develop disease while other foals in the same environment do not. One possible explanation for this phenomenon is that differences in immune function among foals determine susceptibility to infection. Recently, it has been demonstrated that the ratio of CD4+/CD8+ T cells in blood samples from foals at 2 weeks of age was significantly lower in foals that subsequently developed *R. equi* pneumonia than in foals that did not develop the disease (Chaffin et al., 2004). In adult horses, the production of IFNγ by CD4+ and CD8+ T cells is required for pulmonary clearance of virulent *R. equi* (Hines et al., 2003). Furthermore, experiments conducted in mice demonstrated that both CD4+ and CD8+ T cells are important for protection against *R. equi*, with CD4+ cells being the primary cell type involved in protection (Nordmann et al., 1992; Kanaly et al., 1993, 1995, 1996; Ross et al., 1996).

Although it is generally accepted that adaptive immune responses are critical for resistance to infection with *R. equi* (Hines et al., 2003; Kanaly et al., 1993, 1995, 1996; Kasuga-Aoki et al., 1999; Kohler et al., 2003), there is also evidence indicating that innate immunity plays an important role. Innate immune responses of mononuclear blood cells play a role in the pathogenesis of *R. equi* (Darrah et al., 2004); however, several lines of evidence indicate that neutrophils also are critical for protection. Neutrophils are known to provide protection against a number of other intracellular pathogens (Dalrymple et al., 1995; Pedrosa et al., 2000; Godaly and Young, 2005). Some foals <1 week of age have markedly reduced bactericidal capacity for *R. equi* than other foals (Martens et al., 1988). Phagocytic function of foal neutrophils is less than that of adult horses (Demmers et al., 2001; McTaggart et al., 2001). Although evidence exists that this reduced phagocytic function is reversed by addition of adult sera (indicating reduced opsonic capacity of foal sera), foal neutrophils have also been demonstrated to have reduced phagocytic capacity relative to that of adult neutrophils, even when both groups are treated with adult sera (Gröndahl et al., 1999; Demmers et al., 2001). Blood concentrations of neutrophils at 2 weeks and 4 weeks of age were significantly lower among foals that developed *R. equi* pneumonia compared to foals at the same farms that did not develop disease (Chaffin et al., 2004). Additionally, it was recently reported that neutrophils play a protective role in mice infected with *R. equi* (Martens et al., 2005).

The mechanism by which neutrophils protect against intracellular infections such as *R. equi* remains unclear. Although neutrophils from horses are capable of killing *R. equi* following phagocytosis (Martens et al., 1988), non-phagocytic mechanisms also may play a role. In other species, neutrophils produce a variety of cytokines and chemokines that modulate the immune response by recruiting and activating other effector cells of the immune system (Appelberg, 2006). Consequently, neutrophils provide a crucial link between innate and adaptive cellular immune responses. To the authors' knowledge, the non-phagocytic role of inflammatory cytokine expression by neutrophils in response to infection with *R. equi* has not been reported. Thus, the purpose of this study was to compare the expression of selected pro-inflammatory cytokines by adult equine neutrophils stimulated *in vitro* by isogenic strains of virulent and avirulent *R. equi*. Cytokines examined included interleukin (IL)-6, IL-8, IL-12p35, IL-12p40, interferon gamma (IFNγ), tumor necrosis factor alpha (TNFα), and IL-23p19. Cytokine expression of adult equine neutrophils was studied because adult horses are known to be resistant to infection with *R. equi* unless immunocompromised, and it was considered important to understand the response of the resistant phenotype in order to conduct subsequent studies of foal neutrophils (Kohler et al., 2003). We hypothesized that cytokine expression by adult equine neutrophils would be significantly increased by exposure to live *R. equi* (virulent and avirulent isolates) and rhGM-CSF, and that expression would not differ significantly among the different stimuli.

2. Materials and methods

2.1. Reagents

RPMI-1640, Hanks balanced salt solution (HBSS), HEPES, phosphate-buffered saline (PBS), cell-culture-grade distilled water, RNase/DNase free distilled water, and $NaHCO_3$ were obtained from Invitrogen (Invitrogen Corp). Ficoll–Paque plus was obtained from Amersham Biosciences (Amersham Bioscience). Human recombinant granulocyte–macrophage colony stimulating factor (rhGM-CSF), bovine serum albumin (BSA), and sodium chloride (NaCl) were purchased from Sigma (Sigma–Aldrich). Primary working buffer consisted of the following: 1× HBSS, 10 mM HEPES, and 8.9 mM $NaHCO_3$. Lysis buffers A and B consisted of the following: 0.2 and 1.6% NaCl, respectively, 20 mM HEPES, and 1% BSA. The pH of all buffers and reagents was adjusted to 7.4 with NaOH.

2.2. Bacteria

A virulent plasmid-bearing strain (ATCC 33701 P+) and an avirulent plasmid-cured strain of (ATCC 33701 P−) *R. equi* were grown in *R. equi* minimal media overnight at 37 °C with rocking. The bacteria were then centrifuged at 2000 × *g* for 10 min. The bacterial pellets were washed 1× with RPMI 1640, and then resuspended at an approximate concentration of 1×10^8 CFU/mL RPMI 1640. The bacteria were aliquoted and frozen at −80 °C. Prior to use, the bacteria were thawed and 10-fold serial dilutions were performed in PBS. The *R. equi* were quantitatively cultured on trypticase soy agar plates supplemented with 5% sheep red blood cells. Prior to infecting equine neutrophils, the *R. equi* were opsonized at 37 °C for 1 h with 5% fresh-frozen serum obtained from a hyperimmunized mare.

2.3. Neutrophil purification

Whole blood was obtained from healthy adult horses using acid–citrate–dextrose (ACD) as an anticoagulant. After allowing the erythrocytes to sediment at room temperature for 1 h, the leukocyte rich plasma was layered onto Ficoll–Paque (specific gravity 1.077 ± 0.001 g/mL) and centrifuged at 500 × g for 20 min. The neutrophil pellet was

washed 2 times with primary working buffer, and the residual erythrocytes were lysed 2 times with a hypotonic lysis protocol as follows: each neutrophil pellet was resuspended in 7.5 mL of lysis buffer A (0.2% NaCl, 1% BSA, 20 mM HEPES). After a brief incubation of 60 s at room temperature, 7.5 mL of lysis buffer B (1.6% NaCl, 1% BSA, 20 mM HEPES) was added to the mixture. The tubes were then centrifuged at 300 × *g* for 10 min. After discarding the supernatant containing lysed erythrocytes, the pellet was resuspended in RPMI-1640. The neutrophils were >98% viable as indicated by exclusion of trypan blue. Furthermore, random samples of purified neutrophils were submitted to the Texas A&M Veterinary Teaching Hospital's clinical pathology laboratory for differential cell counts to ensure that the neutrophil purification method effectively removed contaminating lymphocytes and monocytes. Contaminating cell types composed <2% of the preps and included monocytes, lymphocytes, and an occasional eosinophil.

2.4. Neutrophil stimulation

Neutrophils ($\sim 2 \times 10^7$ mL^{-1}) were incubated for 2 and 4 h at 37 °C with the following: 100 ng rhGM-CSF (positive control), virulent *R. equi* (MOI 10:1), or avirulent *R. equi* (MOI 10:1). For each time-point, an unstimulated neutrophil control was included (negative control). An equal volume of RPMI-1640 was added to each of the unstimulated neutrophil controls so that the volume in every sample was the same. In order to determine the baseline level of cytokine expression as a point of reference, a 0-h unstimulated sample was obtained for every experiment. Following stimulation, all neutrophils were pelleted by centrifugation at 4 °C for 5 min at 2000 × *g*, and the supernatant was discarded.

2.5. RNA and cDNA preparation

RNA was extracted immediately from each neutrophil pellet using the RNeasy mini kit (Qiagen). The concentration and purity of the RNA was assessed by spectrophotometry. Aliquots of RNA were prepared in DNase/Rnase-free dH_2O to a final concentration of 0.05 μg/μL and frozen at −80 °C until subsequent cDNA synthesis. Prior to initiating cDNA synthesis, each aliquot of RNA was treated with amplification grade DNase I according to the manufacturer's protocol (Invitrogen Corp.). cDNA was subsequently synthesized with SuperScript III First-Strand synthesis System for RT-PCR (Invitrogen Corp.) using the manufacturer's protocol. In order to avoid synthesis of *R. equi*-derived cDNA, the cDNA synthesis was primed with oligo $(dT)_{20}$ primers provided with the cDNA synthesis kit. Upon completion of cDNA synthesis, all cDNA was stored at −80 °C until use.

2.6. Primer selection and real-time PCR conditions

Primer and probe sequences and their respective GenBank accession numbers are listed in Table 1. Intron-spanning equine β2 M, IL-12p35, IL-12p40, and IL-23p19 specific primer/probe premixes were designed using the Assays-by-Design software program (Applied Biosystems). Primer/probe sequences specific for equine IFNγ were obtained from a previous publication (Garton et al., 2002). Each 25-μL real-time PCR reaction contained

Table 1
Oligonucleotide primer and probe sequences for amplification of various equine cytokines and the β2 M control.

Gene (GenBank accession #)	Primer/probe	Sequence 5′–3′
β2M (X69083)	Forward	CGGGCTACTCTCCCTGACT
	Reverse	GGGTGACGTGAGTAAACCTGAAC
	Probe	CCGTCCCGCGTGTTC
IFNγ (D28520) Garton et al. (2002)	Forward	AAGTGAACTCATCAAAGTGATGAATGA
	Reverse	CGAAATGGATTCTGACTCCTCTTC
	Probe	TCGCCCAAAGCTAACCTGAGGAAGC
TNFα (M64087) Garton et al. (2002)	Forward	GCTCCAGACGGTGCTTGTG
	Reverse	GCCGATCACCCCAAAGTG
	Probe	TGTCGCAGGAGCCACCACGCT
IL-6 (U64794)	Forward	GAAAAAGACGGATGCTTCCAATCTG
	Reverse	TCCGAAAGACCAGTGGTGATTTT
	Probe	CAGGTCTCCTGATTGAAC
IL-8 (AY184956)	Forward	GCCACACTGCGAAAACTCA
	Reverse	GCACAATAATCTGCACCCACTTTG
	Probe	ACGAGCTTTACAATGATTTC
IL-12p35 (Y11130)	Forward	CCCGGAAAGGCCTCTTCT
	Reverse	ACCTGGTACATCTTCAAGTCCTCAT
	Probe	TAAGGCACAGCGTCATCA
IL-12p40 (Y11129)	Forward	TCACAAGAAGGAAGATGGAATTTGGT
	Reverse	CCGGAATAATTCTTTGCCTCACATTT
	Probe	TTTAAAAGACCAGAAAGAATCC
IL-23p19 (NM_001082522)	Forward	GCTGTGATCCTGAAGGACTCA
	Reverse	CCCTGGTGGATCCTTTGCA
	Probe	CAGGGCTGACTGTTGTC

the following: 2.5 μL plasmid or template cDNA; 1.25 μL 20× primer/probe premix (Applied Biosystems); 12.5 μL 1× TaqMan Universal Master Mix (Applied Biosystems); and molecular-grade water (Gibco, BRL). Amplification and data analysis were carried out on a GeneAmp 7500 Sequence Detection System (Applied Biosystems). The thermal profile consisted of an initial hold at 50 °C for 2 min, followed by a single denaturation at 95 °C for 10 min, and then 40 cycles of 95 °C for 15 s, 60 °C for 60 s. The resulting Ct values were normalized to the endogenous control, β2M, and the relative quantification values were determined using the ddCt method (Livak and Schmittgen, 2001) with time 0 h as the calibrator.

2.7. Plasmid selection and design

Clones containing the cDNA-derived sequences of β2M, IL-6, IL-8, and TNFα were generously provided by the Pratt Laboratory (University of Georgia). Intron-spanning equine cloning primers specific for IFNγ, IL-12p35, IL-12p40, and IL-23p19 were designed using the Primer3 online software program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The gene targets were amplified from equine cDNA (obtained from neutrophils stimulated with *R. equi*), and subcloned into the TOPO-TA (Invitrogen Corp) vector, according to the manufacturer's protocol. Plasmid DNA was purified from clones corresponding to each of the 7 genes using the Fast Plasmid Mini kit (Eppendorf), and the inserts were confirmed through sequencing. Plasmid DNA concentrations were determined spectrophotometrically and DNA stocks were stored at −80 °C.

2.8. Assay validation

Assay validation was performed to determine the inter- and intra-assay variation of the real-time primers and probes, and to demonstrate the reproducibility of the methods used for sample preparation. Primers and probes for some of the cytokines (TNFα, IL-6, and IL-8) were validated in another laboratory (Allen et al., 2007), using methods identical to those described here. To validate the RNA extraction and cDNA synthesis method, 3 RNA extractions were performed on a single unstimulated neutrophil preparation. For each RNA extraction, 2 separate cDNA syntheses were performed so that there were a total of 6 cDNA samples. Real-time PCR was performed in triplicate on all 6 cDNA preparations using the endogenous control, β2M. Results from the real-time PCR were evaluated in terms of the coefficient of variation (CV), which was calculated by dividing the mean Ct value by the respective standard deviation.

To confirm the product size and specificity of each primer/probe set, all PCR products from a single real-time PCR assay were run on an agarose gel. The same PCR products were purified using the Stratprep PCR purification kit (Stratagene) and bidirectional sequencing was performed using the real-time PCR primers. The derived sequences were compared with those used to design primers/probes and with a BLAST search of GenBank sequences (http://www.ncbi.nlm.nih.gov) to confirm target specificity.

Inter-assay variability was determined for each gene of interest by running the triplicate plasmid DNA dilutions (30 reactions/gene) in 2 separate real-time PCR assays. Intra-assay variability was determined for each gene of interest by running 10 replicates of every other dilution (50 reactions/gene) in a single real-time PCR assay.

2.9. Statistical analysis

Ratio data were transformed logarithmically for analysis because they were not distributed normally. Data were analyzed using linear mixed-effects models (Pinheiro and Bates, 2000); to account for repeated measures on individuals, horse was modeled as a random effect and time, stimuli, and their respective interaction terms were modeled as fixed effects. Model fit was assessed by graphical methods to determine whether there was evidence of poor fit or violation of model assumptions. Post-hoc testing of between-group differences was made using the method of Scheffé. A significance level of $P < 0.05$ was used. Statistical analyses were performed using S-PLUS (version 7.0; Insightful, Inc.).

3. Results

3.1. Assay validation

To validate the method of sample preparation, 6 cDNA samples (2 from each of 3 RNA samples) were analyzed concurrently by real-time PCR. The mean CVs for RNA and cDNA preparation across all sets were 2.8% (range 1.4–4.9%) and 2.3% (range 1.4–3.7%), respectively. The CVs determined for the intra- and inter-assay variation of each gene are listed in Table 2.

Gel electrophoresis was performed on the PCR products to verify the specificity and size of the amplicon generated from each primer/probe set (picture not shown). Bidirectional sequencing and a BLAST database search of each of the sequences generated confirmed that all primer/probe sets amplified the intended gene.

3.2. Cytokine expression of adult equine neutrophils

There were no significant changes in expression of IFNγ or IL-12p35 by neutrophils following any stimulus relative to unstimulated neutrophils. Although expression of IFNγ tended to be increased after 4 h for all neutrophils, this difference was not significant when accounting for effects of stimulus and time.

There were significant effects of stimulus and time on expression of TNFα by neutrophils (Fig. 1a). Relative to unstimulated neutrophils, expression of TNFα by rhGM-

Table 2
Inter- and intra-assay CVs.

Gene	Inter-assay CV	Intra-assay CV
β2M	2.24% (range 0.81–3.87%)	0.76% (range 0.22–1.94%)
IL-12p35	2.49% (range 0.11–11.4%)	0.81% (range 0.62–0.98%)
IL-12p40	2.45% (range 0.55–7.94%)	0.5% (range 0.32–0.87%)
IFNγ	3.16% (range 1.31–4.11%)	0.53% (range 0.23–0.97%)
IL-23p19	2.37% (range 1.27–3.8%)	1.77% (range 0.61–3.64%)

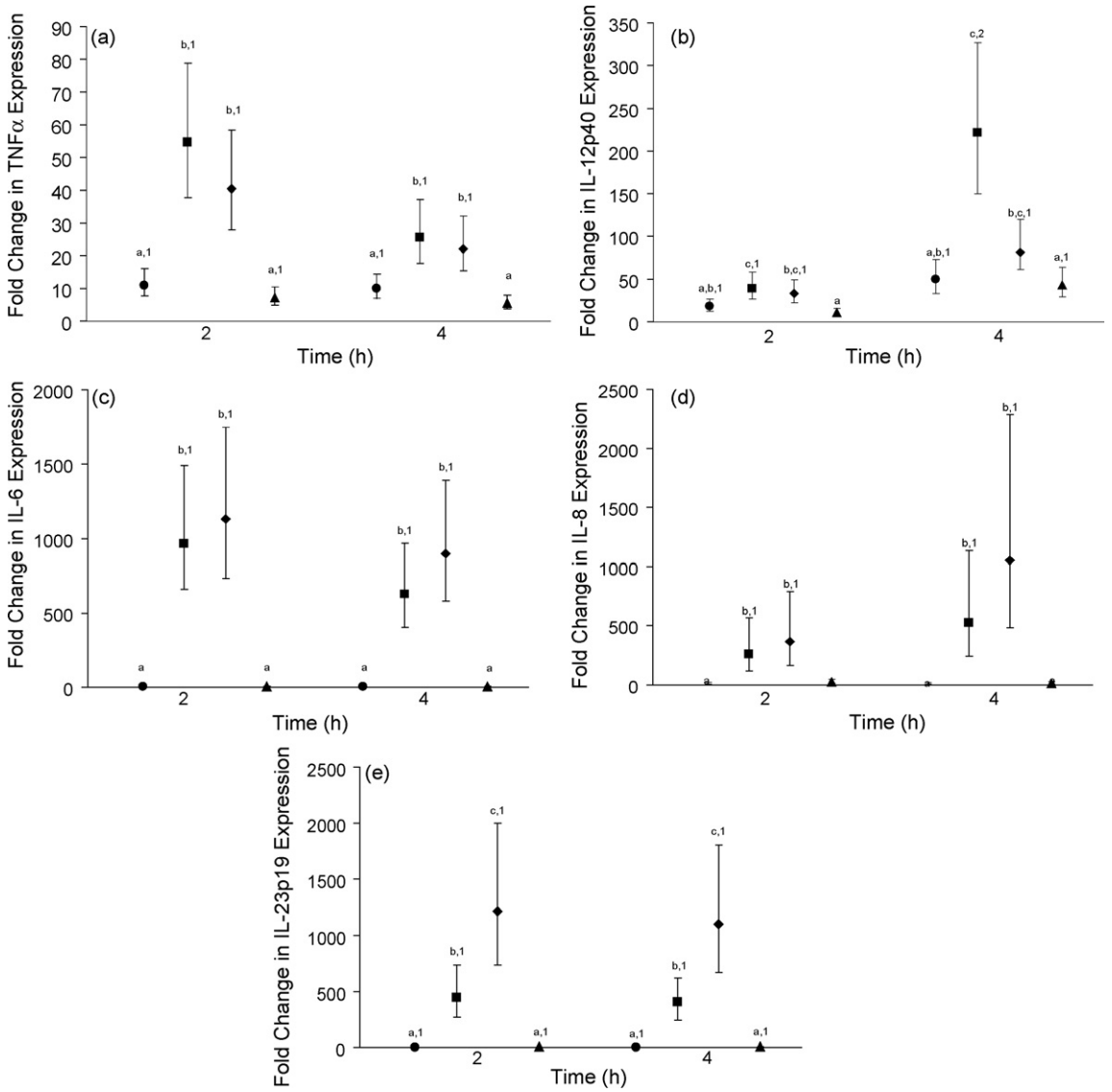

Fig. 1. Expression of (a) TNFα, (b) IL-12p40, (c) IL-6, (d) IL-8, and (e) IL-23p19 mRNA in unstimulated (●) neutrophils and neutrophils stimulated with avirulent (■) *R. equi*, virulent (◆) *R. equi*, and rhGM-CSF (▲). All values were normalized to the endogenous control, β2M, and fold-changes were calculated using baseline control values (unstimulated cells at time 0). Values represent the mean fold changes in expression (relative to baseline) of 5 adult horses and thin vertical lines extending to thin horizontal lines represent the time-specific 95% confidence intervals for each stimulus. Stimuli with different letters differed significantly ($P < 0.05$) from one another at a given time. Stimuli with a number 1 differed significantly ($P < 0.05$) from baseline.

CSF-stimulated neutrophils was not significantly different at both times. Relative to unstimulated and rhGM-CSF-stimulated neutrophils, expression of TNFα by virulent and avirulent *R. equi*-stimulated neutrophils was significantly ($P < 0.05$) greater at 2 and 4 h; however, there was no significant difference at either time in expression of TNFα between neutrophils stimulated with virulent or avirulent *R. equi*. Expression of TNFα by all neutrophils increased significantly ($P < 0.05$) at both times relative to baseline, with the exception of neutrophils stimulated with rhGM-CSF for 4 h which were not significantly different than baseline.

There were significant effects of stimulus and time on expression of IL-12p40 by neutrophils (Fig. 1b). Relative to unstimulated neutrophils, expression of IL-12p40 by avirulent *R. equi*-stimulated neutrophils was significantly ($P < 0.05$) greater at 2 and 4 h; however, there was no significant difference in expression of IL-12p40 at either time-point by virulent *R. equi*-stimulated neutrophils relative to unstimulated neutrophils. Relative to rhGM-CSF-stimulated neutrophils, expression of IL-12p40 by virulent and avirulent *R. equi*-stimulated neutrophils was significantly ($P < 0.05$) greater at 2 and 4 h. There was no significant difference at either time in expression of IL-12p40 between neutrophils stimulated with virulent or avirulent *R. equi*. Expression also did not differ significantly at either time-point between unstimulated and rhGM-CSF-stimulated neutrophils. With the exception of neutrophils

stimulated with rhGM-CSF for 2 h, expression of IL-12p40 by all neutrophils increased significantly ($P < 0.05$) at both times relative to baseline. There was significantly ($P < 0.05$) higher expression of IL-12p40 by neutrophils stimulated with avirulent *R. equi* for 4 h, than those stimulated with avirulent *R. equi* for 2 h.

There were significant effects of stimulus and time on expression of IL-6 by neutrophils (Fig. 1c). Relative to unstimulated and rhGM-CSF-stimulated neutrophils, expression of IL-6 by virulent and avirulent *R. equi*-stimulated neutrophils was significantly ($P < 0.05$) greater at 2 and 4 h; however, there was no significant difference at either time in expression of IL-6 between neutrophils stimulated with virulent or avirulent *R. equi*. Relative to baseline, there was no significant difference at either time in expression of IL-6 by unstimulated or rhGM-CSF-stimulated neutrophils. Relative to baseline, expression of IL-6 by neutrophils stimulated with virulent and avirulent *R. equi* was significantly ($P < 0.05$) greater at 2 and 4 h.

There were significant effects of stimulus and time on expression of IL-8 by neutrophils (Fig. 1d). Relative to unstimulated and rhGM-CSF-stimulated neutrophils, expression of IL-8 by virulent and avirulent *R. equi*-stimulated neutrophils was significantly ($P < 0.05$) greater at 2 and 4 h; however, there was no significant difference at either time in expression of IL-8 between neutrophils stimulated with virulent or avirulent *R. equi*. Relative to baseline, expression of IL-8 was increased significantly by neutrophils stimulated with rhGM-CSF for 2 h and by neutrophils stimulated with virulent and avirulent *R. equi* for 2 and 4 h.

There were significant effects of stimulus and time on expression of IL-23p19 (Fig. 1e). Relative to unstimulated and rhGM-CSF-stimulated neutrophils, expression of IL-23p19 by neutrophils stimulated with virulent and avirulent *R. equi* was significantly ($P < 0.05$) greater at 2 and 4 h. At 2 and 4 h, there was significantly ($P < 0.05$) greater expression of IL-23p19 by neutrophils stimulated with virulent *R. equi* than those stimulated with avirulent *R. equi*. There was no significant difference in expression between unstimulated and rhGM-CSF stimulated neutrophils at any time-point. Expression of IL-23p19 by all neutrophils (irrespective of stimulus) increased significantly ($P < 0.05$) at both times relative to baseline.

4. Discussion

During primary infection with many intracellular bacteria, the host relies on innate immune responses to contain the replication of organisms until specific adaptive immunity develops (Pedrosa et al., 2000; Bennouna et al., 2003). Thus, innate immunity is likely important for controlling early *R. equi* infections. Evidence exists that immaturity of innate immune responses and individual variability in those responses may contribute to increased susceptibility to infection by *R. equi* (Boyd et al., 2003; Breathnach et al., 2006; Chaffin et al., 2004; Darrah et al., 2004). It is increasingly apparent that neutrophils play an important non-phagocytic role in controlling intracellular infections, particularly early in the course of disease (Pedrosa et al., 2000). The *in vitro* bactericidal capacity and the protective role of neutrophils against *R. equi* in mice experimentally infected with *R. equi* have been demonstrated previously (Martens et al., 1988, 2005). However, the non-phagocytic role of inflammatory cytokine expression by neutrophils in response to infection with *R. equi* has not been reported.

We examined the expression of 7 pro-inflammatory cytokines that are known to play important roles in immunity to infectious disease. Adult equine neutrophils were stimulated for 2 and 4 h with *R. equi* (virulent and avirulent) and rhGM-CSF. These incubation times were chosen based on the short life-span of the neutrophil (Pedrosa et al., 2000), reports that neutrophils contain stores of preformed cytokines that are rapidly released and subsequently replenished (Denkers et al., 2003) (although these studies suggest the possibility that transcriptional studies alone will not reflect the entire contribution of neutrophils to the immune response to *R. equi* or other bacteria) and studies done in heterophils demonstrating that cytokine expression increases significantly after 2 h stimulation and plateaus after 4 h stimulation (Kogut et al., 2003; Swaggerty et al., 2004). Maximum expression of many inflammatory cytokines within a 4-h period has also been demonstrated to occur in *R. equi*-stimulated macrophages (Giguere and Prescott, 1998). Furthermore, preliminary time-course experiments conducted in our laboratory demonstrated that the expression of these cytokine mRNAs was most consistently elevated between 2 and 4 h of stimulation, whereas cytokine mRNA expression was not consistently elevated after 1 and 24 h stimulation. As discussed below, the times used to evaluate expression likely may not have been optimal for all of the cytokines studied. A wide range of MOIs has been reported in the *R. equi* literature (Martens et al., 1988; Hondalus and Mosser, 1994; Giguere and Prescott, 1998). An MOI of 10:1 was chosen in order to maximize the stimulating effects of *R. equi* on neutrophils while minimizing any potential adverse effects that might arise from adding too many bacteria. A single MOI was studied because preliminary studies indicate this MOI effectively stimulated cytokine expression by equine neutrophils and financial limitations precluded evaluating additional MOIs.

The precise biological role of IL-6 is unclear. Some studies suggest that IL-6 is a Th2 cytokine (Rincon et al., 1997), while others suggest it is a Th1 cytokine (Leal et al., 1999; Saunders et al., 2000). Evidence for the latter, however, is stronger. Mice deficient in IL-6 appear to be highly susceptible to infection with the intracellular pathogens *Listeria monocytogenes* (Dalrymple et al., 1995) and *M. tuberculosis* (Ladel et al., 1997). In this study, we demonstrated that equine neutrophils exposed to virulent and avirulent *R. equi* displayed a significant increase in expression of IL-6, and that increase was sustained throughout the period of observation. If indeed IL-6 promotes a Th1-type response, then expression of this cytokine in response to rhodococcal infection would likely be beneficial to the host, particularly if it were to promote IFNγ production early in the course of disease (Boyd et al., 2003; Breathnach et al., 2006). Additional studies will need to be conducted in order to clarify the nature of the role of

IL-6 in *R. equi* infection, but the data we report here strongly support such a role.

IL-8 is a strong chemoattractant for neutrophils, monocytes, and T cells (Baggiolini et al., 1995; Zhang et al., 1995), and has been shown to enhance killing of *M. tuberculosis* by neutrophils (Godaly and Young, 2005). In this study, adult equine neutrophils demonstrated a marked increase in expression of IL-8 upon stimulation with *R. equi*. We hypothesize that neutrophils play a vital role in the early host defense against *R. equi*. It is possible that they do this through the production of IL-8, thereby recruiting additional neutrophils to the site of infection (and thus killing more *R. equi*). Furthermore, the ability of IL-8 to attract T lymphocytes may be important for facilitating antigen presentation at the immunologic synapse and initiating a cell-mediated immune response (Baggiolini et al., 1995).

IFNγ is critical for induction of a Th1-type cell-mediated immune response (Langrish et al., 2004). There is unequivocal evidence of the protective nature of IFNγ in bacterial infections such as *M. tuberculosis* (Flynn et al., 1993). Likewise, several studies have shown that IFNγ is important in protection against and pulmonary clearance of *R. equi* in experimentally infected mice (Kanaly et al., 1993, 1995). Furthermore, PBMCs and pulmonary lymphocytes obtained from neonatal foals produce relatively low amounts of IFNγ, which may explain their susceptibility to infection with *R. equi* (Boyd et al., 2003; Breathnach et al., 2006). In the present study there was an effect of time on expression of IFNγ; however, this effect was most likely attributable to stimulation of the neutrophils by processing. The expression of IFNγ by adult equine neutrophils was not significantly increased by stimulation with virulent or avirulent *R. equi*. One possible explanation for failure to induce a significant response is that maximal expression of IFNγ likely occurred after the time-points used in our study, viz., 2 and 4 h. We selected these time-points based on preliminary data (unpublished) indicating that cytokine expression was most consistently measured within a 4-h time period, and a previous report that demonstrated a 450% increase in IFNγ mRNA expression by human neutrophils stimulated for 1 h with LPS + IL-12 + IL-15 (Ethuin et al., 2004). In another report, however, neutrophils infected with *Nocardia asteroides* produced significant amounts of IFNγ as late as 3 days post-infection (Ellis and Beaman, 2002). Moreover, a previous publication demonstrated that expression of IFNγ mRNA by equine PBMC's was 24-fold higher after 24 h of ConA stimulation than 4 h of ConA stimulation (Giguere and Prescott, 1999). Thus, it is possible that expression of IFNγ mRNA by equine neutrophils did not occur at the time-points we measured. Alternatively, equine neutrophils may not be a major source of this cytokine, a finding consistent with previous observations (Joubert et al., 2001).

The importance of TNFα in the course of disease arising from infection with *R. equi* has been demonstrated in mice. Depletion of TNFα in these mice resulted in a lethal course of infection despite the mice being given a sub-lethal dose of virulent *R. equi* (Kasuga-Aoki et al., 1999). In this study, expression of TNFα by equine neutrophils was significantly increased in response to stimulation with virulent and avirulent *R. equi* following both 2 and 4 h of incubation.

Interleukin 12 is a heterodimer composed of the p35 and the p40 subunits (Langrish et al., 2004). Animal studies have shown that IL-12 is critical for the control of many intracellular infections, such as those caused by *Mycobacteria* spp. and *Salmonella* spp. (Fieschi et al., 2003). In this study, there was very little expression of IL-12p35 detected by real-time PCR. The slight expression that was present may have been due to contaminating lymphocytes and macrophages, which constituted less than 2% of the neutrophil preparations. As with IFNγ, it is possible that expression of IL-12p35 mRNA did not occur at the time-points evaluated. Despite the lack of differential expression of IL-12p35, there was differential expression of IL-12p40. This finding is consistent with a recent publication that demonstrated the differential expression of IL-12p40, but not IL-12p35, by adult equine macrophages and dendritic cells in response to stimulation with cytosine-phosphate-guanosine oligodeoxynucleotides (CpG-ODN) (Flaminio et al., 2007). Although the distinct roles of the p35 and p40 subunits are not clear, the p40 subunit is a known component of another important heterodimer (composed of the IL-12p40 and IL-23p19 subunits), IL-23 (Harrington et al., 2006). Thus, we investigated the possibility that neutrophil stimulation with *R. equi* induced the production of IL-23 rather than IL-12, by measuring the differential expression of IL-23p19. Indeed, we found that expression of IL-23 was significantly increased upon exposure to *R. equi*. This could have important immunological implications, because the production of IL-23 drives the development of an alternative T-cell subset characterized by the production of the pro-inflammatory cytokine IL-17, which acts in part to mobilize neutrophils from the bone marrow and induce the acute phase response via IL-6 and TNFα production (Langrish et al., 2004; Harrington et al., 2006). Thus, an entirely different T-cell subset may play a crucial and/or complementary role in the outcome of infection with *R. equi*. Interestingly, the virulent strain of *R. equi* induced significantly greater expression than did the avirulent strain. Because adult horses are generally refractory to disease caused by *R. equi*, the importance of the observed difference between virulent and avirulent *R. equi* with respect to pathogenesis and host/agent interactions is unknown. It remains to be determined whether this difference occurs in foals; however, it will be important to examine the susceptible phenotype with respect to IL-23. If indeed foals respond differently, this knowledge may shed light on the pathogenesis of *R. equi*. Clearly, these observations merit further testing.

We selected rhGM-CSF as a positive control on the basis of a previous report that demonstrated the activity of rhGM-CSF on equine cells (Hammond et al., 1999), and lack of a source of equine GM-CSF. The addition of rhGM-CSF, however, failed to stimulate cytokine expression in adult equine neutrophils. One possible reason for this is that rhGM-CSF displays some degree of species-specificity: it is active in stimulating cells of dogs but not those of mice (Lee et al., 1985; Mayer et al., 1990). Recently (after our studies were initiated), it was reported that equine GM-CSF (eqGM-CSF) is structurally and biologically different at

the N-terminus and the C-terminus from other mammalian homologues (Mauel et al., 2006), a finding that could explain the lack of stimulation of equine neutrophils by rhGM-CSF observed in this study. It is also possible that a different source of rhGM-CSF could result in sufficient stimulation of cytokine mRNA expression.

The ability of live *R. equi* to stimulate the expression of pro-inflammatory cytokines by equine neutrophils strongly suggests that neutrophil derived cytokines contribute importantly to the cytokine milieu that drives Th1 polarization and development of an adaptive immune response. Additional studies are necessary to correlate cytokine mRNA expression with cytokine secretion, to evaluate the quality and quantity of cytokine expression profiles of young foals, and to assess temporal differences in these profiles between foals that are susceptible to disease caused by *R. equi* and foals that are not. This study is the first study to describe cytokine expression by *R. equi*-infected equine neutrophils, and importantly the potential role of IL-23 in immunity to infection with *R. equi*. This study will serve as precedent for future studies involving the interaction of the developing immune system of foals and *R. equi*.

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