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Short communication

Activation of peripheral blood monocytes results in more robust production of IL-10 in neonatal foals compared to adult horses

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ABSTRACT

Foals are particularly vulnerable to infection by *Rhodococcus equi* during the first 2 weeks of life whereas mature horses are not. While an innate immunodeficiency likely accounts for this clinically relevant vulnerability, the factors that contribute to infection by *R. equi* have not been fully elucidated. In this study, we demonstrate that cells of the monocyte lineage, including monocytes, macrophages, and dendritic cells, that have been activated with LPS and IFN-γ, respond with a statistically significant, greater amount of cytokine mRNA production of IL-10, IL-12p35, and IL-12p40 than unstimulated control cells. Interestingly, activation of neonatal cells resulted in a twofold log increase in baseline cytokine mRNA expression of IL-10 compared with adult cells. In contrast, no significant differences in mean cytokine mRNA expression of IL-12p35 and IL-12p40 were detected, suggesting that the defect in chromosomal remodeling that prevents IL-12p35 gene transcription as a cause for decreased IL-12 synthesis in human neonates is not a likely occurrence in equine neonates. Collectively, these differences indicate that *in vivo* activation of equine cells of the monocyte lineage may result in different autocrine and paracrine cellular responses that vary according to age, with potential impact on regulation of adaptive and innate immune responses.

1. Introduction

It has been well documented across species that the early post-natal period is characterized by an increased susceptibility to infectious diseases (Ridge et al., 1996). This vulnerability is especially apparent for diseases caused by intracellular pathogens, suggesting a functional deficit in cell-mediated immunity (Ridge et al., 1996). While immature T-cell responses may contribute to immunologic immaturity in several species (Harris et al., 1992), it is also likely that functional deficits in antigen presenting cells (APC) and/or macrophages contribute to reduced cell-mediated immune responses during the neonatal and juvenile periods (Trivedi et al., 1997).

Across species, T helper 1 lymphocytes secrete gamma interferon (IFN-γ), interleukin 2 (IL-2), and tumor necrosis factor-β, all of which promote cellular immune responses. Evidence suggests that interleukin 12 (IL-12) favors a Type 1, interferon gamma (IFN-γ) T cell response, whereas its paucity favors a Type 2 polarized cytokine response. Monocytes, macrophages, and dendritic cells are the principal cell types of humans that produce IL-12 (Marodi, 2002; Lee et al., 1996). Type 1 responses are critical for effective cell-mediated immunity to intracellular pathogens and are characterized by high IL-12 and low IL-10. Interestingly, previous studies in humans have demonstrated a protracted maturation of IL-12 synthetic capacity during childhood; in fact, there is a slow maturation of IL-12 synthetic capacity in children as they age (Upham et al.,

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2002). IL-12p70 exists as a heterodimer, IL-12p35 together with IL-12p40. Recent studies implicate a defect in chromosomal remodeling that prevents IL-12p35 gene transcription as one cause for decreased juvenile IL-12 synthesis (Goriely et al., 2004). Collectively, these studies suggest that age-related differences can affect expression of IL-12, a cytokine with immunoregulatory potential.

IL-10 is a key immunoregulatory cytokine, helping maintain a balance between pathology and protection. It modulates the host inflammatory response to microbial products, such as LPS and peptidoglycan, which can lead to septic shock and multi-organ dysfunction (Standiford et al., 1995; Greenberger et al., 1995; Kato et al., 1995; van der Poll et al., 1995; Cusumano et al., 1996; Florquin et al., 1994; Bean et al., 1993). In addition, IL-10 inhibits the expression of most inducible cytokines and secondary mediators that contribute to inflammation. These include the chemokines important in recruitment of monocytes, neutrophils, dendritic cells, and T lymphocytes (Reddy et al., 2001). In addition, IL-10 suppresses the transcription of both the IL-12p35 and IL-12p40 heterodimers (Zhou et al., 2004).

To gain insight into the role cells of the monocyte lineage play in development of cell-mediated immune responses in neonatal foals, we enriched PBMC for cells of the monocyte lineage for *in vitro* propagation. The resultant initial cell population therefore included a predominance of monocytes, dendritic cells, and few lymphocytes. Within hours of *in vitro* culture, the monocytes became adherent and subsequently developed into macrophages. We demonstrated that both neonatal and adult cells of the monocyte lineage respond to *in vitro* stimulation of LPS and INF-γ. In both cases, cells stimulated with LPS/IFN-γ responded with a statistically significant, greater amount of cytokine mRNA production of IL-10, IL-12p35, and IL-12p40 than unstimulated controls. However, following stimulation, the fold-change of expression of IL-10 was significantly greater in neonates than in adults, suggesting an intrinsic bias of foal cells to respond to LPS/IFN-γ stimulation with production of a distinctly different immunoregulatory cytokine profile than adults.

2. Materials and methods

2.1. Subjects

10 healthy foals less than 48 h of age were used as subjects for isolation of PBMC. This age-group of foals was selected as (1) it is most likely to demonstrate functional differences when compared with adults; (2) the rate of functional maturation of equine APC is unknown; (3) infection with *Rhodococcus equi* may occur within the first 2 weeks of life (Horowitz et al., 2001). For comparison, 6 geldings, all at least 4 years or older were used. The procedures and experimental techniques were approved by the Institutional Animal Care and Use Committee of Iowa State University.

2.2. Blood samples

180 ml of whole blood were obtained by jugular venipuncture from the foals. 450 ml were similarly obtained from the geldings. Buffy coats were collected by centrifugation of whole blood in 50 ml conical tubes at 800 g for 30 min at 25 °C. The resultant buffy coats for each animal sampled were aliquoted into two 50 ml conical tubes, diluted with 50 ml autologous plasma, and centrifuged at 800 g for 30 min at 25 °C. Buffy coat cells were layered onto a double gradient of Histopaque 1077 and 1119 (Sigma–Aldrich, St. Louis, MO) and the mononuclear cell and granulocyte fractions were obtained using a previously described protocol (Raabe et al., 1998; Smith et al., 1998). To remove platelets from the mononuclear cell fraction, a rinse was performed. The mononuclear cell fraction was then characterized by flow cytometry using forward/side scatter profiles and the following cell-type specific fluorescent markers: VMRD E18A/equine B cell, VMRD HB88A/equine CD2, VMRD DH59B/equine CD172a.

2.3. Cell enrichment

1×10^8 mononuclear cells were subjected to AutoMACs separation by first resuspending the cells in 1 ml of cold MACs buffer (PBS with 0.5% bovine serum albumin and 2 mM EDTA). Enrichment of monocytes and circulating dendritic cells was performed with a primary antibody (25 μg/ml VMRD DH59B/equine CD172a) and 100 μl/ml goat anti-mouse IgG microbeads (Miltenyi Biotec, Auburn, CA) according to the company's protocol. Cells were characterized before and after enrichment by flow cytometry using forward/side scatter profiles and the following cell-type specific fluorescent markers: VMRD E18A/equine B cell, VMRD HB88A/equine CD2, VMRD DH59B/equine CD172a.

2.4. Cell culture

Enriched cells were cultured in 12-well plates with media alone or with media containing 50 ng/ml equine IFN-γ (R&D Systems, catalog # 1586-HG) with 10 μg/ml LPS O26:B6 (Sigma catalog # L8274) added 3 h later. Following a 24 h incubation, cells were harvested by adding 500 μl of Marligen lysis buffer (Rapid Total RNA Purification System, Marligen Biosciences, Ijamsville, MD) and the resultant lysate was frozen in 1.5 ml eppendorf tubes at −80 °C until needed for RNA extraction.

2.5. RNA extraction

Total RNA was extracted with the Rapid Total RNA Purification System (Marligen Biosciences, Ijamsville, MD) according to the manufacturer's protocol. Briefly, cell lysates were thawed, homogenized by aspiration through a 21 g needle, precipitated with 70% ethanol, and the material was exposed to an RNA binding column; RNA was eluted in 50 μL of RNase-free water. The concentration of the RNA was measured spectrophotometrically and treated with 1 U of DNase I (Ambion TURBO DNA-free System, Austin, TX) per μg of RNA according to product specifications. Sample purity (260/280 O.D. ratio) was between 1.8 and 2.1 for all RNA samples.

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2.6. Quantitation of cytokine transcripts

To measure expression of cytokine mRNA of IL-12p35, IL-12p40, and IL-10, real-time one-step quantitative RT-PCR was performed with each RNA sample using a previously described approach (Kawashima et al., 2006; Gallup and Ackermann, 2006). Prior to qPCR analysis, the DNase treated RNA samples were diluted 1:10 with nuclease-free water and a test plate was run on an equivolumetric mixture of the samples (termed "stock I") to determine which RNA dilution range provided the best signal (lacked inhibition and exhibited log-linear behavior and high amplification efficiencies) for targets of interest. The 1:10 samples were then further diluted on a per-sample, per-target basis (according to results from the test plate analysis) to their ideal ng/μL concentrations for final qPCR analysis. Samples were set up in triplicate or quadruplicate 25 μl reactions using the Invitrogen One-step (Invitrogen Corp., Carlsbad, California) Master Mix in 96-well qPCR reaction plates (ABgene, Rockford, IL). Samples were run using the MX3005P qPCR System (Stratagene, La Jolla, CA) for detection and relative quantification of the targets. After reverse transcription for 15 min at 55 °C and denaturation for 2 min at 95 °C, 45 PCR cycles (95 °C for 15 s and 60 °C for 30 s) were performed. Forward and reverse primers specific for each cytokine were used at concentrations of 775 nM (IDT, Coralville, IA). Taqman probes labeled with reporter dye 6-carboxyfluorescein (FAM) and dark quencher (DQ) dye Iowa Black™ (IDT, Coralville, IA) were used at 150 nm. Primer pairs and probes were designed with Primer Express 2.0 software (Applied Biosystems) based on GenBank sequence data for the cytokines of interest and for the housekeeping gene (Table 1). The negative, no-template control (NTC) wells contained nuclease-free water (Ambion).

2.7. Analysis of gene expression

All samples were run in triplicate or quadruplicate and quantities of transcripts were derived using the standard-curve quantitation method (Ginzinger, 2002). Briefly, a threefold serial dilution of a positive control (stock I) was used to generate a standard curve for each template (Gallup and Ackermann, 2006). Ct values for each template were plotted as a function of concentration (log scale); the slope and intercept of a fitted standard curve were estimated through linear regression. Quantities of unknown samples were obtained by interpolating unknown concentrations from the standard curves. In addition, PCR efficiencies were calculated based on slopes of fitted standard curves.

2.8. Statistical analysis

An exploratory analysis was carried out in order to characterize the statistical properties of qPCR levels of target and reference genes. Thus, normality of data was checked through Kolmogorov–Smirnov tests and inspection of qq-norm plots. Statistical summaries and summary plots were inspected for random variability due to animals or assays. Statistical inference was done through the application of a linear-mixed model and *t*-tests for data normally distributed, and through the non-parametric Wilcoxon signed-rank test for data not normally distributed. In the case of *t*-tests, the assumption of equality of variances between groups to be tested was checked through an *F*-test

3. Results and discussion

3.1. Characterization of cells

Whole blood was obtained by jugular venipuncture. DH59B recognizes equine CD172a found on both granulocytes and cells of the monocyte lineage (Tumas et al., 1994). Granulocytes were removed using a histopaque gradient; removed granulocytes are shown (Fig. 1A). The remaining population of mononuclear cells (Fig. 1B) was further enriched for monocytes through positive selection, with the lymphocyte discard population shown (Fig. 1C). The enriched population of cells achieved with selection with DH59B (Fig. 1D) consistently yielded a population $\geq$90% positive for DH59B. The enriched population of cells contained $\leq$7% positive for E18A and $\leq$2% positive for HB88A (data not shown). These cells were plated and additional non-adherent cells discarded following overnight incubation.

3.2. Statistical analysis

Statistical inference was done by using a linear-mixed model and *t*-tests for data with a normal distribution, and by the non-parametric Wilcoxon signed-rank test for data that did not have a normal distribution. Analysis of the log-transformed data for normality indicated that levels of IL-10 and IL-12p35 (from control and stimulated cells) were normally distributed whereas levels of IL-12p40 and GAPDH were not.

To determine the effect of APC activation on expression of the cytokines of interest, results were normalized to both the control samples (unstimulated cells) as well as to GAPDH in a separate analysis. Data normalized by GAPDH grouped by date of assay corroborated previous signs of interassay variability, as well as variability from animal to

Table 1
Nucleotide identity of primers and probes used to amplify cytokine mRNA by quantitative RT-PCR.

Target	Sense primer	Antisense primer	Probe
IL-12p35	5′-GACAGCCATTGACAAGCTGATG-3’	5′-TTCAAGGGAGGGCTTTTGTG-3′	5′-6-FAM-AGGCCCTGAACTTCAACAGTGAGACTGTG-DQ-3′
IL-12p40	5′-GGCCAGATCCGTGTCCAA-3′	5′-GGATACGGATGCCCATTCG-3′	5′-6-FAM-CCAGGGACCGCTACTACAGCTCATCCT-DQ-3′
IL-10	5′-GATCTCCCAAATCCCATCCA-3′	5′-AGGAGAGAGGTACCACAGGGTTT-3′	5′-6-FAM-CCAAGGAGCTGATTCAGCTCTCCCAGAA-DQ-3′
GA3PDH	5′-CCCACCCCTAACGTGTCAGT-3′	5′-TCTCATCGTATTTGGCAGCTTTC-3′	5′-6-FAM-TGGATCTGACCTGCCGCCTGG-DQ-3′

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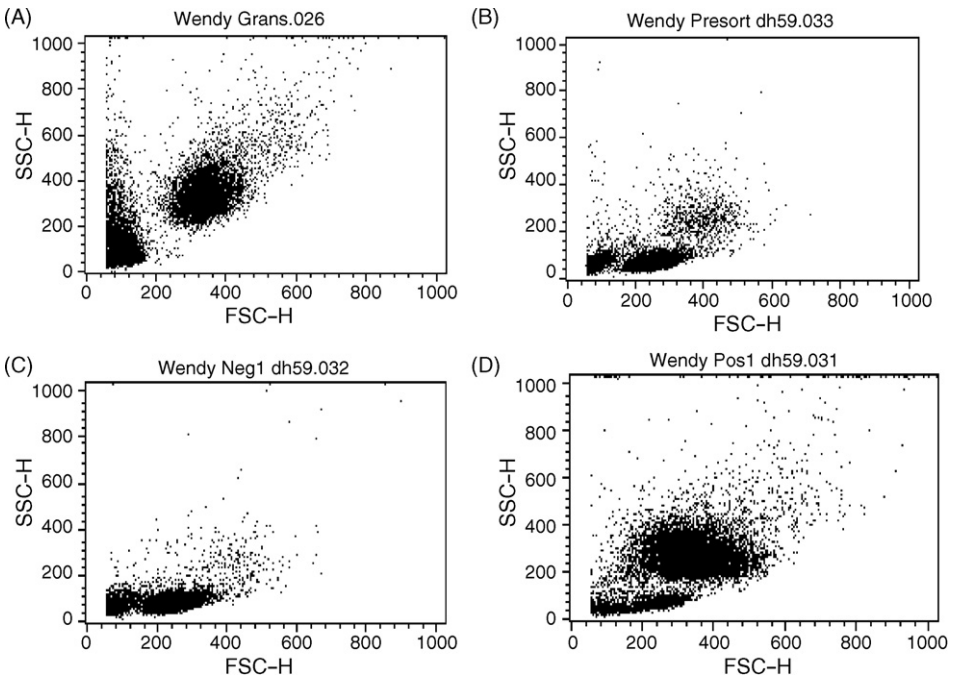

Fig. 1. Enrichment of equine PBMC for circulating monocytes and dendritic cells. Buffy coat cells from peripheral blood were layered on a double Histopaque gradient (1077 and 1119). The population of granulocytes is shown (A). AutoMACs separation and enrichment of remaining CD172a positive cells was performed with a primary antibody (25 μg/ml VMRD equine DH59B) and 100 μg/ml goat anti-mouse IgG microbeads (Miltenyi Biotec, Auburn, CA). The resultant enriched monocytes and dendritic cells were characterized by flow cytometry using forward/side scatter profiles and the following cell-type specific fluorescent markers: VMRD E18A/equine B cell, VMRD HB88A/equine CD2, VMRD DH59B/equine CD172a. Equine DH59B negatively selected lymphocytes (C) and positively selected monocytes (D) are readily identified compared with the pre-selected control (B).

animal (data not shown). Hence, a linear mixed model was applied to evaluate data for IL-10 and IL-12p35 which accounted for random effects of experiment and animal, in addition to the fixed effects of treatment, age group and their interaction (mathematical formulation not shown). For IL-10, this model resulted in a significant effect for both treatment and treatment inside age group. For IL-12p35, the linear mixed-model resulted in significant differences for treatment effects only (Fig. 2). This model was not applied to data for IL-12p40 because these data were not normally distributed; the data for IL-12p40 were analyzed using a Wilcoxon signed-rank test (below).

Since interassay variability was dispersed by normalization of treatment samples to control samples, *t*-tests were performed with data which had a normal distribution (IL-10 and IL-12p35). Results showed that means of log ($IL\text{-}10_{IFN+LPS}/IL\text{-}10_{control}$) and means of log ($IL\text{-}12p35_{IFN+LPS}/IL\text{-}12p35_{control}$) for both adults and foals were significantly different from zero (Table 2). These results are consistent with those obtained with the linear mixed-model and indicate a significant effect of treatment. Notably, however, there was a significant difference between log ($IL\text{-}10_{IFN+LPS}/IL\text{-}10_{control}$) of adults and foals ($p$-value < 0.0105) indicating that the magnitude of the response of neonatal foal cells to stimulation was greater than that of the adult with respect to IL-10 mRNA. Therefore, the mixed linear model and the *t*-tests both indicated a significant response to treatment for IL-10 and IL-12p35 as well as a significant difference in response due to age for IL-10 with foals responding with a greater than two-fold log increase in IL-10 upon stimulation with IFN-γ and LPS.

A Wilcoxon signed-rank test for data not normally distributed showed that log ($IL\text{-}12p40_{IFN+LPS}/IL\text{-}12p40_{control}$) is significantly different from zero for both age groups (Adults, p-value < 0.0005 and Foals, p-value < 0.0000), indicating a significant effect for treatment. Overall, the same results were also obtained with normalization of IL-10, IL-12p35, or IL-12p40 by the reference gene GAPDH (data not shown) further supporting the results obtained by normalization to control samples (unstimulated cells). Means of log ($GAPDH_{IFN+LPS}/GAPDH_{control}$) were not significantly different from zero for both age groups.

Functional deficits in APC and/or macrophages have been cited as aspects of cell-mediated immunity that contribute to ineffective immune responses in neonates (Ridge et al., 1996; Trivedi et al., 1997). In the equine species, indirect evidence indicates that infection with *Rhodococcus equi* occurs during the first few weeks of life (Horowitz et al., 2001) supporting the notion that the foal lacks effective cell-mediated immune responses during the neonatal period. In contrast to foals, rhodococcal disease is rarely observed in immunocompetent adults (Vengust et al., 2002). To overcome intracellular infections, the infected macrophage must be stimulated by cytokines, such as IFN-γ, that are elaborated by a Th1 cell. Activated monocytes, macrophages, and dendritic cells elaborate cytokines that are an integral component of initiation of effective innate and adaptive immune responses. In this

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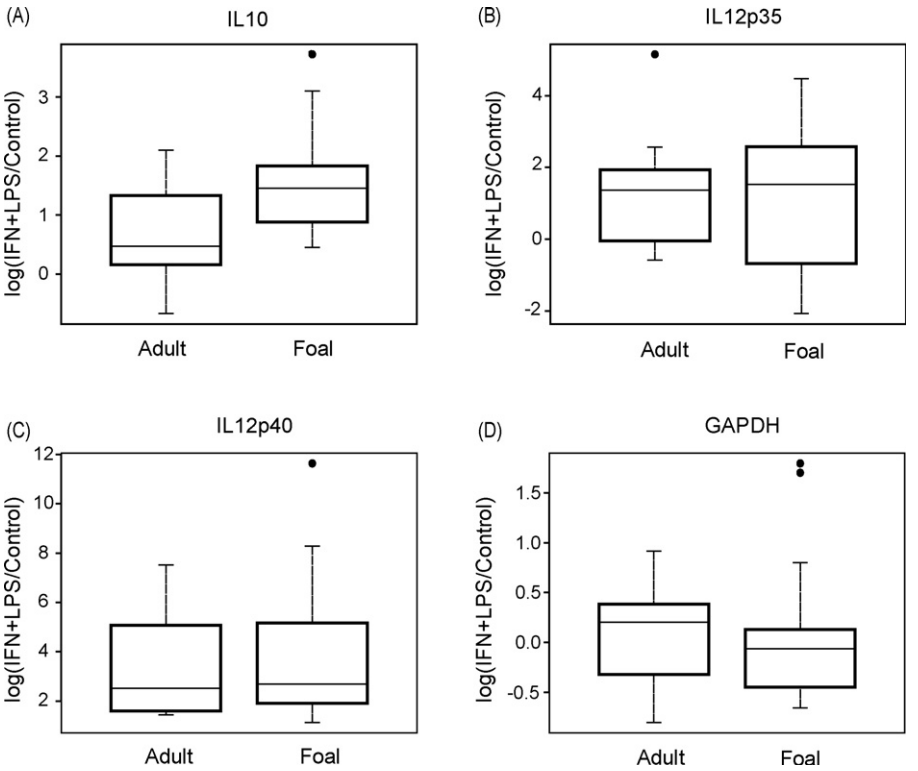

Fig. 2. Box plots showing log fold change of the indicated cytokine mRNA and housekeeper (GAPDH) from equine adult (adult) and neonatal foal (foal) cells of the monocyte lineage stimulated with LPS and IFN-γ. The median is represented by the horizontal bar within the box; the interquartile range by the vertical extent of the box; the range of values of data not considered outliers by the minimum and maximum whiskers; and outliers by circles.

study, we compared cells of the monocyte-lineage between neonatal foals and adults that were activated *in vitro* with LPS and IFN-γ for expression of cytokine mRNA for IL-10, IL-12p35, and IL-12p40. Compared with control cells, stimulated adult and neonatal cells of the monocyte lineage all responded with an increase in the expression of these cytokine mRNAs. However, the fold-increase of expression of IL-10 was significantly greater in neonates than in adults. Collectively, these results suggest that activated neonatal monocytes, macrophages, and dendritic cells may drive a different immunoregulatory response than adult cells of the monocyte lineage.

Our results are interesting in that fold change in expression of cytokine mRNA for IL-12p35 and IL-12p40 following stimulation with LPS/IFN-γ was similar; however, fold change of expression of cytokine mRNA for IL-10 was significantly higher in neonatal foals. It has been demonstrated recently that IL-10 suppresses the transcription of both the IL-12p35 and IL-12p40 heterodimers, the latter through inhibition of recruitment of RNA polymerase II to the p40 promoter (Zhou et al., 2004; Rahim et al., 2005). In addition, in human neonates, a defect in chromosomal remodeling that prevents IL-12p35 gene transcription has been shown as a contributing cause for decreased IL-12 synthesis in human neonates (Goriely et al., 2004). Since the fold-change of expression of IL-12p35 and IL-12p40 was similar between foals and adults, it appears that any down-regulatory effect that IL-10 may be having on IL-12 within this population of equine cells is equivalent and that the defect in chromosomal remodeling that prevents IL-12p35 gene transcription in human neonates (Goriely et al., 2004) may not occur in equine neonates.

Table 2
t-Test results for log ($IL\text{-}10_{IFN+LPS}/IL\text{-}10_{control}$) and log ($IL\text{-}12p35_{IFN+LPS}/IL\text{-}12p35_{control}$).

Target gene (IFN+LPS/control)	Age group	Mean	95% C.I.	*p*-Value
log (IL-10)	Adult	0.697	(0.278, 1.117)	0.0028
log (IL-10)	Foal	1.421	(1.145, 1.697)	0.0000
log (IL-12p35)	Adult	1.199	(0.539, 1.859)	0.0013
log (IL-12p35)	Foal	1.123	(0.393, 1.852)	0.004

IL-10 down-regulates many of the effector functions of macrophages, including the release of reactive nitrogen and oxygen intermediates (Cunha et al., 1992; Niiro et al., 1992; Wu et al., 1993; Cenci et al., 1993; Kuga et al., 1996). Presence of IL-10 also results in the down-regulation of expression of MHC II and costimulatory molecules on macrophages and dendritic cells (Joss et al., 2000). These cumulative effects can contribute to the T helper 2 bias and dampening of (pro)inflammatory responses frequently ascribed to IL-10. IL-10 also supports the maturation of monocytes to macrophages and favors the phagocytic activity of both cell types (Allavena et al., 1998; Buchwald et al., 1999). These collective effects could contribute to a favorable environment for intracellular, macrophage-tropic pathogens. In short, with regard to cells of the

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monocyte lineage, IL-10 plays a significant immunoregulatory role in APC function.

The effects of IL-10 have been studied in models involving infection with bacterial, fungal, and protozoal agents. In all cases, resistance to infection can be improved with reduction in IL-10 levels (Reddy et al., 2001). In most cases, even normal levels of IL-10 result in a relative impairment of disease resistance, while administration of exogenous IL-10 further impairs resistance to disease. In murine systems, IL-10 has suppressed the intracellular killing of bacteria in neutrophils, including *Klebsiella*, *Streptococcus*, and *Mycobacterium* spp. (Greenberger et al., 1995; Denis and Ghadirian, 1993; van der Poll et al., 1996). Moreover, innate and adaptive immune responses of mice to infection with *Listeria*, *C. albicans*, *T. gondii*, and *T. cruzi* have been enhanced with an IL-10 knockout background or anti-IL-10 treatment (Neyer et al., 1997; Gazzinelli et al., 1996; Li et al., 1999; Holscher et al., 2000; Hunter et al., 1997). Thus, elevated levels of IL-10 have been causally associated with enhanced severity of disease.

Interestingly, few *in vivo* studies have chronicled the development of expression of cytokines of the neonatal foal's response to bacterial infection. However, in one study of septic neonates, Pusterla et al. found that expression of IL-10 in whole blood was highest in non-survivors, indicating a propensity for IL-10 expression by leukocytes that encounter bacteria in peripheral blood (Pusterla et al., 2006). Similarly, Giguere et al. (1999) found that foals infected at approximately 3 weeks of age with virulent *R. equi* 103^{+} had increased levels of IL-10 and IL-12p40, but not IL-12p35, in lung tissue at 3 and 14 days post-inoculation compared to foals infected with the avirulent, plasmid-cured *R. equi* 103^{-} and uninfected controls (Giguere et al., 1999). Our *in vitro* results support these *in vivo* findings in that LPS and IFN-γ stimulated APC from neonates responded with markedly increased expression of IL-10 cytokine mRNA compared with adult APC and were responsive to cell stimulation as measured through IL-12p35 and IL-12p40 cytokine mRNA expression as well. These observations suggest that neonatal and young foals respond to stimuli of cells residing in lung as well as peripheral CD172a(+) mononuclear cells, with increased levels of IL-10. Collectively, these results indicate an age-related differential regulation of immune responses and that our experimental system may provide insight into aspects of APC maturation in the horse.

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