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## Foals are interferon gamma-deficient at birth

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### Abstract

The increased vulnerability of foals to specific pathogens such as *Rhodococcus equi* is believed to reflect an innate immunodeficiency, the nature of which remains poorly understood. Previous studies have demonstrated that neonates of many species fail to mount potent Th1 responses. The current research investigates the ability of circulating and pulmonary lymphocytes of developing foals to produce interferon gamma (IFN $\gamma$ ). Peripheral blood mononuclear cells (PBMC) were prepared from up to 10 horse foals at regular intervals throughout the first 6 months of life. Bronchoalveolar lavage (BAL) samples were collected at 1, 3 or 6 months of age from three groups of five foals. The PBMC and BAL cells were stimulated in vitro and IFN $\gamma$  production was measured by intracellular staining. In addition, RNA was extracted from freshly isolated and in vitro stimulated PBMC and BAL cells for quantitation of IFN $\gamma$  gene expression by real time PCR. Newborn foals exhibited a marked inability to express the IFN $\gamma$  gene and produce IFN $\gamma$  protein. This deficiency was observed in both circulating and pulmonary lymphocytes. However, IFN $\gamma$  gene expression and protein production increased steadily throughout the first 6 months of life, reaching adult levels within the first year of life. These findings suggest that foals are born with an inherent inability to mount a Th1-based cell mediated immune response which may contribute to their susceptibility to intracellular pathogens.

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**Keywords:** Equine; Real time PCR; BAL; IFN; Cytokine; Flow cytometry; Neonate

### 1. Introduction

The inability of neonates of many species to mount effective immune responses has long been recognized. In particular, neonates commonly fail to mount cellular immune responses which provide effective defense against intracellular pathogens (Greenough, 1996). Foals receive limited immune protection during

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the neonatal period from maternal antibodies acquired through ingestion of colostrum. However, little is known about the development of cellular immune defenses in young foals. It is assumed that young foals are similarly immunodeficient, given that they are highly vulnerable to a variety of pathogens, such as *Rhodococcus equi* (Prescott, 1991) and rotavirus (Powell et al., 1997). However, many gaps exist in our understanding of neonatal foal immunology. Identifying the underlying basis of neonatal immunodeficiency in the foal is an important step towards promoting their health and survival. Previous studies have investigated the development of humoral immunity in foals (Sheoran et al., 2000), and problems with vaccination in the face of maternal antibodies (Cullinane et al., 1994; Wilson et al., 2001). In a study on cellular immune function in foals, Flaminio et al. (2000) demonstrated minor age-dependent changes in lymphocyte populations in both the peripheral and lung compartments, and concluded that certain leukocyte functions of newborn foals were similar to those observed in adult horses. However, no analysis of cytokine production by foals has been reported.

Contrasts in cytokine profiles are commonly observed between neonates and adults of many species and can serve as indicators of important differences in immune function (Marodi, 2002b). For instance, Th1 immune responses, characterized by the production of interferon gamma (IFN $\gamma$ ), have repeatedly been shown to be deficient in newborn humans (Wilson et al., 1986; Chipeta et al., 1998; Joyner et al., 2000; Gasparoni et al., 2003; Protonotariou et al., 2004) and mice (Adkins and Hamilton, 1992; Adkins et al., 2000). This immunodeficiency may be due to suboptimal antigen presenting cell function resulting in low level Th1 cytokine production by naïve CD4<sup>+</sup> T cells (Trivedi et al., 1997; Upham et al., 2002). In addition, neonatal macrophages may demonstrate hyporesponsiveness to IFN $\gamma$  activation (Marodi et al., 1994; Marodi, 2002a). However, it is now widely accepted that under appropriate conditions of stimulation, neonatal T cells can produce IFN $\gamma$  (Qureshi and Garvy, 2001; Siegrist, 2001; Vekemans et al., 2001; Adkins et al., 2004). Therefore, neonates appear to be Th1 immunocompetent, but have a high threshold for activation of Th1 responses.

Th1 cytokines such as IFN $\gamma$  are central to the induction of antigen-specific cellular immune responses (O'Garra, 1998). Importantly, the production of IFN $\gamma$  is a critical component of the protective immune response to *R. equi*, a gram-positive facultative intracellular bacterium that infects macrophages and can cause pyogranulomatous pneumonia in young foals. This was initially demonstrated in a mouse model, where it was shown that clearance of the bacterium was dependent on IFN $\gamma$ -producing CD4<sup>+</sup> T lymphocytes (Kanaly et al., 1995, 1996). More recently, Hines et al. (2003) demonstrated that the ability of adult horses to clear virulent *R. equi* infection is dependent on IFN $\gamma$  production by CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes, with concomitant generation of non-MHC-restricted *R. equi*-specific CTL responses (Patton et al., 2004) also playing a role. Interestingly, Patton et al. (2005) have subsequently shown that foals are incapable of mounting *R. equi*-specific CTL responses at 3 weeks of age, but appear to be capable of doing so by 8 weeks of age. The purpose of the current research is to investigate the ability of resting or in vitro-stimulated foal lymphocytes to synthesize IFN $\gamma$ . We demonstrate that both peripheral blood and pulmonary lymphocytes from young foals fail to express the IFN $\gamma$  gene, or synthesize IFN $\gamma$  protein at levels observed in adults. The deficiency in IFN $\gamma$  production is most pronounced in foals of less than 3 months of age. Demonstration of a lack of IFN $\gamma$  production by young foals has important implications for neonatal foal care and vaccination, and may help explain the increased susceptibility of foals to infectious diseases. In addition, these data encourage further research to investigate the underlying cause for Th1 deficient responses in foals, and to develop ways to expedite the acquisition of this immune phenotype.

## 2. Materials and methods

### 2.1. Animals

Fifteen mixed-breed horse foals, bred at the Equine Research and Resources Facilities of the College of Agriculture, University of Kentucky, were used in this study. They were conventionally reared alongside their dams on pasture. Four adult ponies, aged 6–11

years were used as adult controls for IFN $\gamma$  production. All animals were treated in accordance with the Guide for the Care and Use of Agricultural Animals in Agricultural Research, U.S. Department of Agriculture. The experimental techniques and procedures were approved by the Institutional Animal Care and Use Committee of the University of Kentucky.

## 2.2. Collection and processing of blood and bronchoalveolar lavage (BAL) samples

Heparinized blood was collected by jugular venipuncture at regular intervals from each foal. Samples from newborn foals were all collected within 72 h of birth. Heparinized blood was overlaid on Ficoll-Paque Plus<sup>TM</sup> (Amersham Biosciences, Piscataway, NJ) for isolation of peripheral blood mononuclear cells (PBMC). PBMC were washed three times in sterile PBS (pH 7.2), and enumerated using a ViCell-XR instrument (Beckman Coulter, Miami, FL). Aliquots of freshly isolated PBMC ( $3 \times 10^6$ ) were immediately resuspended in RNA-STAT 60 (Tel-Test Inc., Friendswood, TX) for subsequent extraction of RNA and analysis of cytokine gene expression in freshly isolated, unstimulated cells. The remaining cells were resuspended at  $3 \times 10^6 \text{ ml}^{-1}$  in medium (RPMI 1640 [Gibco, Grand Island, NY], supplemented with 2.5% fetal equine serum [FES, Sigma, St. Louis, MO], 2 mM glutamine [Sigma], 100 U/ml penicillin/streptomycin [Sigma], 55  $\mu\text{M}$  2-mercaptoethanol [GIBCO, Grand Island, NY]) and cultured *in vitro* (see below).

For BAL collections, 15 foals were randomly assigned to three groups of five individuals. BALs were collected from a single group of foals at 1, 3 or 6 months of age. This sampling design avoided any possible inflammatory changes to the lung environment that may have been caused by repeated sampling of individual foals (Traub-Dargatz et al., 1988). BAL tubes were prepared by inserting polyethylene tubing (0.08 in. inner diameter; Cole Palmer, Vernon Hills, IL) into outer tubing (3/16 in. inner diameter; Cole Palmer). A 5.5 in. 16 gauge intravenous catheter (Abbott Laboratories, Sligo, Ireland) was inserted into and glued to the inner tubing. The assembled tubing was placed in a sterilization pouch and gas sterilized in an AN74 instrument (H.W. Andersen Products, Inc., Haw River, NC).

Foals were intravenously administered a sedative cocktail containing xylazine (0.15 mg/kg; Butler Co., Dublin, OH), acepromazine (0.01 mg/kg; Butler) and butorphanol tartrate (Torbugesic<sup>®</sup>, 0.01 mg/kg; Fort Dodge Animal Health, Fort Dodge, IA). Sterile BAL tubes were lubricated at the distal end with sterile lubricating jelly and passed into the lung until gently seated in a bronchiole. Luer-lock 60 ml syringes (Tyco Health Care, Mansfield, MA) were filled with sterile saline (0.9% NaCl; Abbott Laboratories, Chicago, IL) and attached to the catheter on the proximal end of the inner BAL tube. The saline was slowly instilled into the lung and immediately withdrawn. This was repeated until at least 150 ml BAL fluid (BALF) had been collected, which was typically 50–70% of the volume of saline instilled. A small sample of BALF was reserved for cytologic analysis (Dip Quick, Jorgensen Laboratories Inc., Loveland, CO). The remainder was centrifuged at  $400 \times g$  for 10 min, the pellet was resuspended in 10 ml PBS, and the cells were enumerated (ViCell XR, Beckman Coulter). BAL cells were then resuspended in medium at  $3 \times 10^6 \text{ ml}^{-1}$ .

## 2.3. Induction and measurement of IFN $\gamma$ production

Aliquots of  $3 \times 10^6$  PBMC or BAL cells from each foal were placed in 24-well plates in 1 ml medium alone, or in 1 ml medium supplemented with phorbol 12-myristate 13-acetate (PMA; 25 ng/ml; Sigma) and ionomycin (1  $\mu\text{M}$ ; Sigma). Brefeldin A (10  $\mu\text{g/ml}$ ) was added to both medium- and PMA-stimulated cultures to ensure the intracellular accumulation of nascent proteins synthesized during the incubation. Plates were incubated for a total of 4 h at 37 °C, 5% CO<sub>2</sub> in air. After 2 h of incubation, 0.5 ml ( $1.5 \times 10^6$  cells) was removed to a 1.5 ml microcentrifuge tube, centrifuged at  $1000 \times g$  in a microcentrifuge for 2 min, and the cell pellet was resuspended in RNA-STAT 60 (Tel-Test) for measurement of cytokine gene expression in cultured cells. The remaining cells in the 24-well plate were incubated for a further 2 h and then transferred to duplicate wells of a 96 well V-bottom microtiter plate (Nunc Inc., Naperville, IL). The plates were centrifuged at  $500 \times g$  for 5 min, and each cell pellet was fixed in 100  $\mu\text{l}$  2% paraformaldehyde (Sigma), and stored

overnight at 4 °C. Fixed cells were washed once in PBS-saponin (PBS-S; PBS supplemented with 1% FBS, 0.1% saponin [Sigma] and 0.1% sodium azide [Sigma]), and then incubated on ice for 30 min with 100 µl CC302 (FITC-conjugated mouse-anti-bovine IFN $\gamma$ ; Serotec, Raleigh, NC) or an isotype control antibody (FITC-conjugated mouse IgG1; Caltag Laboratories, Burlingame, CA) at a concentration of 1 µg/ml in PBS-S. CC302 has previously been shown to cross-react with equine IFN $\gamma$  (Pedersen et al., 2002). Unbound antibody was removed by washing the cells twice with PBS-S, and each cell aliquot was resuspended in FACS buffer for flow cytometric analysis.

#### 2.4. Flow cytometric analysis

Mononuclear cells were identified and gated based on forward and side-scatter parameters, and at least 50,000 gated events were acquired using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). The data were analyzed using Cell Quest<sup>®</sup> software (Becton Dickinson). Markers were set on isotype control antibody-labeled PMA-stimulated samples from each foal such that 1% cells were IFN $\gamma$  positive. These markers were then used to determine the percentage of IFN $\gamma$ -producing mononuclear cells in the corresponding PMA-stimulated cell cultures labeled with anti-IFN $\gamma$ -specific antibody (CC302). Results are expressed as the percentage of mononuclear cells that produced IFN $\gamma$  in response to PMA-stimulation, after subtraction of the isotype control detection (1% by definition). Mean fluorescence intensity (MFI) of the IFN $\gamma$  signal in IFN $\gamma$ -producing cells was also recorded for each sample.

#### 2.5. Relative quantitation of cytokine gene expression by real time PCR

Total cellular RNA was extracted from freshly isolated, or PMA and ionomycin-stimulated PBMC or BAL cells preserved in RNA-STAT 60 (Tel-Test), following the manufacturer's protocol. One half microgram total RNA was brought to 49.5 µl in nuclease-free water (Qiagen, Valencia, CA), and 30.5 µl reverse transcription master mix (0.5 µl [20 U/µl] avian myeloblastosis virus [AMV] reverse transcriptase [Promega, Madison, WI], 1 µl oligo dT

primer [0.5 µg/µl; Promega], 1 µl RNAsin [40 U/µl, Promega], 4 µl dNTP [10 mM; Promega], 8 µl AMV buffer [Promega], and 16 µl MgCl<sub>2</sub> [25 mM, Promega]) was added to each sample. The reactions were incubated at 42 °C for 15 min and 95 °C for 5 min in a thermocycler. The resultant cDNA was diluted 1:1 with nuclease-free water to allow for measurement of several cytokine genes by real time PCR.

Cytokine gene expression was measured in cDNA samples using an Applied Biosystems 7500 sequence detection system (Applied Biosystems, Foster City, CA). Intron-spanning equine IFN $\gamma$  and beta-glucuronidase ( $\beta$ -GUS)-specific primer/probe sets were designed for this purpose (Assays-by-Design, Applied Biosystems). The selected primers and probes failed to amplify genomic DNA and reverse transcription-negative RNA samples. PCR reactions were incubated at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Each reaction contained 20 µl master mix (12.5 µl TaqMan<sup>®</sup> Universal PCR Master Mix [Applied Biosystems], 1.25 µl 20 $\times$  assay mix for the gene of interest [primer/probe set; Applied Biosystems], 6.25 µl nuclease-free water [Qiagen]), and 5 µl cDNA template. All reactions were performed in duplicate wells. Changes in cytokine gene expression were calculated by relative quantitation using the  $\Delta\Delta C_T$  method (Livak and Schmittgen, 2001) where  $\Delta\Delta C_T = (\text{IFN}\gamma C_T - \beta\text{-GUS } C_T)_{\text{foal of interest}} - (\text{mean IFN}\gamma C_T - \text{mean } \beta\text{-GUS } C_T)_{\text{newborn foal group ("the calibrator")}}$ . Fold changes for each individual foal at each age were calculated as  $2^{-\Delta\Delta C_T}$ . Results are expressed as the mean fold change in IFN $\gamma$  gene expression by the group of foals at each age.

#### 2.6. The effect of colostrum on IFN $\gamma$ production

Paired whole blood samples were collected within 30 min of birth (pre-nursing), and again 36 h later from five foals born at the Equine Reproduction Laboratory, Colorado State University. All blood samples were collected by jugular venipuncture directly into a single PAXgene<sup>™</sup> tube (Qiagen) for each foal. This resulted in the immediate solubilization of blood cells and stabilization of cellular RNA. Total RNA was subsequently extracted from each sample using the PAXgene<sup>™</sup> blood RNA kit (Qiagen), following the manufacturer's protocol. Reverse transcription of RNA and quantitative real

time PCR reactions were performed as described above.

### 2.7. Statistical analysis

All data were analyzed by one-way analysis of variance (ANOVA). When data were not normally distributed, ANOVA was performed on log-transformed data (ANOVA-LOG). If log-transformed data was not normally distributed, non-parametric analysis (ANOVA on RANKS) was performed. Post-hoc pairwise comparisons of normally distributed data were performed using the Holm–Sidak test. All age-related differences in cytokine production were considered significant at  $p \leq 0.05$ .

## 3. Results

### 3.1. *IFN $\gamma$ production by PMA & ionomycin-stimulated foal PBMC*

PBMC prepared from newborn foals demonstrated a near complete inability to synthesize IFN $\gamma$  following *in vitro* stimulation with PMA and ionomycin (Fig. 1a and b). However, IFN $\gamma$ -production in response to polyclonal stimulation was acquired over time. A significant and steady increase in the frequency of IFN $\gamma$ -producing cells occurred as the foals aged ( $p = 0.032$ ; ANOVA; Fig. 1a). The mean percentage of cells producing IFN $\gamma$  began to increase immediately after birth, and was significantly elevated in foals at 3, 4, 5 or 6 months of age, when compared to newborns ( $p \leq 0.014$ ; Holm–Sidak). It was also significantly higher in 3-, 4-, 5- and 6-month-old foals compared to 1-week-old foals ( $p \leq 0.03$ ), and in 3-, 4- and 6-month-old foals compared to 2 week old foals ( $p \leq 0.05$ ). Two-week-old foals also demonstrated a lower frequency of IFN $\gamma$  producing cells than 5-month-old foals, but this difference was not statistically significant ( $p = 0.07$ ). The increase in the frequency of IFN $\gamma$ -producing cells in 1-month-old foals compared to newborns approached significance ( $p = 0.068$ ). A significant deficiency in the mean frequency of IFN $\gamma$ -producing cells was observed in foals aged 3 weeks or younger, when compared to adult ponies ( $p < 0.05$ ; Dunn's method). No significant difference was observed between the fre-

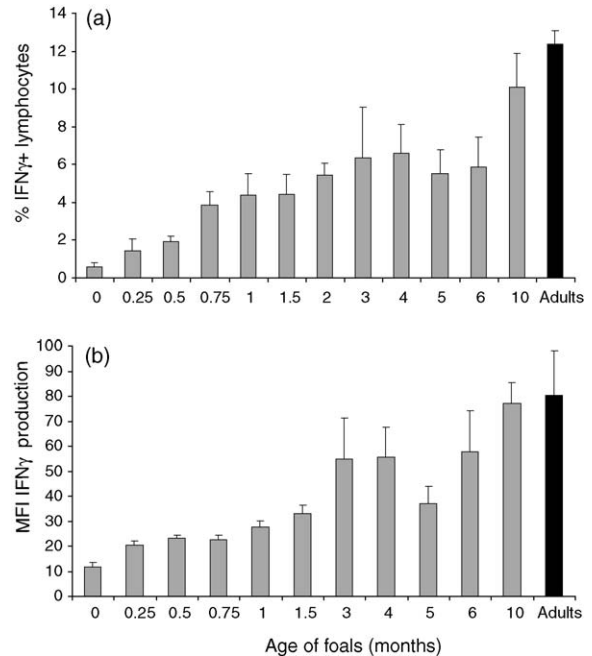


Fig. 1. Production of IFN $\gamma$  by PMA and ionomycin-stimulated foal PBMC increases with age. PBMC were stimulated *in vitro* with PMA and ionomycin in the presence of brefeldin A for 4 h as described in Section 2. PBMC from a group of four adult ponies were repeatedly isolated in parallel with foal samples and served as a positive control for IFN $\gamma$  production. The mean frequency of IFN $\gamma$ -producing cells increases with age (a). Similarly, the mean amount of IFN $\gamma$  produced by individual foal PBMC stimulated with PMA and ionomycin increases with age (b). Mean fluorescence intensity (MFI) corresponds to the mean amount of IFN $\gamma$  produced per cell as determined by flow cytometry. Mean values (+S.E.) are reported. The number of foals analyzed at each age were 4, 4, 4, 8, 8, 6, 2, 10, 10, 10, 10 and 10, respectively. Four repeat measures were performed on each adult pony.

quency of IFN $\gamma$  producing cells in foals at or above 1 month of age and adults ( $p > 0.05$ ). However, it appears that 6-month-old foals had not yet reached maximal IFN $\gamma$  synthesis as evidenced by the increase in the frequency of IFN $\gamma$  producing cells by the same foals at 10 months of age. No detectable IFN $\gamma$  was produced by medium-stimulated cells from any experimental foal or adult pony (data not shown).

Analysis of the mean fluorescence intensity (MFI) of IFN $\gamma$  production, which estimates the mean amount of IFN $\gamma$  produced per cell, demonstrated a similar pattern (Fig. 1b). MFI of IFN $\gamma$  produced per foal PBMC also increased with foal age ( $p < 0.001$ ; ANOVA on RANKS). This measurement of IFN $\gamma$

production was significantly higher in PBMC from 3-, 4-, 6- and 10-month-old foals compared to newborn foals ( $p < 0.05$ ; Dunn's method). Therefore, the frequency of IFN $\gamma$  producing cells and the amount of IFN $\gamma$  produced per cell both increased as the foals aged.

### 3.2. IFN $\gamma$ gene expression in PMA and ionomycin-stimulated foal PBMC

IFN $\gamma$  gene expression increased from a low level in newborn foals throughout the first 6 months of life (Fig. 2). The  $\Delta\Delta C_T$  method was used to calculate relative changes in gene expression (Livak and Schmittgen, 2001), with the mean amount of IFN $\gamma$  gene expression by newborn foals serving as the calibrator. Fold increases in IFN $\gamma$  gene expression by developing foals are shown. There was a significant increase in IFN $\gamma$  gene expression by foals as they aged ( $p < 0.001$ ; ANOVA-LOG). A marked increase in IFN $\gamma$  gene expression occurred as early as the first week of life ( $p = 0.002$ ; Holm–Sidak). IFN $\gamma$  gene expression was significantly elevated in foals of all

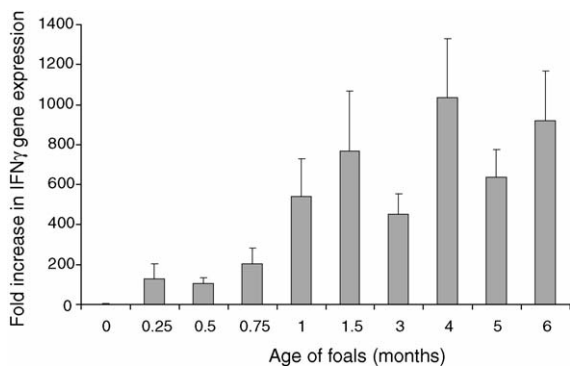


Fig. 2. IFN $\gamma$  gene expression by foal PBMC stimulated with PMA and ionomycin increases with age. Fold increases in IFN $\gamma$  gene expression by foals of various ages compared to newborn foals are shown in bars. PBMC were stimulated in vitro with PMA and ionomycin for the detection of IFN $\gamma$  production by intracellular staining (Fig. 1a and b). After 2 h, aliquots of PBMC were removed and fixed in RNA-STAT 60 for subsequent RNA extraction. Following reverse transcription, real time PCR was performed to quantitate the expression of the IFN $\gamma$  gene. Mean IFN $\gamma$  production by newborn foals was arbitrarily set at 1. Results are shown as mean fold increases (+S.E.) in IFN $\gamma$  gene expression by developing foals compared to newborn foals. The number of foals analyzed at each age were 3, 2, 3, 5, 8, 5, 8, 10, 10 and 10, respectively.

ages compared to newborn foals ( $p \leq 0.002$ ; Holm–Sidak).

### 3.3. IFN $\gamma$ production by PMA and ionomycin-stimulated foal BAL cells

BAL cells harvested from the lungs of 1-month-old foals contained a very low percentage of cells that produced IFN $\gamma$  following in vitro stimulation with PMA and ionomycin (Fig. 3a). The frequency of IFN $\gamma$ -secreting cells increased with foal age ( $p < 0.001$ ; ANOVA-LOG), similar to that observed in PBMC populations. IFN $\gamma$ -producing cells were significantly elevated in 3- and 6-month-old foals compared to 1-month-old foals ( $p < 0.001$ ; Holm–Sidak). In addition, these cells were more frequent in

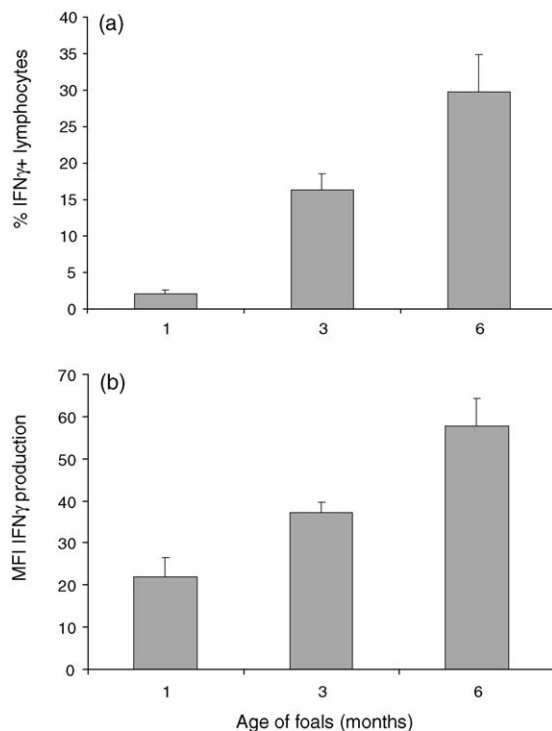


Fig. 3. Production of IFN $\gamma$  by PMA and ionomycin-stimulated foal BAL cells increases with age. BAL cells were stimulated in vitro with PMA and ionomycin in the presence of brefeldin A for 4 h as described for PBMC samples. The increase in the mean (+S.E.) frequency of IFN $\gamma$ -producing BAL cells is shown in (a). The increase in the amount of IFN $\gamma$  produced by individual foal BAL cells is shown in (b). The number of foals analyzed at each age were 4, 5 and 5, respectively.

6-month-old foals compared to 3-month-old foals but this difference was not statistically significant ( $p = 0.1$ ; Holm–Sidak). Analysis of the MFI of IFN $\gamma$  production by BAL cells demonstrated a similar pattern (Fig. 3b). The mean amount of IFN $\gamma$  produced per foal PBMC also increased with foal age ( $p = 0.002$ ; ANOVA). This measurement of IFN $\gamma$  production was significantly greater in BAL cells from 6-month-old foals compared to 1-month-old ( $p < 0.001$ ; Holm–Sidak) or 3-month-old foals ( $p = 0.013$ ; Holm–Sidak). The increase in the amount of IFN $\gamma$  produced by 3-month-old compared to 1-month-old foals approached significance ( $p = 0.058$ ; Holm–Sidak). Therefore, the frequency of IFN $\gamma$  producing cells and the amount of IFN $\gamma$  produced per cell both increased with age in the lung environment.

#### 3.4. IFN $\gamma$ gene expression by PMA and ionomycin-stimulated foal BAL cells

IFN $\gamma$  gene expression in cells harvested from the lungs of foals increased with age ( $p = 0.012$ , ANOVA). Mean fold increases in IFN $\gamma$  gene expression by foal BAL cells are shown in Fig. 4. IFN $\gamma$  gene expression by BAL cells was significantly elevated in 6-month-old foals compared to 1-month-old ( $p = 0.006$ ; Holm–Sidak) or 3-month-old foals ( $p = 0.016$ ; Holm–Sidak). No significant difference

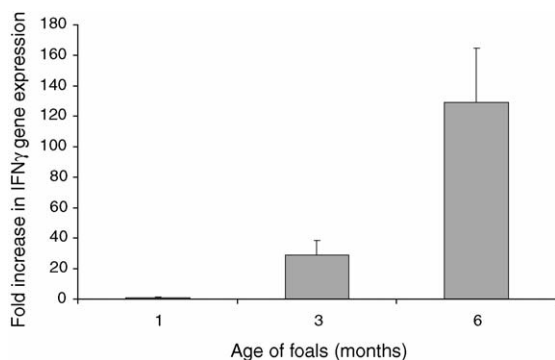


Fig. 4. Expression of the IFN $\gamma$  gene in PMA and ionomycin-stimulated foal BAL cells increases with age. BAL cells were stimulated and IFN $\gamma$  gene expression was determined as per PBMC in Fig. 2. Results are shown as mean fold increases ( $\pm$ S.E.) in IFN $\gamma$  gene expression by 3- or 6-month-old foals compared to 1-month-old foals. The number of foals analyzed at each age were 4, 5 and 5, respectively.

was observed in IFN $\gamma$  gene expression by BAL cells from 1- and 3-month-old foals ( $p = 0.47$ ).

#### 3.5. Relationship between IFN $\gamma$ gene expression and IFN $\gamma$ protein production

All three measures of IFN $\gamma$  production in foals (% IFN $\gamma$  producing cells [Fig. 1a], MFI of IFN $\gamma$  production [Fig. 1b] and IFN $\gamma$  gene expression [Fig. 2]) demonstrate very similar patterns. Fig. 5 illustrates the relationship of the mean amount of IFN $\gamma$  produced by PMA and ionomycin stimulated cells (MFI) in foals and the mean fold increase in IFN $\gamma$  gene expression measured in the same samples. These complementary measures of IFN $\gamma$  production are shown to be in very close agreement ( $p = 0.0035$ ; Pearson correlation coefficient = 0.822). Similarly % IFN $\gamma$ -producing cells and IFN $\gamma$  gene expression ( $p = 0.0017$ ,  $r = 0.854$ ), and % IFN $\gamma$ -producing cells and MFI IFN $\gamma$  production ( $p = 0.0003$ ,  $r = 0.904$ ) are highly correlative (data not shown).

#### 3.6. Effect of colostrum ingestion on IFN $\gamma$ gene expression

RNA was prepared from PAXgene<sup>TM</sup> whole blood samples collected by jugular venipuncture of five foals, prior to nursing (30 min after birth), and again

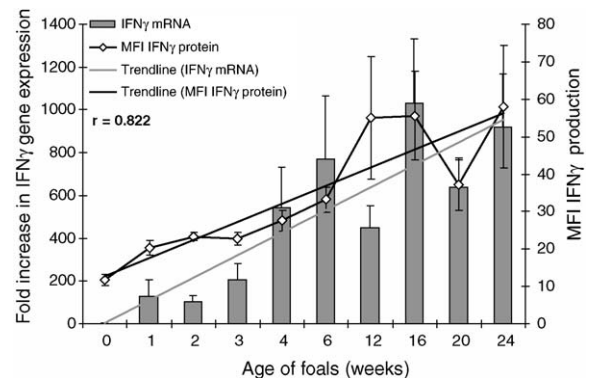


Fig. 5. There is a significant correlation between IFN $\gamma$  gene expression and IFN $\gamma$  protein production ( $p = 0.0035$ ). Mean ( $\pm$ S.E.) fold increases in IFN $\gamma$  gene expression are shown in bars (as in Fig. 2), with a trendline (grey line). MFI ( $\pm$ S.E.) of IFN $\gamma$  production (as in Fig. 1b) is displayed with open diamonds, with a trendline (black line). The significance of the correlation, and the Pearson correlation coefficient for the relationship between these two measures of IFN $\gamma$  is displayed.

Table 1  
IFN $\gamma$  gene expression detected in paired whole blood samples prepared from foals, pre- and post-colostrum ingestion

Pre-colostrum	Post-colostrum
und.	36.140
35.970	36.375
und.	36.434
und.	34.644
und.	35.246

Results are shown as threshold cycle values ( $C_T$ ). und. = IFN $\gamma$  mRNA undetectable after 40 cycles. Pre-colostrum samples collected 30 min after birth, and post-colostrum samples collected 36 h later.

36 h later. IFN $\gamma$  gene expression was assessed in each blood sample by real time PCR, and results are shown in Table 1. IFN $\gamma$  mRNA was undetectable in four of the five foals prior to nursing, but had increased to detectable levels in all foals 36 h of age.

#### 4. Discussion

Lymphocytes from newborn foals demonstrate a profound deficiency in both IFN $\gamma$  gene expression and IFN $\gamma$  protein production in vitro. This failure to synthesize normal adult levels of IFN $\gamma$  is observed both in circulating lymphocytes, as well as those harvested from the lung via bronchoalveolar lavage. The ability of foal lymphocytes to produce IFN $\gamma$  is acquired steadily as the foals develop. The mean levels of IFN $\gamma$  gene expression and IFN $\gamma$  protein production each increase in an age-dependent fashion, beginning as early as 1 week of age, with production of IFN $\gamma$  approaching normal adult levels when foals reach approximately 3 months of age. Flow cytometric analysis of IFN $\gamma$  production indicates that both the frequency of IFN $\gamma$ -producing cells as well as the amount of IFN $\gamma$  produced per cell (MFI) increase steadily in developing foals. This finding suggests that the lack of IFN $\gamma$  production by neonatal foals does not simply reflect a diminished frequency of Th1-lineage effector or memory T cells in their circulation or lungs. If that were the case, the relatively few memory cells present in young foals would be expected to produce IFN $\gamma$  at adult levels, an assumption that is clearly contradicted by these data. Therefore, there appears to be an inherent deficiency in IFN $\gamma$  production by

foal T cells that is independent of lymphocyte differentiation status.

A highly significant positive correlation was observed between the age-dependent increase in IFN $\gamma$  gene expression and the amount of IFN $\gamma$  protein produced by lymphocytes from developing foals (MFI;  $p = 0.0035$ ;  $r = 0.822$ ), indicating that IFN $\gamma$  production by foals is directly proportional to the level of IFN $\gamma$  gene transcription. Furthermore, this suggests that the age-dependent deficiency in IFN $\gamma$  production by foals is mediated at the level of gene transcription. The molecular basis for this remains unknown but may involve chromatin structure (Goriely et al., 2004). Gupta et al. (2005) have recently shown that IFN $\gamma$  production by human neonates is similarly regulated at the level of gene expression.

Th1 deficiency in neonates has long been recognized (Wilson et al., 1986) and is well described in mice and humans (Adkins et al., 2004). The demonstration by the current research that foals are similarly IFN $\gamma$ -deficient may help explain their increased susceptibility to intracellular pathogens. It seems likely that the lack of inherent IFN $\gamma$  production by stimulated foal peripheral blood or pulmonary lymphocytes may contribute to their vulnerability to *R. equi* infection and disease (Hines et al., 2003; Kohler et al., 2003). Patton et al. (2005) demonstrated that young foals were incapable of mounting non-MHC-restricted anti-*R. equi* CTL responses observed in immune adults. Further research is required to establish the basis for this immunodeficiency in foals, but it is likely to be related to Th1 deficiency and the lack of mature cellular immune function.

The underlying cause for Th1 deficiency in neonates remains unclear. Perhaps the commonly held belief that successful pregnancy is a Th2 phenomenon (Lin et al., 1993; Al-Shammri et al., 2004; Bulla et al., 2004) results in Th1 suppression that persists in the neonate post-partum. However much research remains to be done in this area, particularly in light of the controversy surrounding the importance of Th2 bias in successful maintenance of pregnancy (Bonney, 2001; Chaouat, 2003; Chaouat et al., 2004). Given that foals acquire passive immunity from post-natal ingestion of colostrum, and furthermore, that colostrum has been shown to contain a wide range of immunomodulatory molecules including soluble cytokines (Wagstrom et al., 2000;

Bottcher et al., 2003; Yamanaka et al., 2003), we investigated whether colostrum itself was responsible for the observed IFN $\gamma$  suppression in young foals. For this purpose, paired pre- and post-nursing RNA samples were prepared from whole blood lysates (PAXgene<sup>TM</sup>, Qiagen) from five individual foals. It was shown that IFN $\gamma$  gene expression was extremely low in foals prior to colostrum ingestion indicating that IFN $\gamma$  deficiency is not dependent on colostrum intake, and was already increasing in those foals 36 h later (Table 1). These data do not support a direct suppressive effect of colostrum on IFN $\gamma$  production by neonatal foals. However, the effects of long term nursing on immune maturation of foals remains poorly understood.

The stimuli that induce the age-associated increase in IFN $\gamma$  production observed in foals are also unclear. It is presumed that myriad exposures to environmental antigens or pathogens mediate this response through stimulation of the foals' innate immune system (Karlsson et al., 2002) and activation of adult-like APC function (Siegrist, 2001). Pathogen-associated molecular patterns (PAMPs) such as LPS, peptidoglycan or double-stranded RNA that are associated exclusively with replicating prokaryotes or viruses, result in activation of Toll-like receptor-mediated (TLR-mediated) pathways (Beutler, 2004) causing immune maturation. Perhaps cumulative exposures of foals to such PAMPs mediate an inherent increase in IL-12 and IFN $\gamma$  production and acquisition of Th1 immune responsiveness. This theory is supported by the fact that when stimulated with strong Th1 polarizing antigens or adjuvants, neonatal T cells are capable of producing IFN $\gamma$  in vitro (Siegrist et al., 1998; Vekemans et al., 2001; Adkins et al., 2004; Marchant and Goldman, 2005). The mean increases in IFN $\gamma$  production for the group of foals studied in the current research are presented in this manuscript. However, it is clear that the rate of increasing IFN $\gamma$  production varies between foals, which may reflect a variation in the magnitude of PAMP-exposure of different individuals.

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