

# Interferon-gamma, interleukin-4 and interleukin-10 production by T helper cells reveals intact Th1 and regulatory T<sub>R</sub>1 cell activation and a delay of the Th2 cell response in equine neonates and foals

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**Abstract** – Cytokines produced by T helper (Th) cells are important in orchestrating the immune response during health and disease. Recent reports indicated that cytokine mRNA expression in foals is often quantitatively lower than that of adult horses suggesting that foal T cells are not fully mature. Here, peripheral blood mononuclear cells from foals and adult horses were stimulated with phorbol 12-myristate 13-acetate and analyzed for intracellular interferon-gamma (IFN- $\gamma$ ), interleukin-4 (IL-4) and IL-10 production, representing the Th1, Th2 and regulatory T<sub>R</sub>1 cell phenotypes respectively, by flow cytometry. In agreement with previous reports, all three cytokines were quantitatively reduced in foals compared to adults. However, the balance between Th1 and Th2 cytokines (IFN- $\gamma$ /IL-4 ratio) showed a clear Th1-biased response in foals by 6 and 12 weeks of life, while similar IFN- $\gamma$ /IL-10 ratios were found in foals and adult horses. By day 5 after birth, intracellular IFN- $\gamma$  production by foal CD4<sup>+</sup> and CD8<sup>+</sup> T cells resembled that in adults. Overall, IL-4 production was low in foals. IL-4<sup>+</sup> cells peaked at day 5 of age when IL-4 was mainly produced by IgE<sup>+</sup> cells. Relative percentages of IL-4<sup>+</sup> Th2 cells were significantly lower in foals at all time points. The data suggested that equine neonates and young foals have an impaired Th2 response, that the immune response of foals is Th1 biased, that IFN- $\gamma$  production by Th and cytotoxic T cells is qualitatively similar to adult horses, and regulatory IL-10 production by T cells is developmentally mature in foals during the first three months of life.

cellular immune response / cytokine / neonatal immunity / T cell differentiation / horse

## 1. INTRODUCTION

After activation T helper (Th) cells develop into different subsets called Th1, Th2, Th17 and regulatory T cells (Treg). These T cell subsets express different cytokines and mediate distinct effector functions during immune responses. Th1 cells are characterized by

interferon-gamma (IFN- $\gamma$ ) production and are required in defending against infection with many intracellular pathogens [36]. Th2 cells produce interleukin-4 (IL-4), IL-5, IL-13 and IL-31. They promote the development of antibody mediated immune responses against extracellular bacteria and parasites [27]. Tregs have major functions in maintaining peripheral tolerance, immune regulation, and in limiting inflammation during autoimmune diseases

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[39]. Treg cells express transforming growth factor beta (TGF- $\beta$ ) and/or IL-10 [14]. The most recently discovered Th cell subset is Th17 characterized by the secretion of IL-17. Th17 cells promote inflammation, play a crucial role in the clearance of pathogens during the host immune defense, and also induce inflammatory processes in autoimmune diseases [17].

In horses, Th cell subset detection was hampered by the lack of reagents to key cytokines characterizing the different Th cell responses. During the past few years, reagents to detect the production of several equine cytokines became available including monoclonal antibodies (mAb) to equine IL-4 [42] and IL-10 [43]. In addition, various groups have previously shown that a mAb to bovine IFN- $\gamma$  cross-reacts with equine IFN- $\gamma$  [32, 41]. This antibody enabled the detection of intracellular IFN- $\gamma$  production during infection with equine herpesvirus type 1 (EHV-1) and equine influenza virus [6, 30]. Besides the lack of reagents to cytokines, Th cell phenotyping in horses is still restricted by the availability of T cell markers. Although antibodies to CD3, CD4 and CD8 were developed and characterized by two workshops on equine leukocyte antigens [18, 21], reagents for T cell receptors, natural killer cells or CD25 to better identify Treg cells are still missing. Thus far, the only Treg cell subsets that were characterized in horses were (1) a population of CD4<sup>+</sup> T cells that simultaneously produces IFN- $\gamma$  and IL-10 [43], and (2) a population of CD4<sup>+</sup> foxp3 expressing cells that was recently characterized in peripheral blood mononuclear cells (PBMC) and endometrial cup cells [8]. The CD4<sup>+</sup>/IFN- $\gamma$ <sup>+</sup>/IL-10<sup>+</sup> Treg cell subset was named T<sub>R</sub>1 cells according to a similar cell population identified in humans and mice [14].

Many investigations have focused on differences in cytokine mRNA expression between equine PBMC isolated from neonates, foals and adult horses. Several parameters of the humoral and cellular immune response of foals were found to be decreased compared to that of adult horses [5, 7, 23, 33] causing some authors to conclude that the foal immune system is "immunodeficient". It has also been suggested that the reduced ability of equine neonates to

produce IFN- $\gamma$  and other pro-inflammatory cytokines enhances their susceptibility to intracellular pathogens [5, 7]. For instance, infection with the intracellular bacterium *Rhodococcus equi* causes severe respiratory disease in foals but mild or no clinical signs in adult horses [15]. However, in response to antigen stimulation, foal immune cells increased the gene expression of certain cytokines. After infection with *R. equi*, monocyte-derived dendritic cells from foals increased IL-12 mRNA expression more than cells from adult horses [11]. Foal PBMC also showed a clear up-regulation of IFN- $\gamma$  mRNA, a decreased IL-4 gene expression and greater IFN- $\gamma$ /IL-4 transcript ratios in response to *R. equi* infection compared to adult horses [16].

Similarly, IFN- $\gamma$  production in response to EHV-1 infection was almost undetectable in foals but increased with age [29]. However, the IFN- $\gamma$  producing cells in foals and young horses were identified as mainly CD8<sup>+</sup> cytotoxic T cells (CTL) [31] which are considered to be associated with protection from disease [3, 19]. In older horses, the EHV-1 specific T cell response shifted towards a CD8<sup>-</sup> phenotype resulting in a clear decrease in IFN- $\gamma$  producing EHV-1 specific CTL in aged horses [31]. These phenotypic variations in the IFN- $\gamma$  producing effector T cells were believed to offer some explanation for age-dependent differences in the susceptibility to clinical disease induced by *R. equi* or EHV-1 in foals and adult horses.

In addition, mRNA expression studies described a generally decreased ability of neonatal PBMC to express IFN- $\gamma$ , TGF- $\beta$  and IL-1 $\alpha$  transcripts in comparison to cells from adult horses [5]. Other cytokines such as IL-8, IL-12 and IL-23 were found to be increased or similarly expressed in PBMC from neonates compared to older foals [20]. Stimulation of PBMC from foals with lipopolysaccharide (LPS) and IFN- $\gamma$  also resulted in increased IL-10 mRNA expression compared to PBMC from adult horses [35].

These reports indicate that cytokine production and T cell development in foals likely vary depending on the cell type and the antigen-specific stimulus. Because the cytokine network

is complex and interactions between immune cells can be manifold, a better understanding of the cytokine production by different immune cells of the horse is required. Here, we compared cytokine responses in foals and adult horses to obtain a broader understanding on the ontogeny of the Th cell response in healthy foals. We analyzed for the first time IFN- $\gamma$ , IL-4 and IL-10 on a protein and cellular level using intracellular staining and flow cytometric analysis. We also investigated the development of Th1 cells (CD4<sup>+</sup>/IFN- $\gamma$ <sup>+</sup>), Th2 cells (CD4<sup>+</sup>/IL-4<sup>+</sup>), the T<sub>R</sub>1 subpopulation of Treg cells (CD4<sup>+</sup>/IFN- $\gamma$ <sup>+</sup>/IL-10<sup>+</sup>) and CTL (CD8<sup>+</sup>/IFN- $\gamma$ <sup>+</sup>) during the first three months of life. Characterizing differences and similarities of adaptive immunity in foals and adult horses is important in assessing appropriate immune responses in healthy foals, in identifying inappropriate immune regulation in diseased foals, and in developing improved or new vaccination strategies for very young horses.

## 2. MATERIALS AND METHODS

### 2.1. Animals and blood sampling

Heparinized blood sample were obtained via jugular venipuncture from 18 neonatal foals (day 1–2 after birth), 15 foals at day 5, and 15 foals each at 6 and 12 weeks of age, and 15 adult horses using the BD Vacutainer system (Becton Dickinson, Franklin Lakes, NJ, USA). The horses consisted of Warmbloods, Thoroughbreds, and Thoroughbred crosses. The adult horse group was composed of 15 dams of the foals. The mares were between 8 to 22 years of age (median 14 years) and blood samples for this study were obtained at 12 weeks after birth. Foals were born and raised at the Cornell University Equine Park during the 2007 and 2008 foaling seasons. All animals were clinically healthy throughout the study. After birth, the foals suckled colostrum from their dams ad lib. On day 1, a blood IgG quantification was performed using the Snap Foal IgG test (IDEXX laboratories, Westbrook, ME, USA). All foals had serum IgG levels of > 800 mg/dL indicating sufficient passive transfer of maternal immunoglobulins.

All animal procedures were approved by the Cornell University Institutional Animal Care and Use Committee (Ithaca, NY, USA) and were in

accordance with the guidelines established by the National Institutes of Health (Bethesda, MD, USA).

### 2.2. Cell isolation and fixation

PBMC were isolated from heparinized blood by density gradient centrifugation (Ficoll-Paque<sup>TM</sup> Plus, GE Healthcare, Piscataway, NJ, USA). A total of  $6 \times 10^6$  cells/well were cultured in 6-well plates in cell culture medium (DMEM containing 10% (v/v) FCS, 1% (v/v) non essential amino acids, 2 mM L-glutamine, 50  $\mu$ M 2-mercaptoethanol, 50  $\mu$ g/mL gentamycin) with Brefeldin A (10  $\mu$ g/mL; Sigma, St. Louis, MO, USA) to block secretion of intracellular proteins. Aliquots of PBMC were either kept in medium or were stimulated with phorbol 12-myristate 13-acetate (PMA; 25 ng/mL) and ionomycin (1  $\mu$ M; both Sigma) to provoke cytokine production. For a kinetic study on cytokine production, PBMC from four adult mares were stimulated with PMA and ionomycin for 2, 4, 6, 8, 18 and 24 h. All other stimulated samples and medium controls from foals and adult horses were incubated for 4 h in a CO<sub>2</sub> incubator. After incubation, the cells were washed in PBS and fixed in 2% formaldehyde for 20 min at room temperature.

### 2.3. Intracellular cytokine staining and phenotyping of T cells

Intracellular staining was performed in saponin buffer (PBS, supplemented with 0.5% (w/v) BSA, 0.5% (w/v) saponin and 0.02% (w/v) NaN<sub>3</sub>). Monoclonal antibodies to equine IL-4 [42], equine IL-10 [43], and bovine IFN- $\gamma$  (MorphoSys, AbD Serotec, Oxford, UK) were used for intracellular staining. The later antibody was previously found to cross-react with equine IFN- $\gamma$  [32, 41]. A murine IgG1 isotype control was included in the procedure using an aliquot of the PBMC. After staining of around  $1 \times 10^6$  fixed PBMC for 20 min at room temperature, cells were washed twice with saponin buffer and once with PBS/BSA (PBS, supplemented with 0.5% (w/v) BSA and 0.02% (w/v) NaN<sub>3</sub>). For cell surface staining of T cells, monoclonal antibodies to equine CD4 (HB61A, VMRD, Pullman, WA, USA), equine CD8 (CVS8, kindly provided by Dr Paul Lunn) and equine CD3<sup>1</sup> were used. Staining of IgE<sup>+</sup> cells was performed with monoclonal anti-IgE 176 [40]. Cell surface staining was performed in PBS/BSA for 10 min at room temperature. Afterwards, the cells were washed once with PBS/BSA

<sup>1</sup> Wagner et al., unpublished data.

and measured by flow cytometry using a FACSCalibur flow cytometer (BD Biosciences, San Diego, CA, USA). A total of 30 000 events were measured per sample. All antibodies were conjugated to Alexa fluorochromes A647 or A488 (Molecular Probes, Invitrogen, Eugene, OR, USA) according to the protocol provided by the supplier, except for the FITC conjugated anti-bovine IFN- $\gamma$  antibody.

#### 2.4. Statistical analysis

A Shapiro–Wilk normality test was performed on each data set and showed that several variables were not Gaussian distributed. Thus, non-parametric test were used for data analysis. The foal groups at various time points were composed of animals living in the same environment but did not represent sequential samples. Cytokine production in the foal and adult groups and the cytokine ratios between age groups were compared by a Kruskal–Wallis test followed by Dunn’s pairwise tests. For example, the total percentages of cytokine producing cells were compared at the lowest age to 2nd lowest, 2nd lowest to 3rd lowest, etc. For the cytokine ratios and the relative percentages of cytokine producing CD4<sup>+</sup> and CD8<sup>+</sup> cells, we compared each of the foal groups to the adults. The analysis was performed 2-tailed and with 95% confidence intervals. We used 2-sided alpha = 0.05 for significance and did not make additional adjustments for multiplicity beyond those implicit within each Kruskal–Wallis test and its Dunn’s test. The statistical calculations were performed using the GraphPad Prism program, version 5.01 (GraphPad Software, La Jolla, CA, USA).

### 3. RESULTS

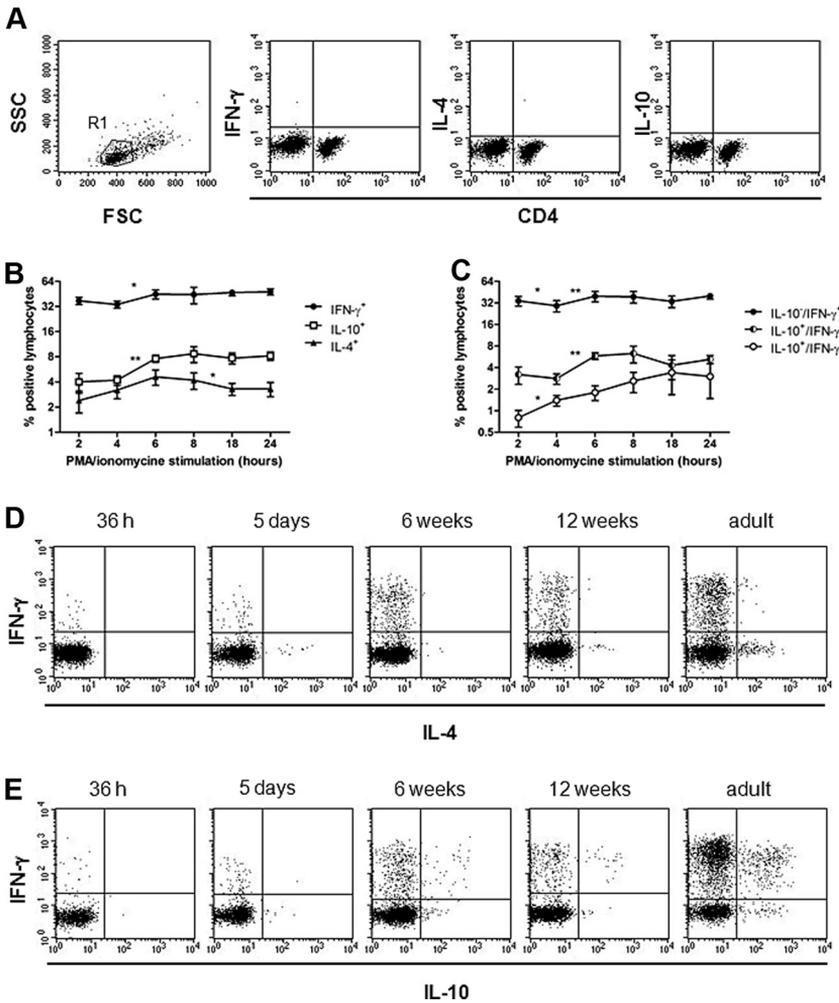
#### 3.1. Cytokine production and T cell marker expression in PBMC stimulated with PMA and ionomycin

PBMC from four adult horses were stimulated with PMA and ionomycin for 2 to 24 h to identify the optimal stimulation time for cytokine production. Intracellular cytokines were detectable at all time points of stimulation (Fig. 1B) but not in non-stimulated cells ( $\leq 0.4\%$  at all time points). The total percentages of IFN- $\gamma$  or IL-10 producing cells showed a significant increase between 4 and 6 h of stimulation (IFN- $\gamma$   $p = 0.03$ ; IL-10  $p = 0.002$ )

and reached a plateau afterwards. The percentage of total IL-4 producing cells also showed a tendency to increase during the first 6 h without being significantly different from the previous time point. Between 8 and 18 h, IL-4 decreased slightly but significantly ( $p = 0.03$ ). For IFN- $\gamma$  and IL-10, we also analyzed individual populations producing only one or both cytokines during 24 h of stimulation (Fig. 1C). Similar to total IFN- $\gamma$  and IL-10 producing cells, the single IFN- $\gamma$ <sup>+</sup> or IFN- $\gamma$ <sup>+</sup>/IL-10<sup>+</sup> cells increased significantly between 4 and 6 h of stimulation. Single IL-10<sup>+</sup> cells increased significantly between 2 and 4 h and then showed a tendency to increase without significant differences from one to the next time point and with increasing variations between horses as indicated by the high standard deviations at 18 and 24 h.

In addition, CD3, CD4 and CD8 expression was compared in stimulated and non-stimulated PBMC (Tab. I). In non-stimulated cells, the percentages of CD3<sup>+</sup>, CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes did not differ during 24 h of incubation. Previous reports have shown that PMA and ionomycin stimulation reduced the expression of CD4. Here, we observed that CD4 and CD8 expression decreased in PMA stimulated lymphocytes compared to non-stimulated cells, while CD3 expression was not effected by the treatment. Within the CD3<sup>+</sup> cells, the percentages of cells expressing CD8 were up to two-fold higher in non-stimulated samples than after PMA stimulation. Significant differences in the stimulated samples were observed between 2 and 4 h ( $p = 0.0498$ ), and 4 and 6 h ( $p = 0.0079$ ). Longer stimulation did not alter the percentage of CD8<sup>+</sup> cells any further. In contrast, the expression of CD4 decreased constantly in stimulated cells, with the exception of a slight increase between 4 and 6 h of stimulation ( $p = 0.0248$ ). The ratios of CD4<sup>+</sup> T cells in non-stimulated versus stimulated cells ranged from 1.37 at 2 h, 2.41 at 4 h, up to 9.11 at 24 h.

Because one major goal of this study was to investigate cytokine production in Th and T<sub>R</sub>1 cells, CD4 detection was crucial for the analysis by flow cytometry. All following experiments used PMA and ionomycin stimulation for 4 h



**Figure 1.** Flow cytometric analysis of IFN- $\gamma$ , IL-4 and IL-10 producing PBMC after stimulation with PMA and ionomycin. PBMC were incubated in the presence of the secretion blocker Brefeldin A. Then, they were fixed and intracellular cytokine staining was performed. (A) Non-stimulated PBMC from a 6 week old foal after 4 h of incubation in medium with Brefeldin A. The left plot shows the gating (R1) on peripheral blood lymphocytes that was used for analysis of the data. The remaining three plots show a two-color staining of non-stimulated lymphocytes using anti-CD4 and different anti-cytokine antibodies. Typically, cytokines were not detected in equine peripheral blood lymphocytes in the absence of stimulation. Isotype controls for cytokine staining generally resulted in less than 0.05% of detectable cells. (B and C): PBMC from 4 adult horses were stimulated with PMA and ionomycin for up to 24 h and cytokine expression was measured at various time points. (B) Total percentages of IFN- $\gamma$ , IL-10 and IL-4 producing cells in the lymphocyte population, (C) Percentages of IFN- $\gamma^+$ /IL-10 $^+$ , IFN- $\gamma^+$ /IL-10 $^-$  and IFN- $\gamma^-$ /IL-10 $^+$  cells during 24 h of stimulation. The data in B and C represent means and standard deviations. Differences in cytokine expression from one to the next time point were compared by Student's *t*-tests. \*\* *p* = 0.001 to < 0.01; \* *p* = 0.01 to 0.05; (D) PMA and ionomycin stimulated cells from foals of different ages and adult horses. Double staining of IFN- $\gamma$  and IL-4 or (E) IFN- $\gamma$  and IL-10. The dot plots show one representative image from a foal or horse of the respective age group.

**Table 1.** Flow cytometric analysis of T cell marker expression in equine PBMC ( $n = 4$ ) after stimulation with PMA and ionomycin (PMA) or in non-stimulated cells (med).

Cell population (%) <sup>a</sup>	Time of stimulation (hours)					
	2	4	6	8	18	24
CD3 <sup>+</sup> (med) <sup>b</sup>	77.6 ± 9.4	79.3 ± 8.3	81.7 ± 7.4	79.9 ± 7.4	86.4 ± 5.9	86.3 ± 3.1
CD3 <sup>+</sup> (PMA) <sup>b</sup>	84.4 ± 3.6	83.5 ± 6.2	85.1 ± 6.3	86.9 ± 4.0	87.5 ± 3.6	87.5 ± 3.2
Ratio CD3 <sup>+</sup> med/PMA	0.92	0.95	0.96	0.92	0.99	0.99
CD4 <sup>+</sup> (med) <sup>c</sup>	76.3 ± 1.3	75.7 ± 1.6	76.5 ± 1.1	76.7 ± 1.2	77.1 ± 2.2	75.8 ± 2.2
CD4 <sup>+</sup> (PMA) <sup>c</sup>	55.7 ± 3.5	31.4 ± 5.1	37.8 ± 6.0	26.8 ± 8.7	14.0 ± 10.4	8.3 ± 3.5
Ratio CD4 <sup>+</sup> med/PMA	1.37	2.41	2.03	2.86	5.50	9.11
CD8 <sup>+</sup> (med) <sup>c</sup>	17.1 ± 1.2	18.2 ± 1.8	17.4 ± 1.2	17.2 ± 1.8	16.2 ± 1.3	15.4 ± 1.1
CD8 <sup>+</sup> (PMA) <sup>c</sup>	10.7 ± 2.0	8.8 ± 1.1	11.0 ± 1.5	11.5 ± 1.4	9.0 ± 1.2	8.9 ± 1.8
Ratio CD8 <sup>+</sup> med/PMA	1.60	2.06	1.58	1.49	1.8	1.73

<sup>a</sup> The data in the table show means and standard deviations.

<sup>b</sup> For analysis of CD3<sup>+</sup> cells, a gate was set on lymphocytes and the percentages of CD3<sup>+</sup> cells were determined.

<sup>c</sup> CD4 and CD8 cells: The percentages show the relative amounts of CD4<sup>+</sup> or CD8<sup>+</sup> cells within the population of CD3<sup>+</sup> cells (= 100%).

to obtain sufficient cytokine production, while CD4 expression was still in an acceptable range. Although cytokine production and CD4 and CD8 expression were slightly higher in adult horses after 6 h compared to 4 h, we did not perform stimulations for 6 h because the cell recovery rate clearly decreased during the first 8 h of stimulation with PMA (data not shown) and cells of neonates were often very sensitive to the treatment resulting in even higher cell losses.

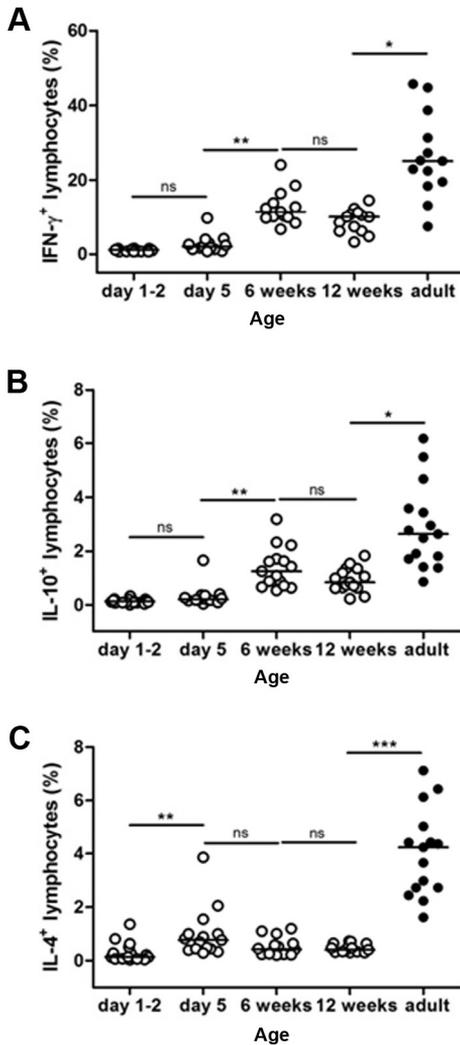
### 3.2. Cytokine production in foals at different ages

To analyze the production of IL-4, IFN- $\gamma$  and IL-10 cytokines in foals during the first three months of life, PBMC were stimulated in vitro and intracellular cytokine production in lymphocytes was analyzed by flow cytometric analysis. Non-stimulated PBMC were run as experimental controls and did not contain detectable amounts of IL-4, IFN- $\gamma$  and IL-10 (Fig. 1A) with the only exception being a small but distinct population of IL-4 producing cells evident in non-stimulated samples from 5-day old foals. PMA treatment of PBMC obtained from newborn and day 5 old foals resulted in

low numbers of cytokine producing cells (Fig. 1D and E). Then, the total percentages of cells producing IFN- $\gamma$  (Fig. 2A) and IL-10 (Fig. 2B) increased until 6 weeks of age and stayed at a similar level by 12 weeks of age. In contrast, the IL-4 production peaked in foals at day 5, decreased slightly but not significantly by 6 weeks and did not differ between 6 and 12 weeks of age (Fig. 2C). For all three cytokines analyzed in the foals, adult levels were not reached by 12 weeks of age.

### 3.3. Ratios of IFN- $\gamma$ /IL-4 and IFN- $\gamma$ /IL-10 in foals and adult horses

Cytokines are mediators of the cellular immune response and the balance of Th1, Th2 and Treg cells crucially influences the initiation and outcome of the immune response. One could hypothesize that comparable ratios of respective cytokines might result in similar immune responses in foals and adults, even if total numbers of cytokine producing cells differ with age. To analyze the relative concentrations of cytokines in foals and adult horses, we calculated the ratios of IFN- $\gamma$ /IL-4 (Fig. 3A) and IFN- $\gamma$ /IL-10 (Fig. 3B) using the total cytokine production in equine lymphocytes stimulated



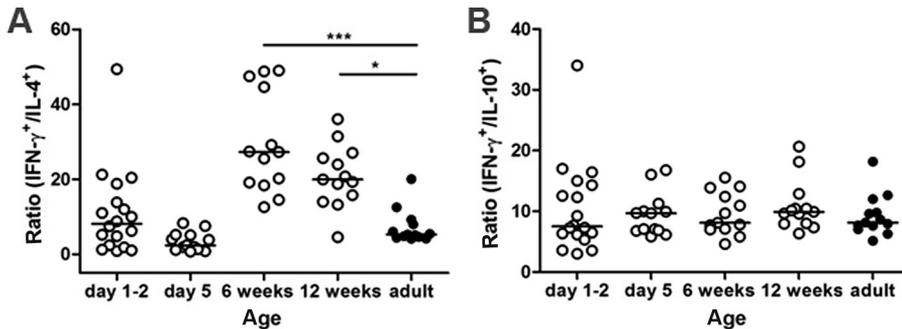
**Figure 2.** Total percentages of (A) IFN- $\gamma$ , (B) IL-10 and (C) IL-4 producing lymphocytes in neonates ( $n = 18$ ), foals until 12 weeks of age ( $n = 15$ ) and in adult horses ( $n = 15$ ). PBMC were stimulated with PMA and ionomycin. The cells were stained for intracellular cytokine production and were analyzed by flow cytometry. The percentages include all cells producing the respective cytokine including double positive cells. The horizontal lines within the data sets represent medians. \*\*\*  $p < 0.001$ ; \*\*  $p = 0.001$  to  $< 0.01$ ; \*  $p = 0.01$  to  $0.05$ ; ns = not significant.

with PMA and ionomycin. The IFN- $\gamma$ /IL-4 ratios in neonates at day 1–2 and day 5 after birth were similar to adult horses. At 6 and 12 weeks of age the IFN- $\gamma$ /IL-4 ratios in foals were significantly increased compared to adult horses. In contrast, the IFN- $\gamma$ /IL-10 ratios were not significantly different in neonates or foals compared to adult horses ( $p = 0.696$ ).

### 3.4. Phenotyping of IFN- $\gamma$ <sup>+</sup> T cells

To investigate whether phenotypic differences of IFN- $\gamma$  producing T cells could be identified between foals and adult horses, cells were double stained for IFN- $\gamma$  and with CD4 or CD8 to identify Th cells and CTL respectively (Fig. 4). The percentages of IFN- $\gamma$ <sup>+</sup> cells within the two T cell populations were determined. Compared to adults, 1–2 days old foals had significantly fewer CD4<sup>+</sup>/IFN- $\gamma$ <sup>+</sup> cells (Fig. 4B) and a slightly increased but not significantly higher percentage of CD8<sup>+</sup>/IFN- $\gamma$ <sup>+</sup> cells (Fig. 4C). Five days old foals had a trend towards greater percentages of CD4<sup>+</sup>/IFN- $\gamma$ <sup>+</sup> cells and fewer CD8<sup>+</sup>/IFN- $\gamma$ <sup>+</sup> cells compared to adult horses, but these differences were not significant. The percentages of CD4<sup>+</sup> or CD8<sup>+</sup>/IFN- $\gamma$ <sup>+</sup> cells of older foals were similar to adult horses.

A third T cell population that simultaneously produces IFN- $\gamma$  and IL-10 are T<sub>R</sub>1 cells. These regulatory T cells also express CD4. IFN- $\gamma$ <sup>+</sup>/IL-10<sup>+</sup> cells were clearly detectable in foals at 6 and 12 weeks of age (Fig. 4D) and represented the majority of the IL-10<sup>+</sup> cells in foals and adult horses (Fig. 1C and E). Previous studies and data obtained here showed that  $84.3 \pm 3.3\%$  of the IFN- $\gamma$ <sup>+</sup>/IL-10<sup>+</sup> lymphocytes are CD4<sup>+</sup> T<sub>R</sub>1 cells (Fig. 4E). Within the total CD4<sup>+</sup>/IFN- $\gamma$ <sup>+</sup> cell population of adult horses, T<sub>R</sub>1 cells accounted for approximately 9%, while 91% of the CD4<sup>+</sup>/IFN- $\gamma$ <sup>+</sup> lymphocytes produced IFN- $\gamma$  only, and thus represented Th1 cells. In foals, the relative amounts of IFN- $\gamma$ <sup>+</sup>/IL-10<sup>+</sup> cells within the total IFN- $\gamma$ <sup>+</sup> cells was 2.4% at 5 days, 6.9% at 6 weeks and 6.7% at 12 weeks of age. However, a



**Figure 3.** Ratios of (A)  $\text{IFN-}\gamma^+/\text{IL-4}^+$  and (B)  $\text{IFN-}\gamma^+/\text{IL-10}^+$  lymphocytes in foals up to 3 months of age and adult horses. PBMC were stimulated with PMA and ionomycin. The cells were stained for intracellular cytokine production and measured by flow cytometry. The total percentages of  $\text{IFN-}\gamma$ ,  $\text{IL-4}$  and  $\text{IL-10}$  producing lymphocytes were determined and ratios were calculated for the respective cytokines. The horizontal lines within the data points show the medians. \*\*\*  $p < 0.001$ ; \*  $p = 0.01$  to  $0.05$ .

significant lower amount of  $\text{IFN-}\gamma^+/\text{IL-10}^+$  cells was only found for foals at day 5 of age compared to adult horses ( $p < 0.001$ ). The relative amounts of  $\text{IFN-}\gamma^+/\text{IL-10}^+$  cells from older foals did not differ from those of adult horses.

### 3.5. Phenotyping of $\text{IL-4}^+$ cells

$\text{IL-4}$  producing cells in non-stimulated PBMC from foals directly after birth were previously shown to be  $\text{CD4}^-$  cells. The  $\text{IL-4}^+$  cells had  $\text{IgE}$  bound to their surface and were characterized as basophils. To determine whether  $\text{IL-4}$  production could be provoked in T cells from neonates and foals, PBMC were stimulated with PMA and ionomycin and double stained for intracellular  $\text{IL-4}$  and cell surface  $\text{CD4}$  or  $\text{IgE}$ .

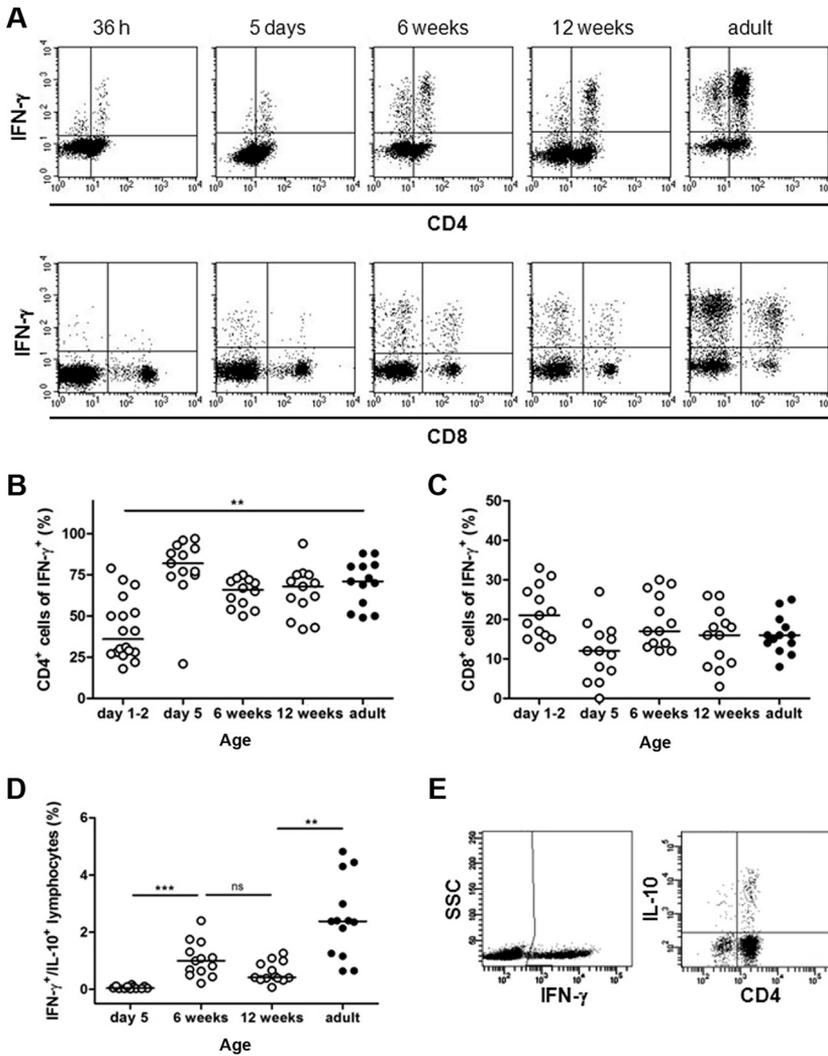
PMA stimulation of PBMC from adult horses consistently induced  $\text{IL-4}$  production in  $\text{CD4}^+$  T cells (Fig. 5) resulting in 87% of  $\text{CD4}^+/\text{IL-4}^+$  cells and 5% of  $\text{IgE}^+/\text{IL-4}^+$  cells. Although the total percentages of  $\text{IL-4}^+$  cells in foals were significantly lower than in adult horses at all time points (Fig. 2C), the percentage of  $\text{CD4}^+/\text{IL-4}^+$  cells consistently increased from 8% at birth to 62% at 12 weeks of age (Fig. 5B). In contrast, the percentage of  $\text{IgE}^+/\text{IL-4}^+$  cells progressively decreased from 95% in newborn foals to 17% at 12 weeks of age. In summary, PMA induced  $\text{IL-4}$  production

by Th cells was almost undetectable in newborn foals, when  $\text{IL-4}$  was mainly produced by  $\text{IgE}^+$  cells. Then,  $\text{IL-4}$  production by Th cells increased slowly with age. At 12 weeks of life, the relative percentage of  $\text{CD4}^+ \text{IL-4}$  producing cells had still a tendency of being reduced compared to adult horses. However, this difference was not significant anymore.

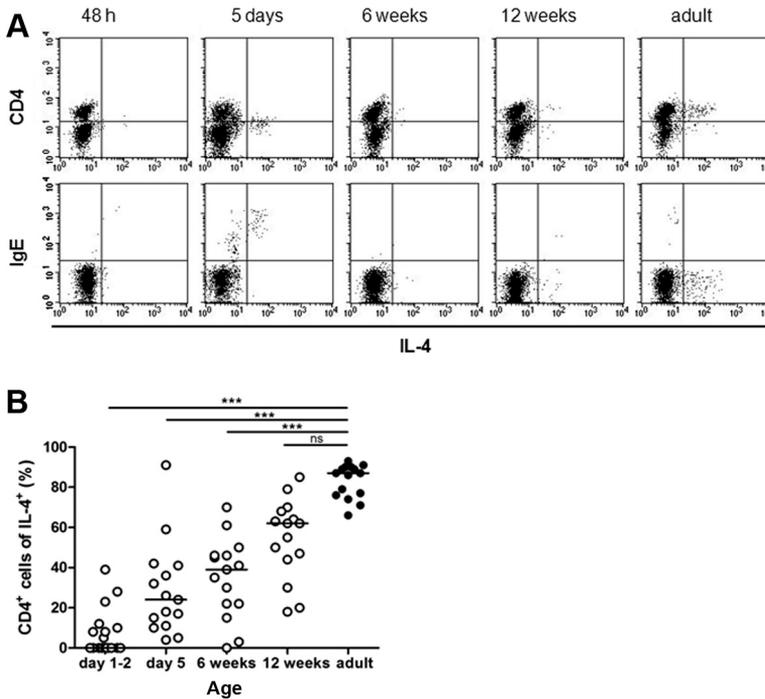
## 4. DISCUSSION

Cytokines are potent regulators of innate and adaptive immune responses. Their fine-tuning is crucial for the successful development of immunity and protection from disease. However, the interactions of cytokines with cells are manifold and result in a complex network of activating and suppressive mechanisms [1, 25, 27, 37, 38].

Cytokines produced by different Th cell subsets play major roles in orchestrating the adaptive immune response during infectious disease. The inability of foals to respond to infection or vaccination comparable to that of adult horses has often been interpreted to reflect “immunodeficiency” and was believed to be a reason for the increased susceptibility of foals to intracellular pathogens such as *R. equi* [5, 7, 20]. Other authors have suggested that foal immune system exhibits a Th2-bias [5, 23, 26]. This



**Figure 4.** Flow cytometric analysis of CD4<sup>+</sup> and CD8<sup>+</sup> IFN- $\gamma$  producing lymphocytes. PBMC from foals of different age groups and from adult horses were stimulated with PMA and ionomycin. The cells were stained for intracellular IFN- $\gamma$  and cell surface CD4 or CD8 expression. (A) Flow cytometric analysis of one representative horse per age group. (B) Relative percentages of CD4<sup>+</sup>/IFN- $\gamma$ <sup>+</sup> and (C) CD8<sup>+</sup>/IFN- $\gamma$ <sup>+</sup> T cells in foals and adult horses within the IFN- $\gamma$  producing cells (= 100%). (D) Percentages of IFN- $\gamma$ <sup>+</sup>/IL-10<sup>+</sup> lymphocytes in foals and adult horses. (E) A tri-color staining was performed on stimulated PBMC to confirm that the majority of the IFN- $\gamma$ <sup>+</sup>/IL-10<sup>+</sup> cells are CD4<sup>+</sup> T cells. Gates were set on lymphocytes (see Fig. 1A) and on IFN- $\gamma$ <sup>+</sup> cells (left panel). The IFN- $\gamma$ <sup>+</sup> lymphocytes were then analyzed for IL-10 and CD4 staining (right panel). The horizontal lines within the data sets B, C and D represent medians. \*\*\*  $p < 0.001$ ; \*\*  $p = 0.001$  to  $< 0.01$ ; ns = not significant.



**Figure 5.** IL-4 producing CD4<sup>+</sup> and IgE<sup>+</sup> cells from foals and adult horses were measured by flow cytometry. PBMC were stimulated with PMA and ionomycin. The cells were stained for intracellular IL-4 and cell surface CD4 or IgE. (A) A representative image from a horse of each age group is shown. (B) The total number of IL-4<sup>+</sup> cells was normalized to 100% and the relative numbers of IL-4 producing CD4<sup>+</sup> cells (%) were calculated for each horse. Medians are shown as horizontal lines within the data points of each group. \*\*\*  $p < 0.001$ ; ns = not significant.

conclusion might have been drawn from a paradigm in neonatal mice indicating a Th2 dominated immune response, although strong Th1 responses have been induced depending on the experimental conditions [4, 12, 13]. In contrast, data on human neonatal T cells did not support that T cells are Th2 polarized and rather point towards a Th1 or Th0 type response in human neonates [2, 9, 22].

In this study, we analyzed the protein expression of three key cytokines, representing Th1 (IFN- $\gamma$ ), Th2 (IL-4) and T<sub>R</sub>1 (IL-10 and IFN- $\gamma$ ) responses, to investigate the development of Th cell responses in foals during the first three months of life. Previous studies on the induction of adaptive immune responses in foals and adult horses have focused on the expression of mRNA [5, 11, 16, 20, 35] but

failed to provide detailed information about the cellular source of cytokine production. For example, IFN- $\gamma$  mRNA up-regulation in PBMC could originate from several cell types such as Th cells, CTL or NK cells and would not necessarily represent a Th1 response. Similarly, other authors have analyzed the production of a single cytokine (IFN- $\gamma$ ), at the protein level with or without regard to the cell type [7, 29, 31]. This work has shown that IFN- $\gamma$  protein and mRNA expression after PMA stimulation of PBMC from foals attained adult levels at around one year of age [7]. We also observed reduced IFN- $\gamma$  production in neonates and older foals compared to adult horses after PMA stimulation. Similarly, the IL-10 and IL-4 production was clearly reduced in neonatal and foal PBMC when compared to adult cells.

This showed that the PMA induced production of all three cytokines, IFN- $\gamma$ , IL-4 and IL-10, is quantitatively lower in foals than in adult horses.

PMA is a structural analog to diacylglycerol and activates protein kinase C (PKC). PMA can activate different cell populations via this pathway, including T cells, B cells, basophils, mast cells and various other cell types. In T cells, the same intracellular signaling pathway is used following antigen-specific T cell receptor (TCR) activation resulting in T cell differentiation and cytokine production. Similarly, PMA can activate T cells independent of their TCR specificity by directly inducing PKC and cytokine production [45]. Thus, PMA stimulation of PBMC results in a polyclonal cytokine response of the naïve T cell pool, yielding insight into the general potential of these cells to produce cytokines. However, the quantitatively reduced cytokine response to PMA in foals compared to adult horses does not necessarily mean that antigen-specific T cell activation would also result in a non-protective immune response in foals. For example, by comparing responses to *R. equi* infection in healthy foals and adult horses, monocyte-derived dendritic cells from foals responded with increased IL-12 mRNA expression, the innate cytokine which triggers Th1 cell development [11]. Healthy foals also showed an up-regulation of IFN- $\gamma$  mRNA, a decreased expression of IL-4 and increased IFN- $\gamma$ /IL-4 transcript ratios in response to *R. equi* [16]. In another pathogen model, the IFN- $\gamma$  production in response to EHV-1 was found to be low in foal PBMC but the EHV-1 induced IFN- $\gamma$  producing cells were mainly protective CD8<sup>+</sup> CTL [29, 31]. In contrast, aged horses which have a higher susceptibility to EHV-1 infection, showed a clear decrease in IFN- $\gamma$  producing EHV-1 specific CTL [31]. Thus, the antigen-specific T cell response to a specific pathogen can still be sufficient and protective in the foals even if the response varies quantitatively from adult horses.

Twenty years ago, Mosmann and Coffman [27] published their famous studies on the induction of Th1 and Th2 pathways in the *Leishmania major* model in mice. Since then,

it is well accepted that the balance of Th cell cytokines is crucial for the outcome of the adaptive immune response and the development of immunity and protection to infectious diseases. Today, this general concept still has important implications for vaccine development. Classic vaccine formulations were often found to be less effective in young foals. To develop protective vaccine strategies for foals early in life, it is important to understand differences and similarities of neonatal and adult immune responses in healthy horses. One way to analyze qualitative parameters of the adaptive immune response is to determine the balance of cytokines produced by Th cells. Despite the quantitative reduction of IFN- $\gamma$  produced by foal CD4<sup>+</sup> cells compared to adult cells, our data demonstrated that the balance of Th1/Th2 cytokines in foals was highly dependent on age and biased towards the Th1 phenotype. A reduced Th1/Th2 cytokine profile compared to adults was only observed on day 5 after birth and was mainly due to the peak in IL-4 production by IgE<sup>+</sup> cells. In 6 and 12 weeks old foals, the T cell response showed a clear Th1 dominated cytokine profile. In addition, adult-like IFN- $\gamma$ /IL-10 ratios and the IFN- $\gamma$  production by Th cells or CTL were evident within 5 days of birth. Collectively these data demonstrated that T cells from equine neonates and foals are competent in mounting Th1, CTL and T<sub>R</sub>1 responses that are qualitatively similar to those observed in adult horses.

In addition, we found that equine neonates and foals had an impaired Th2 response that did not reach adult-like levels even by 3 months of age and that the IL-4 production in neonates and very young foals originated mainly from IgE<sup>+</sup> basophils and not from T cells. These data challenge the dogma that equine neonates and foals mount Th2 biased immune responses [5, 23, 26]. Similarly, non-stimulated neonatal basophils isolated around day 5 after birth produced IL-4 and increased IL-4 secretion from these cells was observed after stimulation with anti-IgE [44]. Recent reports derived from humans or experimental mouse models provide increasing evidence that basophils are the most important innate inducers of Th2 cell differentiation during allergic conditions and responses

to parasites by providing the early IL-4 signal that initiates the polarization of Th cells towards the Th2 phenotype [10, 24, 28, 34]. Similarly, basophil derived IL-4 could provide the innate trigger to initiate Th2 differentiation in equine neonatal T cells. However, although IL-4 from basophils was detectable during the first week of life, we observed a clear delay in the development of IL-4 producing Th cells in foals. Thus, an immediate or broad influence of the early IL-4 production by basophils on the development of Th2 immunity is rather unlikely.

Overall, the T cell response in neonatal foals to PMA reported here and by others using antigen specific stimuli [16, 29, 31] more closely resembled immune responses in human as compared to murine neonates. Human neonatal T cells were also found to develop weak IL-2 and IFN- $\gamma$  responses and proliferated poorly in response to TCR mediated stimulation. However, if human neonatal T cells were stimulated by TCR-independent pathways, such as cross-linking of CD3 plus recombinant IL-6 or IL-12 or anti-CD28 antibody, adult-like T cell responses were observed [2].

In summary, our results provide a new point of view on the development of Th cell responses and the balances of Th1, Th2 and T<sub>R</sub>1 cytokines in healthy equine neonates and foals.

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