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Research paper

The effect of age on foal monocyte-derived dendritic cell (MoDC) maturation and function after exposure to killed bacteria

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ABSTRACT

Neonatal foals are uniquely susceptible to certain infections early in life. Dendritic cells (DC) are vital in the transition between the innate and adaptive immune response to infection, but DC biology in foals is not fully characterized. Monocyte-derived DC represent a suitable in vitro model similar to DC that differentiate from monocytes recruited from circulation. We hypothesized that foal monocyte-derived DC (MoDC) would exhibit age-dependent phenotypic and functional differences compared to adult horse MoDC. MoDC generated from 9 horses (collected once) and from 8 foals (collected at 1, 7, and 30 days-of-age) were exposed to killed whole cell *Escherichia coli* or *Staphylococcus aureus* bacteria. MoDC expression of MHC class II (MHC class-II), CD86, and CD14 were measured by flow cytometry, and supernatant cytokine concentrations of IL-4, IL-17, IFN-γ, and IL-10 were quantified with a validated immunoassay. The percentage of MoDC expressing MHC class-II and CD86 was lower and CD14 was higher for cells generated from 1-day-old foals compared to cells generated from adult horses ($P \leq 0.0001$). Bacterial exposure increased the percentage of cells expressing CD86 at all ages ($P < 0.0001$). Bacteria-exposed MoDC from 1-day-old foals produced significantly less IL-4, IL-17, and IFN-γ than adult MoDC produced in response to bacterial exposure ($P \leq 0.04$). Following bacterial exposure, foal MoDC phenotype and cytokine secretion were different than those of mature horses. These differences could reduce the ability of foals to generate a protective immune response against bacterial infection.

1. Introduction

Bacterial sepsis is the most frequent cause of death in foals within the first week of life and is associated with significant economic losses in the equine industry (Brewer and Koterba, 1988; Marsh and Palmer, 2001; Peek et al., 2006). Foals are vulnerable to infection with a number of opportunistic pathogens such as *Rhodococcus equi*, *Cryptosporidium* spp., *Candida albicans* and *Pneumocystis jiroveci*, all of which are considerably less likely to cause infection in adult horses (Boyd et al., 2003). This age-related susceptibility to infection suggests important limitations in foal host defense mechanisms (Flaminio et al., 2009; Mosser and Hondalus, 1996). Induction of an appropriate T lymphocyte response to a given pathogen is one aspect in the immune response that may limit the ability of the neonate to overcome infection (Boyd et al., 2003; Kovarik and Siegrist, 1998). It is the cytokine milieu generated by cells of the innate immune system, including dendritic cells (DC), that fundamentally dictates the type of T cell response generated (Balkwill and Burke, 1989; Boyd et al., 2003).

Dendritic cells (DC) are important antigen presenting cells (APC) that play a pivotal role in activating and polarizing the adaptive immune response. In peripheral tissues, immature DC continuously survey the environment for pathogen and damage associated molecular patterns and capture peptide antigen for presentation. Immature DCs lack the mature complement of, and density of, critical cell surface proteins to optimally induce primary T cell responses (Cella et al., 2000; Flaminio et al., 2007). The interaction with foreign antigen by DC induces their differentiation and activation, and initiates DC maturation. Phenotypic changes observed during the transition to fully matured DC

Abbreviations: APC, antigen presenting cells; DC, dendritic cells; MoDC, monocytederived dendritic cells

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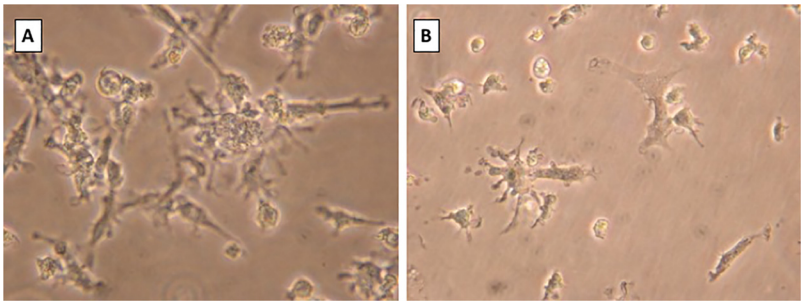

Fig. 1. Monocyte-derived dendritic cells after 4 days in culture isolated from an adult horses (A) and 1 day old foal (B). Foal cells exhibit a more immature phenotype characterized by a rounder shaped with truncated and fewer dendrites.

include: 1) decreased CD14 expression, indicating differentiation from a monocyte into a DC (Mauel et al., 2006; Merant et al., 2009; Ziegler et al., 2016); 2) decreased endocytic capability (Auray et al., 2010; Cavatorta et al., 2009; Merant et al., 2009; Steinbach et al., 2005); 3) increased antigen presentation capacity (expression of MHC class-II); and 4) increased expression of co-stimulatory molecules such (e.g., CD86) (Flaminio et al., 2009; Merant et al., 2009). Only those DC that have fully matured are capable of optimally priming naïve T cells in lymphoid tissues (Moyo et al., 2013).

Studies in human infants have documented substantial differences in the maturation and function of neonatal DC *ex vivo* relative to those generated from adult blood monocytes, (Hunt et al., 1994; Langrish et al., 2002; Petty and Hunt, 1998; Velilla et al., 2006) with implications in progression of neonatal sepsis (Elsayh et al., 2013). However, studies investigating DC biology in foals are limited and primarily focus on induced monocyte derived dendritic cells (MoDC). An increased understanding of the age-dependent changes in foal DC function during both health and disease is imperative to establishing better preventative and therapeutic strategies for neonatal infections in foals. The objective of this study was to determine the effect of age on the maturation and function of equine neonatal DC during *ex vivo* exposure to killed bacteria. We hypothesized that foal MoDC would exhibit phenotypic and functional differences related to the age of the foal and when compared to adult horse MoDC.

2. Materials and methods

2.1. Animals

Horses and foals belonging to the University of Georgia and client-owned horses and foals were used in this study. The University's Institutional Animal Care and Use Committee and Clinical Research Committee approved study protocols and owner consent was obtained for client-owner animals prior to enrollment. Nine healthy adult mixed-breed horses (6 males, 3 females, age 2–16 years) were sampled once during the spring, and 8 healthy Quarter Horse foals (5 males, 3 females) were sampled at 1, 7, and 30 days of age. All animals were confirmed to be systemically healthy prior to sampling by normal physical examination and absence of hematologic abnormalities on CBC. All foals were full term (> 330 days gestation), born via unassisted vaginal delivery, and were confirmed to have adequate transfer of passive immunity (IgG > 800 mg/dL) at 1 day of age prior to enrollment.

2.2. MoDC generation, culture and stimulation

Approximately 120 mL blood was collected via direct jugular venipuncture into syringes containing 1.5 mL of 100 mM EDTA from each adult horse once, and from each foal at 1, 7, and 30 days of age. Peripheral blood mononuclear cells (PBMC) were isolated via single step density centrifugation using 1.077–1.088 lymphocyte separation media (Corning, USA) (Hart et al., 2011). PBMC were plated at 5×10^6 cells/mL in 6-well plates (Nunc ®) with serum-free medium (AIM-V, Gibco, USA) supplemented with 2.5 ug/mL amphotericin B and 2 mM L-glutamine. Cells were incubated at 37 °C with 5% CO_2. After 4-hours, the non-adherent cells and media were removed. Fresh serum-free media containing recombinant equine IL-4 (10 ng/mL, R & D Systems, USA) and recombinant equine GM-CSF (50 ng/mL; Kingfisher Biotech, Inc., USA) were added to the adherent cells, which were then incubated as above for 4 days to stimulate MoDC generation. After 48 h in culture, 100% of the medium was discarded and fresh medium and cytokines were added. Control wells contained media and cytokines only. Stimulated wells were exposed to heat-killed, briefly sonicated whole-cell bacterial preparations of *Staphylococcus aureus* (200 µL of 1:50 dilution) or *Escherichia coli* (200 µL of 1:200 dilution). Bacterial whole cell preparations were prepared in the University of Georgia College of Veterinary Medicine Applied Immunology laboratory and stored at −80 °C until use as described previously (Fratto et al., 2017; Nace et al., 2014). Preliminary studies were conducted to determine that these dilutions optimally produced DC maturation while maintaining DC viability (data not shown).

2.3. MoDC maturation assessment via flow cytometric phenotype analysis

On day 4 of culture, adherent and non-adherent MoDC were harvested. A cell scraper (VWR, USA) was used to gently remove adhered cells. Viability was assessed by 0.04% trypan blue exclusion. The average viability of bacteria-exposed and control (non-exposed) adult MoDC was 95% (± 3.5) and 94% (± 5.3), respectively. Foal MoDC viability on average was 87% (± 7.5) for control cells and 82% (± 9.1) for bacteria-exposed cells. In this study we used an approach similar to that described by others, based on the expression of a combination of cell surface markers, to identify and characterize equine MoDC (Flaminio et al., 2007). Fig. 1 illustrates the subjective morphologic differences between MoDC generated from a 1 day old foal and an adult horse. MoDC phenotype was used to delineate MoDC from closely related cell types including monocytes and macrophages (based on substantial CD14 expression) and to classify their stage of maturation (based on the percentage and density of MHC class-II, CD86 and residual level of CD14 expression). As such, MoDC were labeled with the following monoclonal fluorophore-conjugated antibodies: 10 µL FITC-conjugated mouse anti-horse MHC class-II at a 1:160 dilution (Abdserotec, USA), 10 µL Alexa 647-conjugated mouse anti-horse CD14 at a 1:100 dilution (clone 105, Wagner Laboratory, Cornell University) and 40 µL undiluted PE-conjugated mouse anti-human CD86 (BD Bioscience, USA). All antibodies were titrated to determine the minimum saturation binding dilution and minimum cross-color masking prior to this study (data not shown). Cells were analyzed using an Accuri C6 Flow Cytometry with BD C6 data acquisition and analysis software. For each sample the percentage of cells positive for the surface marker tested and the median fluorescence intensity (MdFI) for cells contained in the MoDC gate (defined by forward angle and side

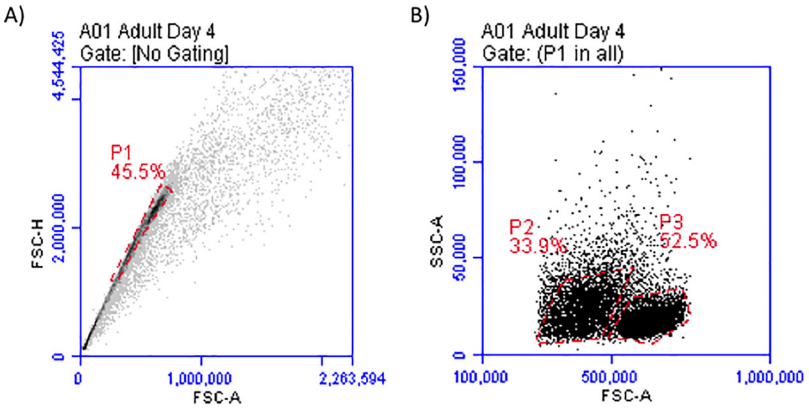

Fig. 2. Gating of equine MoDC generated from whole blood using a representative sample from an adult horse after 4 days in culture. A) Dot plot of forward scatter-height (FSC-H) versus forward scatter-area (FSC-A) outlining the population of interest (P1). B) Dot plot depicting additional gating to delineate the P2 population comprising the most immature MoDC from the P3 population consisting of fully differentiated but immature MoDC using forward scatter-area (FSC-A) and side scatter-height (SSC-A).

scatter) was recorded. To standardize the analysis of cellular phenotype, singlet gating (forward angle height vs forward angle area) was used as a primary gating tool. The singlet gate map that provided the most uniform population of cells while excluding cellular debris and non-single cells (doublets, clumps) is the P1 population and is shown in Fig. 2A. Further, forward angle vs side scatter signal was used to define two populations of cells (P2 and P3) from within the P1 population (Fig. 2B). The smaller population (P2) represents the weakly attached less well-differentiated DC, and the larger population (P3) represents the larger more uniform fully differentiated DC from culture (Fig. 2B). These gates served as the guide to phenotypic analysis. These populations were confirmed by phenotypic assessment of non-adherent cells and those that needed to be scraped from the wells in several cultures (data not shown). The objective of this study was to assess phenotype and function of cells fully differentiated from monocytes and committed to the MoDC lineage. For this reason, only fully- differentiated MoDC within the P3 population were analyzed.

2.4. Functional assays

2.4.1. Cytokine quantification

Prior to harvesting MoDC for surface marker assessment, supernatants from wells containing cells exposed to bacterial antigens and control cells (medium/cytokines only) were collected and stored at −80 °C for batch analysis. Quantification of both inflammatory and anti-inflammatory cytokines (IL-4, IL-10, IFN-γ and IL-17) were determined using a previously validated bead-based fluorescent immunoassay (Horse 5-plex, Cornell University; (Wagner and Freer, 2009). These cytokines were selected to provide insight into the possible polarization of subsequent adaptive responses.

2.4.2. Macropinocytosis and phagocytosis assays

On day 4 of culture, 1×10^5 non-stimulated MoDC were incubated in a 96-well round bottom plate with 50 µL of one of the following: FITC conjugated ovalbumin (5 µg/mL; Molecular Probes, USA), bodipylated *S. aureus* (5 µg/mL; Invitrogen, USA), or bodipylated *E. coli* (10 ug/mL; Molecular Probes, USA) for 4 h at 37 °C with 5% CO_2. After the incubation period, 40 µL of 0.4% trypan blue was added immediately prior to flow cytometric analysis to quench surface fluorescence. The percentage of positive cells (those showing fluorescent signal) and mean fluorescence intensity (MFI) was recorded for each sample to assess protein pinocytosis or non-opsonized bacterial phagocytosis.

2.5. Statistical analysis

Normality of the data was assessed based on examination of histograms and normal Q-Q plots of the residuals. Constant variance of the data was assessed by plotting residuals against predicted values. Non-parametric data were log transformed to achieve normality. Surface marker data, endocytosis data and cytokine data for IL-10 and IL-17 were analyzed using linear mixed-effects models with horse modeled as a random effect to account for repeated measurements and age and bacterial stimulation modeled as fixed effects. Model fit was assessed using Akaike's information criterion values. Significant portions of the cytokine data were censored. Censored data was analyzed using a Tobit regression to account for this. Thus, IL-4 and IFN-γ were analyzed using a Tobit regression model with random and fixed effects as described above. Two-way interactions were evaluated. For effects found to be significant by an overall F-test, pairwise comparisons were made using the method of Holm-Sidak. For all analyses, $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of age and bacterial stimulation on DC maturation

Percentage and median fluorescence intensity of MoDC from adult horses and foals expressing surface markers MHC class-II, CD14 and CD86 are presented in Fig. 3. The results obtained reflect surface marker expression for cells within a forward angle and side scatter gate that was previously determined to contain fully-differentiated immature MoDC (Fig. 2B, P3). In foals at all ages, the percentage of MoDC expressing MHC class-II was significantly lower than MoDC generated from adult horse blood ($P \leq 0.003$). The median density of MHC class-II expression was not significantly different between MoDC generated from foal blood compared to those generated from adult horse blood. At 1 and 7 days of age, the fraction of MoDC derived from foals that were expressing CD86 was less than that of adult horse MoDC, while the fraction of foal MoDC expressing CD14 was more than that of adult horse MoDC ($P \leq 0.02$). The percentage of foal MoDC expressing CD86 and CD14 were not significantly different from adult cell percentages by 30 days of age ($P = 0.05$). The median density of CD86 expression was increased in MoDC generated from foals at 7 days of age compared to those generated from adult horses ($P = 0.03$). However, the density of CD14 expression was not significantly different between foal and adult horse MoDC.

Bacterial exposure did not significantly alter the percentage ($P \geq 0.13$) or median density ($P \geq 0.18$) of foal or adult horse MoDC expressing surface markers, with the exception of CD86. CD86 was expressed by a higher fraction of MoDC after bacterial stimulation, independent of age ($P = 0.0001$).

3.2. Effect of age on cytokine production

The effect of age on the production of cytokines by MoDC is shown in Fig. 4. Basal production of IL-10 and IFN-γ by control foal MoDC was not significantly different to that of control adult cells. However, basal production of IL-17 and IL-4 in MoDC from foals of all ages was

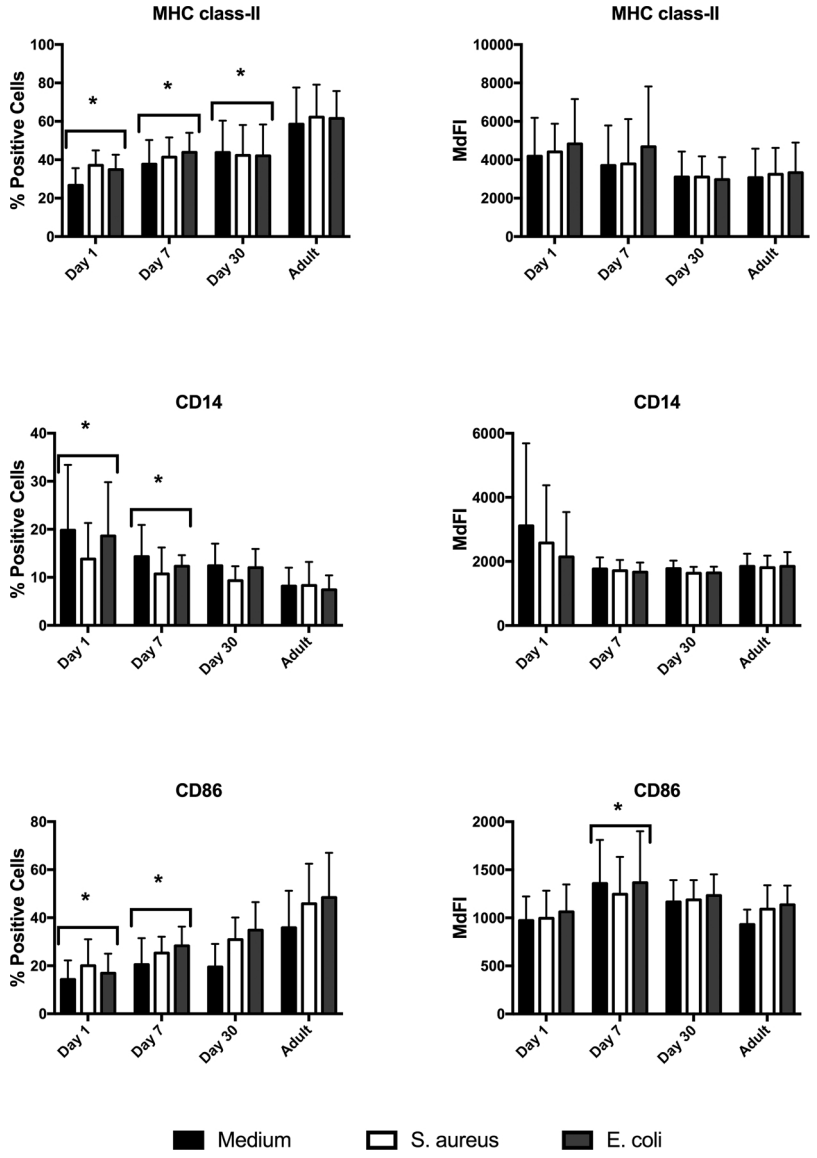

Fig. 3. Percentage (mean ± standard deviation) and median fluorescence intensity (MdFI, mean ± standard deviation) of MoDCs expressing MHC class-II, CD14, and CD86 generated from blood from adult horses (n = 9) once and foals (n = 8) at 1, 7 and 30 days of age in the presence and of absence of bacterial exposure to killed whole-cell preparations of *Escherichia coli or Staphylococcus aureus*. * Denotes statistically significant differences between adult horse and foal MoDCs. ($P < 0.05$).

significantly less than adult horse MoDC ($P \leq 0.04$).

Bacterial exposure of adult MoDC resulted in significantly increased production of all cytokines compared to basal conditions ($P \leq 0.01$) except IL-4, which was not significantly increased after exposure to *S. aureus* ($P = 0.05$). Similar to adult horse MoDC, *E. coli*-exposed MoDC generated from foals at 1 day of age significantly increased production of IL-10 ($P = 0.0001$). In response to exposure to *S. aureus,* MoDC generated from foals at all ages increased production of IL-10 compared to control MoDC from the same foals ($P \leq 0.0001$). The production of IL-10 by foal MoDC at all ages was comparable to that of adults. In contrast, MoDC generated from foals at 1 day of age did not increase IFN-γ and IL-17 production above basal levels after exposure to *E. coli* or *S. aureus*. MoDC from older foals were able to significantly upregulate IFN-γ and IL-17 production after bacterial exposure, but only in response to *E. coli* exposure ($P \leq 0.01$). IL-4, IFN-γ and IL-17 production by stimulated MoDC from foals 7 days of age and younger was significantly less than that of adult horse MoDC ($P \leq 0.01$) after bacterial exposure.

3.3. Macropinocytosis and phagocytosis

Macropinocytosis and non-opsonized phagocytosis activity for MoDC generated from foal and adult horse blood is presented in Fig. 5. To investigate the functional capability of foal MoDC to take up protein antigen and bacteria, MoDC that were the most immature as defined by forward angle and side scatter and confirmed by surface phenotype were evaluated (Fig. 1B, P2). The percentage of MoDC taking up FITC-conjugated ovalbumin by macropinocytosis was greater in MoDC generated from foals of all ages compared to adult horse MoDC ($P \leq 0.009$). The percentage of MoDC generated from foals at 1 day of age that phagocytized bodipylated *S. aureus* was greater than for adult horse MoDC ($P = 0.0001$). The percentage of MoDC phagocytizing bodiplayted *E. coli* was greater for MoDC generated from foal blood collected at 30 days of age compared to MoDC generated from adult horse blood ($P = 0.005$). There was not a significant difference in the amount (MFI) of FITC-ovalbumin, bodiplayted *S. aureus*, or bodiplayted *E. coli* that was taken up by adult horse or foal MoDC ($P \geq 0.36$).

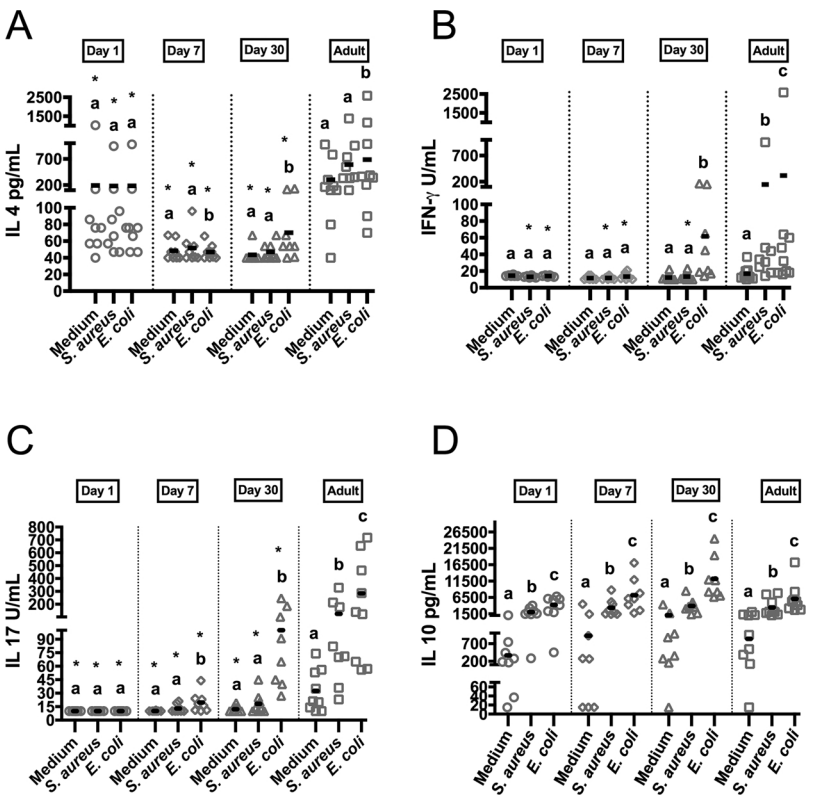

Fig. 4. Cytokine concentration [(A) IL-4, (B) IFN-γ, (C) IL-17, and (D) IL-10] produced by isolated MoDC from adult horses (squares, n = 9) and foals (n = 8) at 1 (circles), 7 (diamonds), and 30 (triangles) days of age following bacterial exposure to killed whole-cell preparations of *Escherichia coli or Staphylococcus aureus* or under basal culture conditions (medium). Each data point represents an individual animal. Horizontal lines represent the mean for that specific age and stimulant condition. *Denotes statistically significant differences between foals and adults. Different letter superscripts denote statistically significant differences between treatment conditions within a given age. ($P < 0.05$).

4. Discussion

The results of this study support our hypothesis that foal MoDC are phenotypically immature and produce an altered cytokine profile, both endogenously and when exposed to bacteria as compared to adult horse MoDC. Distinct phenotypic differences between immature adult MoDC and MoDC derived from foals at 1 day of age were demonstrated in our study, based on an increased percentage of foal MoDC expressing a monocyte-like phenotype. These foal MoDC were characterized by a higher percentage of CD14 positive cells and a lower percentage of cells expressing MHC class-II and CD86 antigen compared to adult MoDC. These findings are in agreement with previous work in foals, (Flaminio et al., 2007, 2009), infants (Lin and Lee, 2014), and mice (Willems et al., 2009). Further, they suggest that neonatal MoDC exhibit phenotypic immaturity across all mammalian species examined.

A smaller fraction of monocytes expressing MHC class-II has been demonstrated prior to the onset of clinically apparent infection in both human adults and infants. This highlights the importance of appropriate antigen-presentation capacity in preventing the onset of disease (Asadullah et al., 1995; Palojarvi et al., 2013). Additionally, loss of MHC class-II and CD86 expression by monocytes and DC has been associated with increased severity of infection in adults (Lin et al., 1993), children (Elsayh et al., 2013), infants (Birle et al., 2003) and mice (Nolan et al., 2009). Results from our study suggest that MoDC from young foals have both phenotypic and functional characteristics that would make them less capable of transitioning into a fully mature DC APC after exposure to bacterial antigens than DC from adult horses. This persistent DC immaturity in foals could ultimately contribute to the development, progression and severity of infection during the neonatal period.

In this study, bacterial stimulation induced an increase in the percentage of MoDC expressing CD86 in both foals and adult horses. However, the percentages of MoDC derived from both foals and adult horses that expressed MHC class-II and CD14 were unchanged from baseline conditions following bacterial stimulation. In addition, no significant difference in the density of MHC class-II, CD86, and CD14 was detected on foal or adult horse MoDC after bacterial exposure. The absence of robust changes in expression density of these proteins is in agreement with some previous studies (Dietze et al., 2008; Flaminio et al., 2007, 2009) but in contrast with the findings of number of others (Cavatorta et al., 2009). The discrepancy in results among studies likely reflects differences in experimental conditions, the bacterial preparation to which the cells were exposed, and the exact MoDC phenotypes generated in response to underlying culture conditions.

In this study, we suspect that culture conditions themselves resulted in partial MoDC maturation. This makes it difficult to detect progressive antigen-induced changes in the percent of cells expressing MHC class-II and CD14 or in the cellular density of MHC class-II and CD14 expression. The expression of CD14 by adult horse MoDC was already low under basal conditions, so no further decrease in expression could be measured based on our detection thresholds. If our culture conditions did induce partial maturation of the foal MoDC, the altered cell surface protein expression in foal MoDC compared to adult horse MoDC under control conditions (without bacterial exposure) might represent true limitations in foal MoDC maturation capacity. Alternatively, foal MoDC might have additional requirements for maturation, including (as yet undefined) environmental signals or cell-cell interactions (Flaminio et al., 2009). Such additional signals were not likely provided under our culture conditions. The reduced fraction of cells expressing MHC class-II and CD86, combined with no observed increase in the fraction of cells expressing these markers in response to bacterial exposure infer endogenous limitations in the ability of foal MoDC to take up, process and present antigen. These limitations of foal MoDC to provide optimal activation of primary naïve T lymphocyte responses during infection in vivo may limit the foal's defense again infection in the neonatal period.

The data presented here also revealed important differences in inflammatory cytokines produced by foal and adult horse MoDC endogenously and after bacterial exposure. The overall balance of inflammatory and anti-inflammatory cytokines produced by DCs following recognition of a pathogen is critical in shaping the subsequent adaptive immune response. In our study, neonatal foal MoDC demonstrated comparable basal production of IL-10 and IFN-γ but decreased

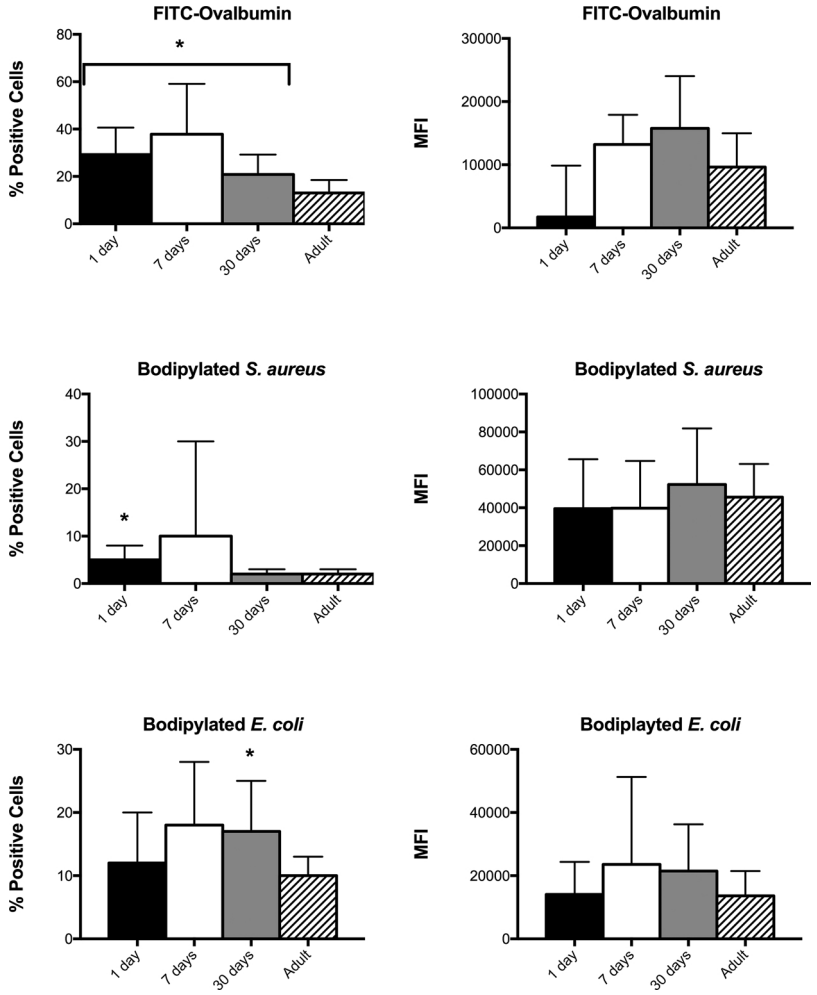

Fig. 5. Percentage (mean ± standard deviation) and mean fluorescence intensity (MFI, mean ± standard deviation) of unstimulated, immature equine MoDC taking up antigen by macropinocytosis (FITC-ovalbumin) or phagocytosis (bodipylated, killed *Staphylococcus aureus* or *Escherichia coli)*. MoDC were generated from adult horses (n = 9) and foals (n = 8) at 1, 7 and 30 days of age. *Denotes statistically significant differences between adult and foal MoDC. ($P < 0.05$)a.

production of IL-4 and IL-17 compared to adult horse cells. This suggests that there may be an intrinsic difference in cytokine responses in foals during homeostasis. Following bacterial exposure, MoDC derived from foals at 1 day of age demonstrated increased production of the anti-inflammatory cytokine IL-10 that was comparable to that induced in adult cells, but foal MoDC failed to show an increase in production of IFN-γ, IL-17, or IL-4 over basal levels. It must be stressed that for all cytokines, except IL-10, many of the foal supernatant samples were below the limit of detection for the assay. Thus, it is difficult to absolutely characterize differences in the production of these cytokines between horses and foals.

IL-10 can suppress the production of inducible cytokines involved in inflammatory and adaptive responses (Sponseller et al., 2009) and is negatively associated with survival in septic foals (Pusterla et al., 2006). In this study, limited bacteria-induced IFN-γ, IL-4, and IL-17 production by MoDC generated from foals at 1 day of age could be mediated by concurrent IL-10 production from foal MoDC. It is possible that foal MoDC might be biased toward the production of IL-10 and have pronounced suppressive effect of IL-10 on neonatal leukocytes. This might explain the lower level of production of IFN-γ and IL-17 by foal MoDC. However, MoDC from adult horses produced similar quantities of IL-10, yet made increased quantities of IFN-γ and IL-17 after bacterial exposure. Alternatively, restricted production of IFN-γ, IL-17, and IL-4 might signify more global molecular limitations in cytokine production due to regulatory mechanisms that are related to the physiological age of foals. Such limitations could include alterations in DNA methylation, access to gene activation master switches in the immune response, or the pathway of protein post-translation modifications available shortly after birth. Further, foals might also require other factors for optimal cytokine responses that were not provided with our culture conditions.

IFN-γ and IL-17 function synergistically to produce a pro-inflammatory response and are involved in immunity against intracellular pathogens (Khader and Gopal, 2010) as well as in immune regulation and activation of adaptive responses. In contrast, IL-4 is the principle cytokine regulating a Th2 immune response. While T cells are considered to be the major sources of IL-17 and IL-4, more recent literature suggests that production of these cytokines by innate immune cells, including DC, is important to establishing an effective immune response (Cua and Tato, 2010; Ma et al., 2015; Onishi and Gaffen, 2010). Previous studies indicate that autocrine effects of IL-17 (Zou and Tam, 2002) and IFN-γ (Pan et al., 2004) support DC maturation with roles in enhancing expression of co-stimulatory and antigen presentation molecules. A role for IL-4 in maturation of DC and regulation of DC secretion of IFN-γ has also been suggested (Webb et al., 2007). The reduced production of these cytokines by foal MoDC endogenously, and in response to bacterial exposure, might reflect limitations in MoDC maturation and have potential implications on the ability of the foal to produce effective immune responses to pathogens.

This study utilized a well-established model in which MoDC were generated by culturing adhered PBMC in the presence of GM-CSF and IL-4 to promote MoDC differentiation. Because a 100% pure population

of MoDC cannot be realistically attained using this model, cytokine production reported here could reflect secretion by other contaminating cell types (monocytes, macrophages, and lymphocytes). As discussed above, both IL-4 and IL-17 are primarily produced by lymphocytes and it is reasonable to assume the possibility that a limited number of contaminating lymphocytes in these cultures contributed to cytokine concentrations reported here. In addition, supplementation of MoDC cultures with reIL-4 to improve MoDC yield and minimize macrophage development might also have contributed to the overall concentration of IL-4 reported here. Studies in equine, (Dietze et al., 2008), murine (Menges et al., 2005), and human (Ruben et al., 2015) models suggest that a population of DC with distinct phenotypic and functional characteristics are produced when culture conditions are supplemented with IL-4. Thus, it is prudent to assume that IL-4 supplementation to MoDC cultures in the study presented here impacted the phenotypic and functional properties of generated MoDC and might also be reflected in our reported IL-4 concentrations. Despite the above limitations, the culture protocol used here permitted detection of significant differences between foal and adult horse MoDC cytokine production in response to bacterial exposure, making the findings reported here still relevant to our understanding of the foal immune response to bacterial infection.

Efficient antigen uptake and presentation by DC is necessary for driving an antigen-specific adaptive immune response. The highly regulated processes of macropinocytosis and phagocytosis are functional attributes that decline as DC mature to prevent undesirable activation of the adaptive immune response against self-antigens (Guermonprez et al., 2002). Thus, in this study, these processes were only evaluated in unstimulated, immature MoDC. Our results revealed an increased number of immature foal MoDC at 1 day of age take up FITC-ovalbumin and *S. aureus* compared to MoDC from adult horses. As discussed previously, the pattern of surface marker indicates it is likely that our culture conditions partially matured the MoDC during generation. Thus, despite some degree of maturation and activation by the culture conditions in this study, foal MoDC remain in a relatively more immature state than adult horse MoDC. The number of foals and horses used in this experiment was relatively small, and increasing the sample size in future experiments would allow for more confidence in defining the endocytic capacity of MoDCs by age. Experiments evaluating endocytic functions following bacterial stimulation might also provide additional insight into age-related changes in MoDC function in foals.

Orchestrating a protective immune response requires multiple functional cellular components, in addition to a multitude of soluble factors including cytokines, hormones, antibodies, vitamins and other proteins. In this study, MoDC were investigated under isolated *ex vivo* conditions, limiting our ability to understand the role of cell-cell and cell-environment interactions that occur in vivo during naturally occurring infection. While the methodology used in this study to generate MoDC is well established, it is unrealistic to assume that a 100% pure population of MoDC is generated. Thus, the surface marker expression and concentrations of cytokines reported here could also reflect surface marker expression and cytokine secretion by low numbers of contaminating cell types (monocytes, macrophages, lymphocytes). Nevertheless, the culture protocol used here permits evaluation of inherent differences in foal MoDC maturation and function by minimizing confounding variables such as plasma factors and thereby provides a platform for future studies that more closely emulate the DC microenvironment in vivo to be performed.

5. Conclusions

The failure to alter surface marker expression and production of key cytokines involved in both cellular and humoral immunity by MoDC derived from foals suggests a state of decreased immune responsiveness to bacterial antigens that extends throughout the first month of life in the foal. While the consequences of foal MoDC immaturity on naïve T cell activation and polarization remain to be elucidated, age-related susceptibility to infection in foals might in part be attributable to the phenotypic immaturity and altered cytokine secretion by foal MoDC described here. Additional studies further elucidating the biology of the whole family of DC in foals will be critical to understanding the underlying mechanisms leading to infection in this highly susceptible population.

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References

Asadullah, K., Woiciechowsky, C., Docke, W.D., Egerer, K., Kox, W.J., Vogel, S., Sterry, W., Volk, H.D., 1995. Very low monocytic HLA-DR expression indicates high risk of infection–immunomonitoring for patients after neurosurgery and patients during high dose steroid therapy. Eur. J. Emerg. Med. 2, 184–190.
Auray, G., Facci, M.R., van Kessel, J., Buchanan, R., Babiuk, L.A., Gerdts, V., 2010. Differential activation and maturation of two porcine DC populations following TLR ligand stimulation. Mol. Immunol. 47, 2103–2111.
Balkwill, F.R., Burke, F., 1989. The cytokine network. Immunol. Today 10, 299–304.
Birle, A., Nebe, C.T., Gessler, P., 2003. Age-related low expression of HLA-DR molecules on monocytes of term and preterm newborns with and without signs of infection. J. Perinatol. 23, 294–299.
Boyd, N.K., Cohen, N.D., Lim, W.S., Martens, R.J., Chaffin, M.K., Ball, J.M., 2003. Temporal changes in cytokine expression of foals during the first month of life. Vet. Immunol. Immunopathol. 92, 75–85.
Brewer, B.D., Koterba, A.M., 1988. Development of a scoring system for the early diagnosis of equine neonatal sepsis. Equine Vet. J. 20, 18–22.
Cavatorta, D.J., Erb, H.N., Flaminio, M.J., 2009. Ex vivo generation of mature equine monocyte-derived dendritic cells. Vet. Immunol. Immunopathol. 131, 259–267.
Cella, M., Facchetti, F., Lanzavecchia, A., Colonna, M., 2000. Plasmacytoid dendritic cells activated by influenza virus and CD40L drive a potent TH1 polarization. Nat. Immunol. 1, 305–310.
Cua, D.J., Tato, C.M., 2010. Innate IL-17-producing cells: the sentinels of the immune system. Nat. Rev. Immunol. 10, 479–489.
Dietze, B., Cierpka, E., Schafer, M., Schill, W., Lutz, M.B., 2008. An improved method to generate equine dendritic cells from peripheral blood mononuclear cells: divergent maturation programs by IL-4 and LPS. Immunobiology 213, 751–758.
Elsayh, K.I., Zahran, A.M., Lotfy Mohamad, I., Aly, S.S., 2013. Dendritic cells in childhood sepsis. J. Crit. Care 28 (881), e887–813.
Flaminio, M.J., Borges, A.S., Nydam, D.V., Horohov, D.W., Hecker, R., Matychak, M.B., 2007. The effect of CpG-ODN on antigen presenting cells of the foal. J. Immune Based Ther. Vac. 5, 1.
Flaminio, M.J., Nydam, D.V., Marquis, H., Matychak, M.B., Giguere, S., 2009. Foal monocyte-derived dendritic cells become activated upon Rhodococcus equi infection. Clin. Vaccine Immunol. 16, 176–183.
Fratto, M.A., Hart, K.A., Norton, N.A., Barton, M.H., Giguere, S., Hurley, D.J., 2017. The effect of free and carrier-bound cortisol on equine neutrophil function. Vet. Immunol. Immunopathol. 183, 16–21.
Guermonprez, P., Valladeau, J., Zitvogel, L., Thery, C., Amigorena, S., 2002. Antigen presentation and T cell stimulation by dendritic cells. Annu. Rev. Immunol. 20, 621–667.
Hart, K.A., Barton, M.H., Vandenplas, M.L., Hurley, D.J., 2011. Effects of low-dose hydrocortisone therapy on immune function in neonatal horses. Pediatr. Res. 70, 72–77.
Hunt, D.W., Huppertz, H.I., Jiang, H.J., Petty, R.E., 1994. Studies of human cord blood dendritic cells: evidence for functional immaturity. Blood 84, 4333–4343.
Khader, S.A., Gopal, R., 2010. IL-17 in protective immunity to intracellular pathogens. Virulence 1, 423–427.
Kovarik, J., Siegrist, C.A., 1998. Immunity in early life. Immunol. Today 19, 150–152.
Langrish, C.L., Buddle, J.C., Thrasher, A.J., Goldblatt, D., 2002. Neonatal dendritic cells are intrinsically biased against Th-1 immune responses. Clin. Exp. Immunol. 128, 118–123.
Lin, S.J., Lee, Y.C., 2014. Effect of influenza A infection on maturation and function of neonatal monocyte-derived dendritic cells. Viral Immunol. 27, 277–284.
Lin, R.Y., Astiz, M.E., Saxon, J.C., Rackow, E.C., 1993. Altered leukocyte immunophenotypes in septic shock. Studies of HLA-DR, CD11b, CD14, and IL-2R

expression. Chest 104, 847–853.
Ma, Y.L., Huang, F.J., Cong, L., Gong, W.C., Bai, H.M., Li, J., Chen, C.X., Zhou, N.Y., Jiang, X.L., Yin, L., Chen, X.P., 2015. IL-4-producing dendritic cells induced during Schistosoma japonica infection promote Th2 cells via IL-4-dependent pathway. J. Immunol. 195, 3769–3780.
Marsh, P.S., Palmer, J.E., 2001. Bacterial isolates from blood and their susceptibility patterns in critically ill foals: 543 cases (1991-1998). J. Am. Vet. Med. Assoc. 218, 1608–1610.
Mauel, S., Steinbach, F., Ludwig, H., 2006. Monocyte-derived dendritic cells from horses differ from dendritic cells of humans and mice. Immunology 117, 463–473.
Menges, M., Baumeister, T., Rossner, S., Stoitzner, P., Romani, N., Gessner, A., Lutz, M.B., 2005. IL-4 supports the generation of a dendritic cell subset from murine bone marrow with altered endocytosis capacity. J. Leukoc. Biol. 77, 535–543.
Merant, C., Breathnach, C.C., Kohler, K., Rashid, C., Van Meter, P., Horohov, D.W., 2009. Young foal and adult horse monocyte-derived dendritic cells differ by their degree of phenotypic maturity. Vet. Immunol. Immunopathol. 131, 1–8.
Mosser, D.M., Hondalus, M.K., 1996. Rhodococcus equi: an emerging opportunistic pathogen. Trends Microbiol. 4, 29–33.
Moyo, N.A., Marchi, E., Steinbach, F., 2013. Differentiation and activation of equine monocyte-derived dendritic cells are not correlated with CD206 or CD83 expression. Immunology 139, 472–483.
Nace, E.L., Nickerson, S.C., Kautz, F.M., Breidling, S., Wochele, D., Ely, L.O., Hurley, D.J., 2014. Modulation of innate immune function and phenotype in bred dairy heifers during the periparturient period induced by feeding an immunostimulant for 60 days prior to delivery. Vet. Immunol. Immunopathol. 161, 240–250.
Nolan, A., Kobayashi, H., Naveed, B., Kelly, A., Hoshino, Y., Hoshino, S., Karulf, M.R., Rom, W.N., Weiden, M.D., Gold, J.A., 2009. Differential role for CD80 and CD86 in the regulation of the innate immune response in murine polymicrobial sepsis. PLoS One 4, e6600.
Onishi, R.M., Gaffen, S.L., 2010. Interleukin-17 and its target genes: mechanisms of interleukin-17 function in disease. Immunology 129, 311–321.
Palojarvi, A., Petaja, J., Siitonen, S., Janer, C., Andersson, S., 2013. Low monocyte HLA-DR expression as an indicator of immunodepression in very low birth weight infants. Pediatr. Res. 73, 469–475.
Pan, J., Zhang, M., Wang, J., Wang, Q., Xia, D., Sun, W., Zhang, L., Yu, H., Liu, Y., Cao, X., 2004. Interferon-gamma is an autocrine mediator for dendritic cell maturation. Immunol. Lett. 94, 141–151.
Peek, S.F., Semrad, S., McGuirk, S.M., Riseberg, A., Slack, J.A., Marques, F., Coombs, D., Lien, L., Keuler, N., Darien, B.J., 2006. Prognostic value of clinicopathologic variables obtained at admission and effect of antiendotoxin plasma on survival in septic and critically ill foals. J. Vet. Internal Med. / Am. Coll. Vet. Internal Med. 20, 569–574.
Petty, R.E., Hunt, D.W., 1998. Neonatal dendritic cells. Vaccine 16, 1378–1382.
Pusterla, N., Magdesian, K.G., Mapes, S., Leutenegger, C.M., 2006. Expression of molecular markers in blood of neonatal foals with sepsis. Am. J. Vet. Res. 67, 1045–1049.
Ruben, J.M., Bontkes, H.J., Westers, T.M., Hooijberg, E., Ossenkoppele, G.J., de Gruijl, T.D., van de Loosdrecht, A.A., 2015. Differential capacity of human interleukin-4 and interferon-alpha monocyte-derived dendritic cells for cross-presentation of free versus cell-associated antigen. Cancer Immunol. Immunother. 64, 1419–1427.
Sponseller, B.A., de Macedo, M.M., Clark, S.K., Gallup, J.M., Jones, D.E., 2009. Activation of peripheral blood monocytes results in more robust production of IL-10 in neonatal foals compared to adult horses. Vet. Immunol. Immunopathol. 127, 167–173.
Steinbach, F., Stark, R., Ibrahim, S., Gawad, E.A., Ludwig, H., Walter, J., Commandeur, U., Mauel, S., 2005. Molecular cloning and characterization of markers and cytokines for equid myeloid cells. Vet. Immunol. Immunopathol. 108, 227–236.
Velilla, P.A., Rugeles, M.T., Chougnet, C.A., 2006. Defective antigen-presenting cell function in human neonates. Clin. Immunol. 121, 251–259.
Wagner, B., Freer, H., 2009. Development of a bead-based multiplex assay for simultaneous quantification of cytokines in horses. Vet. Immunol. Immunopathol. 127, 242–248.
Webb, D.C., Cai, Y., Matthaei, K.I., Foster, P.S., 2007. Comparative roles of IL-4, IL-13, and IL-4Ralpha in dendritic cell maturation and CD4+ Th2 cell function. J. Immunol. 178, 219–227.
Willems, F., Vollstedt, S., Suter, M., 2009. Phenotype and function of neonatal DC. Eur. J. Immunol. 39, 26–35.
Ziegler, A., Everett, H., Hamza, E., Garbani, M., Gerber, V., Marti, E., Steinbach, F., 2016. Equine dendritic cells generated with horse serum have enhanced functionality in comparison to dendritic cells generated with fetal bovine serum. BMC Vet. Res. 12, 254.
Zou, G.M., Tam, Y.K., 2002. Cytokines in the generation and maturation of dendritic cells: recent advances. Eur. Cytokine Netw. 13, 186–199.