MATURATION AND FUNCTION OF EQUINE NEONATAL MONOCYTE-DERIVED DENDRITIC CELLS *EX VIVO*

by

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ABSTRACT

It has been proposed that an increase in susceptibility to infection in neonatal foals is associated with age-related differences in the capacity to mount a competent immune response. Limitations in immune responses in foals have been previously reported. Dendritic cells (DC) are important innate immune cells that provide the functional link between the innate and adaptive immune response and are critical to the activation of naïve T cell. We propose that DC are important to functional immunity in neonates. Evidence in other species suggests that soluble factors (also found in plasma) regulate immune cell function and are different in neonates and adults. Cortisol is a systemic soluble factor that has been shown to inhibit DC function in other species. In neonatal foals, cortisol availability is uniquely regulated and the free fraction of cortisol (active form) is increased in foals compared to adults. To date, DC function in neonatal foals is poorly characterized and the role of systemic soluble factors, on equine DC function has not been investigated.

The overall objective of this project was to increase our understanding of DC biology in foals by investigating a subpopulation of DC that differentiate from blood monocytes within tissues. First, culture conditions were optimized for the generation and maintenance of equine monocyte-derived DC (MoDC) *ex vivo* under serum-free medium conditions. After establishing a set of reproducible and reliable culture conditions, this system was used to investigate intrinsic age-related differences in foal MoDC phenotype and function following exposure to killed bacteria *ex vivo*. Finally, this model was employed to examine the role of soluble factors and cortisol in modulating foal MoDC maturation and function after exposure to killed bacteria. In these studies, MoDC maturation and function were determined by assessing surface marker expression, cytokine production, and endocytic capabilities.

The results from these studies provide a broad assessment of foal MoDC biology after *ex vivo* generation and exposure to killed bacteria. This work provides evidence suggesting that both inherent age-specific differences in MoDC maturation and function and specific components of the environment under which MoDC mature are important components of foal MoDC dysfunction.

INDEX WORDS: Equine, Neonate, Dendritic cell, Plasma, Cortisol, Sepsis

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DEDICATION

To my dad, who always encouraged me to reach for the stars.

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CHAPTER 1

INTRODUCTION

There are significant gaps in our knowledge and understanding of the function and regulation of the neonatal foal immune response. Neonatal foals are uniquely susceptible to bacterial infections, especially to opportunistic infections, early in life. Susceptibility to infection in foals appears to arise as a result of differences in availability and activity of hormones and innate humoral factors, limited function of innate immune cells, and delayed activation of an appropriate adaptive immune response due to immaturity of the cellular components required to effectively make that transition. It is well accepted that dendritic cells (DC) are critical in supporting this transition between innate and adaptive immune responses, but little is known about DC biology in foals. While the availability of tools to examine circulating DC in foals are limited, we can easily generate and evaluate one important subpopulation of DC, those that differentiate from blood monocytes into DC within tissues. These monocyte-derived DC (MoDC) represent an important subpopulation of DC that are present in the mucosal tissues during homeostasis and during periods of inflammation and infection.

This dissertation provides insight into the biology of foal MoDC generated *ex vivo* from blood monocytes and then stimulated with killed bacteria. Specifically, this work describes the methodology used to generate and cultivate equine MoDC in chemically defined medium using only recombinant equine growth factors as additives, and the assessment of MoDC maturation and function using surface marker expression, antigen sampling, and endogenous and induced cytokine production. Further, the studies presented here also examine a model of the extracellular microenvironment by investigating the influence of soluble factors on MoDC maturation and function. This was accomplished by using foal and adult horse plasma to simulate a model of the tissue microenvironment where MoDC are generated *in vivo*. The hormone cortisol, which is increased during infection and differentially regulated in the foals compared to adults, was also examined for its specific effects on MoDC maturation and function.

Chapter 2 provides a review of the literature and is comprised of 8 sections. In Section I, our current knowledge of the global foal immune response, with the exception of DC, is presented. Section II outlines the critical role that dendritic cells play in establishing an effective immune response during infection and in immune tolerance. Current perspective on equine DC function and their role in the immune response are presented in Section III. Section IV reviews the primary soluble factors (also present in plasma) that modulate the immune response. Section V summarizes the impact of cortisol in regulating the immune response. Section VI details our present understanding of cortisol regulation in foals and Section VII reviews effects of cortisol on DC maturation and function across species. Finally, Section VIII summarizes in detail the rationale for the studies presented here.

Chapters 3 – 5 presents a series of three manuscripts that comprise the results of this research. Chapter 3 describes an optimized protocol for generating equine MoDC without the use of serum-supplemented medium, a method that permits MoDC cellular capabilities to be clearly defined without confounding modulation by plasma factors. In Chapter 4, age-related changes in foal MoDC maturation and function are outlined. In addition, the response of foal MoDC to bacterial exposure is examined as a function of age. Chapter 5 investigates the impact of differential sources of plasma, or cortisol, on foal MoDC maturation and function using an *ex vivo* bacterial exposure model.

In Chapter 6, a general discussion of the results in total, and a statement of the conclusions drawn from the three sets of studies conducted – and in composite - are summarized; and proposed future applications of this dissertation research are discussed.

CHAPTER 2

LITERATURE REVIEW

SECTION I: THE NEONATAL FOAL IMMUNE RESPONSE

Sepsis is a common cause of foal morbidity and mortality during the first week of life.[1,2] While neonatal foals are susceptible to infection with a variety of pathogens, they most frequently develop infection caused by opportunistic pathogens or environmental organisms.[3,4] During the first few weeks of life, foals are unable to produce a sufficient quantity of antibody following vaccination to block the development of disease.[5-8] Increased susceptibility to infection and limited responses to vaccinations in foals are proposed to be a result of immaturity of innate [8-11] and adaptive [10,12,13] immune responses as compared to adult animals.

Equine Immune System Development

The majority of development of the equine fetal immune system occurs *in utero*. Immune system development in the fetus is influenced by the maternal immune response during pregnancy.[14] There is a compelling body of evidence in both human and murine models that suggest a T-helper 2 (Th2) response is the predominant immune response during gestation and is essential for maintenance of a healthy pregnancy.[15-18] Some studies suggest that persistence of a Th2-biased response [19] in the neonate after birth is associated with increases in susceptibility to infection observed in foals during the first weeks of life.[20] However, overstimulation of the maternal immune system and development of a T-helper 1 (Th1) response in the dam can lead to early termination of pregnancy and is associated with negative outcomes in neonates.[21-23] The impact of activation of the maternal immune system during pregnancy on fetal immune responses has been assessed utilizing murine models. These studies demonstrated robust pro-inflammatory responses in neonates, the development of autoimmune disease, and neurologic disorders in neonates born to immune-stimulated dams.[15,24,25] When investigating the immune response in neonates, it is important to recognize the impact of the maternal immune response during pregnancy on the development of the immune system of the fetus and its effect on post-natal health.

Neonatal Immune Response at Birth

After birth the neonate must endure a sudden transition from a protected and relatively sterile intra-uterine environment to the outside world with an abundance of antigens and potential pathogens. The immune response of the foal must develop tolerance quickly to environmental flora and commensal organisms, as these microbes colonize the skin, and gastro-intestinal and respiratory tracts. In neonatal foals, T regulatory cells induce and maintain tolerance required to achieve the necessary balance between inflammatory responses and commensal acceptance of microbes. Enhancement of the population of resident macrophages also controls immune responses to microbes in the environment. Through microbe recognition and removal, resident macrophages provide an environment where microbes can be tolerated. However, the foal must still be able to fight infection during this period. Thus, transfer of passive immunity from the dam to the foal through colostrum ingestion is the critical method to circumvent the limited immune defenses in neonates and is the major tool minimizing the risk of infection during the neonatal period.

Colostrum

Adequate and timely ingestion of colostrum by the neonate is an essential element in protection against pathogens.[26-29] Colostrum is composed of several components that act in conjunction with the neonatal immune response. The critical components that are transferred are antibodies, cytokines, and maternal leukocytes.[30] One of the most important components of colostrum is antibody (Ig). IgG, IgA, IgM, and IgE have all been identified in equine colostrum. IgG and IgM are involved in opsonization, complement activation and microbial killing. IgA, in addition to IgM and IgG are important in the removal or neutralization of pathogens.[30] Epitheliochorial placentation in horses prevents *in utero* transfer of maternal antibodies to the fetus. This results in the foal being born with very low, and non-protective concentrations of endogenous IgG and IgM.[28,31] During the first 3 months of life, secondary lymphoid tissues become organized and populated with immune cells facilitating increases in endogenous production of antibody.[30,32]

In addition to Ig, equine colostrum contains maternal cytokines and leukocytes. Inflammatory cytokines IL-6 and TNF-α are undetectable in pre-suckling foal circulation and are passively transferred to the foal from the mare in colostrum.[30,33] Equine colostrum at the time of parturition, primarily contains $CD4^+$ and $CD8^+$ T lymphocytes, with a small number of B cells (<5%), a few macrophages, monocytes, and NK cells.[34] Effects of inflammatory cytokines and maternal immune cells transferred through colostrum ingestion on the development of foal immune responses are poorly understood. Intestinal uptake of colostral leukocytes is described in other species (e.g. cattle, sheep, goats, swine, dogs), but has not been well documented in horses.[35] While it has been postulated that these elements may prime the foal innate immune response immediately after birth,[30] additional studies are needed.

In summary, the transfer of colostral-derived maternal immune elements likely support the foal's initially limited innate immune defenses by providing environmentally-relevant antibodies to augment specific lymphocyte responses.

Neutrophils

Neutrophils are the most abundant cells of the innate immune system [36] and are important for the clearance of bacteria and damaged tissue, B cell stimulation, and regulation of tissue level responses. In most mammals, neutrophil function is limited during the first 2-5 days after birth and is associated with the development of severe bacterial and fungal infection in neonates.[37,38] Prior to the ingestion of colostrum, foal neutrophils exhibit decreased phagocytosis [39] and oxidative killing of bacteria.[40] Immediately following the ingestion of colostrum, foal neutrophil function is transiently improved to adult horse levels.[39] Subsequent to this immediate period, an inherent functional impairment foal neutrophils is observed in the *in vitro* uptake of bacteria, [39,41] but yeast cells can be taken up normally. [42,43] This suggests that transient improvement in neutrophil function after birth could be a result of maternally derived soluble factors, including opsonins, transferred to the foal in colostrum. The oxidative burst activity of foal neutrophils is equally ambiguous, with some studies suggesting comparable activity to adults [41,44] and others documenting decreased activity [45,46] until foals reached 3 months of age.[45] Discordant results could be a result of individual variation in opsonic activity of the plasma used in these studies.[47]

Phagocytosis and oxidative burst activity can be augmented by soluble components in plasma including classical opsonins and complement. The opsonic capacity of foal serum appears to be microbe-dependent. Reduced opsonic capacity of foal serum results in impaired neutrophil phagocytosis of yeast cells and some bacteria, [42,43,46,48] and does not reach adult levels until 3 - 4 weeks of age.[42,45] In contrast, another study observed an adult-like capacity to phagocytose *Actinobacillus equuli* or *Escherichia coli* when foal plasma was utilized as the source of opsonins.[49] In this study, the opsonic capacity of foal serum was dependent upon the quantity of opsonins. This was demonstrated by a greater concentration of foal plasma needed for phagocytosis of *E. coli* than for *A. equuli*.[49] Thus, appropriate neutrophil function in foals is dependent upon the availability of opsonins and is pathogen-specific. In summary, these studies suggest that foal neutrophils exhibit some intrinsic cellular dysfunction that may be at least partially ameliorated by the availability of maternally-derived soluble factors during the neonatal period.

Studies aimed at evaluating opsonic components of foal sera suggest that lack of complement factors, rather than IgG, is one of the critical factors limiting foal neutrophil function.[47,49,50] Low circulating serum concentrations of complement protein C3 have been described in neonatal foals.[51] Further, reduced opsonic capacity of heat-inactivated serum (where complement is destroyed) has been demonstrated.[49,52] Whether foal serum only lacks necessary components to facilitate optimal neutrophil function or if it also contains inhibitory factors suppressing neutrophil function remains to be elucidated.

The ability of foal neutrophils to produce cytokines following antigen exposure has been investigated. Neutrophil gene expression from foals at 24 hours and at 2, 4, and 8 weeks of age following stimulation with virulent *R. equi* has been assessed.[53] Results from this study are consistent with those reported for mature equine neutrophils.[54] Specifically, neutrophils from foals at all ages are able to express IFN-γ, TNFα, IL-6, IL-8, IL-12p40, IL-12p35, and IL-23p19 in response to bacterial exposure. Further, the expression of IL-6, IL-8, IL-12p40, and IL-23p19 was demonstrated to be age-related, with IL-6, IL-8 and IL-23p19 decreasing with age and IL-12p40 increasing with foal age.[53] In another study the ability of foal neutrophils at 2, 14, and 56 days of age to respond to TLR-9 stimulation with CpG oligodinucleotides (CpG-ODN) was assessed.[36] This study demonstrated an increase in IL-6, IL-17 and IL-12p40 gene expression in response to stimulation, and a similar increase in IL-12p40 expression with increasing foal age.

Peripheral Blood Mononuclear Cell Cytokine Production

Several studies have investigated the functional abilities of peripheral blood mononuclear cells (PBMC) in foals. The PBMC cellular fraction of blood is comprised of monocytes, lymphocytes and NK cells. Foal age-driven changes in cytokine mRNA expression by foal PBMC at rest and following stimulation with a range of antigens during the first few months of life have been well described.[55,56] Basal mRNA and protein expression of IFN-γ,[55,56] IL-4,[55] IL-17,[55] and IL-6[56] are low after birth and increase with the age of the foal. Under basal conditions, there appears to be reduced endogenous production of these cytokine by neonatal foals that undergoes progressive enhancement over time. This enhancement of endogenous cytokine activity is likely in response to prior antigenic stimulation following normal environmental exposure and expansion of a population of cells that were activated prior to measurement.[19]

Foal PBMC also demonstrate age-related changes in cytokine expression following exposure to many different mitogens and antigens. Following stimulation of foal PBMC with concanavalin A,[19] *R. equi,*[55,56] CpG-ODN,[56] phorbol 12-myristate 13-acetate (PMA), or ionomycin,[57,58] age-related changes in cytokine gene expression include increases in IFN-γ,[19,56-58] IL-1α,[19] TGF-β1,[19] IL-12p40/35,[56] IL-17,[55] IL-6,[56] IL-4,[58] IL-10,[58] and decreased IL-23p19[56] during the first month of life. However, there is no agreement concerning the effect of age on foal PBMC cytokine production among studies. For example, some report comparable expression of IFN-γ,[55] IL-6,[19] and IL-10[59] in PBMC from neonatal foals relative to PBMC from older foals. An explanation for discordant results is that age-related changes are dependent on the stimulus employed. Nevertheless, the cytokine patterns described above indicate that at birth pathogen recognition pathways can be activated, however, they undergo gradual maturation with foal exposure to the environment.[56] Interpretation of these studies is limited because they do not provide functional comparisons with PBMC from adult horses. Further, the clinical importance of the magnitude of cytokine production required to provide a protective immune response *in vivo* has not been determined.

PBMC cytokine gene expression has been compared between cells from healthy foals and from septic foals at hospital admission and during initial hospitalization by Gold *et al*[60] In this study, there was no difference in the median gene expression of IFN-γ, but there was a 6-fold decrease in expression of IL-4 by PBMC from septic foals relative to PBMC from healthy foals. Although comparable expression of IL-6 by healthy and septic foals was observed at admission, 24 hours later there was a 4-fold decrease in IL-6 expression in septic foal PBMC compared to healthy foal PBMC. Of note, the septic foals were not age-matched to the healthy foals and the median age of control foals was 84 hours, but only 48 hours for septic foals. In a different study, cytokines were measured directly from the blood in both septic and healthy foals[61] and revealed lower expression of TNF-α and TGF-β, but greater expression of IL-8 in septic foals than healthy foals. While there was no difference in the production of IL-10, IL-6 and IL-1β between the two groups of foals, IL-10 expression was greater in the non-surviving group of septic foals.[61]

A study by Jacks *et al*[62] demonstrated that *R. equi* stimulated bronchial lymph node mononuclear cells from healthy and *R. equi* infected foals at ~3 weeks of age significantly increased cellular gene expression of IFN-γ. In this study mononuclear cells from infected foals expressed more IFN-γ, but less for IL-4 compared to mononuclear cells from infected adult horses. Comparable levels of IL-10 and IL-2 mRNA expression by mononuclear was observed for all groups (infected and healthy foals and adult horses). Mononuclear cells from healthy foals stimulated with *R. equi* antigen expressed considerably lower levels of IFN-γ mRNA relative to mRNA expression by mononuclear cells isolated from infected and healthy adult horses after *R.*

equi exposure. These results suggest that despite age-related changes in cytokine gene expression, under the appropriate conditions and with exposure to an appropriate stimulus, PBMC from 3-week-old foals are capable of mounting adult-like IFN-γ responses. This implies the possibility of capacity for functional Th1 response in foals earlier than previously believed.[20,57] It appears that impaired foal PBMC cytokine production is dependent on the antigen involved, and may be related to prior priming of pathogen recognition pathways. This assumption is supported by a publication that documented differential cytokine patterns and magnitudes of cytokine expression in foal PBMC in response to different CpG-ODNs sequences.[56]

In summary, the results of these studies indicate that cytokine profiles generated by foal PBMC are often qualitatively and quantitatively different than responses by adult horse PBMC in most situations. This suggests that foal cytokine production may be regulated differently in foals during the first several weeks of life. The specific cytokine response generate to a particular pathogen subsequently defines the context of the adaptive response that is mounted, and if not appropriate for that pathogen, might limit the ability of the neonatal foal to clear an infection.

Adaptive Immunity

An effective adaptive immune response requires appropriate lymphocyte expansion and suitable polarization for optimal response to each pathogen. Equine fetal lymphocytes residing within the thymus are responsive to plant lectins, which signal through elements of T cell receptor (TCR) context, but do not simulate antigen mediated activation, at 80 days of gestation. Cells circulating in the peripheral blood of the fetus are responsive to plant lectins or phorbol ester and ionomycin stimulation at 120 days of gestation.[31] In response to intrauterine exposure to T2 coliphage, the equine fetus will generate specific neutralizing antibodies of the IgM class.[63] These studies indicate that at birth foal lymphocytes possess a functional TCR complex, and T cell independent B cell responses will generate antigen-specific antibodies of the IgM isotype.

At birth and during the first 2 – 5 days of life, healthy foals have reduced circulating leukocyte counts than mature horses, but foal leukocyte numbers quickly reach and surpass adult horse leukocyte counts.[39] This includes a well-documented age-dependent pattern of expansion of circulating lymphocyte subpopulations in foals from birth to weaning.[39,52,64] Circulating lymphocyte numbers increase linearly until 3 months of age.[65] During this time, lymphocyte numbers in blood more than double due to dramatic increases in $C8^+$ T lymphocytes, and the population of B cells.[52] Expansion of lymphocyte subpopulations in foals is due to active production of fully differentiated functional lymphocytes in the bone marrow and thymus of the neonate, and antigen-dependent responses to environmental and endogenous flora in organized lymphoid foci and tissue, and leading to the development of immunological memory.[52,65]

Immediately after birth, foal lymphocyte proliferation responses to mitogens are decreased relative to adult horse responses.[39] This observation has been attributed to suppressive effects of increased circulating cortisol concentrations from the dam and produced in the foal just before and during parturition.[66] By 1 – 2 weeks of age, foal lymphocytes show comparable or superior non-antigen driven lymphoproliferative responses to TCR-CD3 epsilon-cross linking mitogens (PHA[39,52] and Con A[52,62,67]). Peak proliferative responses of foal lymphocytes after mitogen stimulation coincides with the typical expansion in lymphocyte numbers described above.[52] Of note, greater proliferative responses by foal lymphocytes exposed to PHA were observed, and suggests that the signaling machinery for antigen-specific responses occur in lymphocytes of foals [52] These studies provide additional evidence that foal lymphocytes at birth possess a functional TCR, TCR-complex associated proteins (including the critical adaptor components of CD3 and ability to respond to co-stimulatory molecules), and are able to undergo the process of clonal expansion under *in vitro* conditions.

Several studies have evaluated the ability of foal T cells to produce cytokines, with particular importance placed on the ability to generate Th1 and Th2 cytokines. Isolated $CD4^+$ T cells from the bronchial lymph nodes of *R. equi*-infected foals at 1 month of age demonstrated increased mRNA expression of IL-12p35, IL-6, IL-2, IL-10, and IFN-γ compared to non-infected control foals. This demonstrated the ability of the $CD4^+$ T cells to respond *in vivo* with a primarily pro-inflammatory cytokine response (excluding IL-10) to infection.[68] Despite strong lymphocyte proliferation responses by foal T cells and the ability of foal T cells to respond with new production of cytokine mRNA in response to infection, foal lymphocytes remain phenotypically but functionally different from adult cells.

Major histocompatibility complex class II (MHC-class II) antigen is expressed on all equine lymphocytes, but at a higher surface density on B cells, and activated T cells. The expression of MHC-class II monomorphic antigen by lymphocytes, particularly T cells, was reduced on foals cells compared to cells from adults, but MHC-class II antigen expression increased with age.[52,69] Following stimulation with both TCR activating[67] and direct intracellular signaling pathway activating [58] mitogens, foal $CD4^+$ T cells at 1-2 days of age and 2 weeks of age were able to produced mRNA for IFN-γ, though the magnitude of production appears reduced compared to adults cells. In addition, only proliferating foal T cells were able to produce IFN-γ, whereas both proliferating and non-proliferating adult T cells produced IFN-γ.[67] This dichotomy likely reflects a proportion of adult cells being comprised of memory T cells functioning as bystanders, which are not present in neonatal foals.[67] Foals at birth through 12 weeks of age also had fewer CD4$^+$ IL-4-producing cells compared to adult horses after stimulation with PMA and ionomycin.[58] In fact, the majority of IL-4 produced by lymphocytes from young foals appears to be produced by IgE$^+$ cells.[58] Further, TCR-independent stimulation demonstrated a reduced number of responsive lymphocytes from foals 5 days of age compared to adult horses.[58] When evaluating these findings, it is important to consider the increase in foal lymphocyte number that occurs with advancing age. Thus, the findings reported above might merely be a consequence of decreased absolute numbers of CD4$^+$ T cells in circulation at this age, and may not represent true cellular functional limitations. Still, these studies suggest that progressive maturation of both Th1 and Th2 responses occur in young foals. The effect of the reduced capacity to produce cytokines by foal cells on their ability to protect against infection remains to be determined.

Finally, three studies have specifically evaluated lymphocyte responses of healthy foals compared to sick foals with respiratory disease. In the first study, Flaminio *et al*[65] demonstrated higher circulating T and B cell absolute numbers in sick foals compared to healthy adult horses, but no difference in the distribution of circulating T cell subpopulations. When *R. equi*-infected foals were compared to healthy controls, the sick foals did have an increased circulating fraction of B cells compared to healthy foals.[65] Bronchial lymph node cells from sick foals also displayed greater proliferative responses to Con A relative to cells from adult horses (healthy and sick). Cells from diseased foals, though, had a decreased proliferative response to *R. equi* antigens compared to cells from healthy adult horses.[62] In summary, the combined results of these studies indicate that while foal T cells demonstrate some level of capability for clonal expansion; the response appears to be dependent on antigen exposure, with unknown implications on disease susceptibility.

Antibody Production

The principal function of the humoral immune system is the production of appropriate quantities and classes of antibody to address the location and nature of invaders. As previously discussed, maternal transfer of antibody via colostrum is of paramount importance in providing neonatal protection during the first several weeks of life. During this period, humoral responses in the foal are still developing. At birth, foals are essentially agammaglobulinemic with only very small quantities of endogenous IgM and IgG1 detected in foal serum prior to colostrum ingestion.[30] Immediately after colostral transfer of Ig across the intestinal epithelium, serum concentration of IgG subclasses and IgA in foal circulation are often nearly equivalent to those in the adult horse.[28] Transferred antibody, primarily the IgG4/7 subclass, is found in both colostrum[70] and foal sera at 24 hours or more after suckling.[28]

While maternal antibody is vital for effective opsonization and "targeting" of foreign antigen, these circulating antibodies also are believed to directly suppress *de novo* synthesis of endogenous Ig by the foal. This has been demonstrated in several studies that have identified accelerated antibody production in colostrum-deprived foals[71] or in foals receiving bovine colostrum.[28,52,72] This situation becomes important to the foal when maternal antibodies start to wane before endogenous foal antibody production has reached protective levels. Thus, the foal is still subject to maternal antibody effects (targeting for innate activation and suppression of neo-antibody production) in the face of declining maternal antibody levels. Both effects decline over the first month of life, at which tine about half of the maternal antibody is gone.

In foals that receive adequate passive transfer of antibody, the onset of true endogenous antibody production is generally difficult to determine. IgG1 is produced *in utero* and is not derived from colostrum. Thus IgG1 levels generally reflect production by the foal.[30] Endogenous production of IgA, IgG1 and IgG3/5 likely begins in foals 5 – 8 weeks of age.[30] In contrast IgG4/7 synthesis appears to occur later, with endogenous production presumed to become significant and measurable relative to maternal antibody in circulation at 16 – 20 weeks of age.[28] Delayed production of IgG4/7 could be associated with prolonged suppression by maternal antibody. The quantity of IgG4/7 transferred in colostrum is the highest during the early neonatal period and it is likely to require multiple half-lives (of 32 days each) to remove its impact.[70] IgG4/7 remained substantially lower in foals at one year of age than adults even in the face of increasing endogenous production with age.[28] The implication of sustained lower concentrations of IgG4/7 is unknown. Low concentrations of this subclass might be ameliorated by the equivalent or greater production of other IgG subclasses in foals relative to adults, especially considering the large overlap of the function of IgG subclasses.[30] Similar to IgG4/7, endogenous production of IgE appears to also be delayed anywhere from 6 – 11 months of age in foals.[30] The regulatory pathways that result in prolonged suppression of IgG4/7 and IgE production, but not IgG1, IgG3/5 and IgA, are not known. Serum IgG concentrations peak at 3 months of age and decline to adult levels by 5 months of age. [65]The nadir of serum IgG concentrations in foals generally occurs at 1 – 2 months of age and is associated with an increased susceptibility to infection at this age.[28] Circulating levels of IgM do not generally reach adult levels until 6 months of age.[65]

The ability of the humoral system in the foal to respond to infection is not fully defined. Foals with documented *R. equi* pneumonia had similar concentrations of IgG and IgM to healthy age matched foals. This is an example of the poor ability of foals to mount an appropriate humoral response following infection.[65] In experimental *R. equi* infection, IgG1/2 and IgG4/7 isotypes, but not IgM, were significantly increased in infected foals compared to healthy control foals and infected adults.[62] These results support known isotype-specific limitations of the foal's humoral immune system.

In summary, the innate and adaptive immune responses of foals are distinctly different than those of adult horses. Although immunologically naïve at birth, the foal is capable of mounting an immune response. Some aspects of the foal's immune response have been found to be comparable to adult horses under specific conditions. Other aspects have been found to be very different in content and magnitude. Some aspects of the foal immune response develop more rapidly than others. It is evident that we do not fully understand the mechanisms governing immune development of the foals. Further, we do not have a functional definition at the cellular, molecular or physiological level of protective immunity against infection in the horse. Thus, our understanding of neonatal immune development of the foal is sorely incomplete.

SECTION II: THE ROLE OF DENDRITIC CELLS IN THE IMMUNE RESPONSE

In 1973, Steinman and Cohn were the first to identify dendritic cells (DC) based on their unique morphologic features.[73,74] Since then, an abundance of research has been conducted to further characterize the role of DC during both health and disease. There are several families of DC that have been identified in the body of mammals. They include "professional" DC of several subclasses arising directly in the bone marrow (e.g. plasmacytoid DC, classical DC, epidermal Langerhans cells) as well as monocyte-derived DC (induced and matured from circulating monocytes that extravasate in damaged or infected tissues). DC subclasses have a variety of functional differences, but share many common properties. They sample the environment (particularly in the face of "danger), they process antigen for presentation, they provide danger context activation markers (CD80, CD86, CD40 and others), and they control the activation and differentiation of naïve and memory T cells to.[75] To provide the "bridge" transition between the invaded tissue innate immune response and the adaptive immune response, DC must enter (or be differentiated in) the tissue, sample the invader, process and present antigen components of the invader, and traffic to the organized lymphoid tissues to present the antigen and the signal the type of required response to lymphocytes.

There are three principle pathways utilized by DC to internalize antigen: 1) phagocytosis; 2) macropinocytosis; and 3) receptor-mediated endocytosis.[76] Both phagocytosis and macropinocytosis are actin-dependent processes that result in ruffling of the plasma membrane and ultimately the formation of large internal vacuoles containing particulate or soluble antigen.[77] Phagocytosis is triggered by the engagement of multiple receptors on the cell surface, leading to a cascade of signal transduction events that result in the polymerization of actin and engulfment of the receptor-antigen complex. Numerous receptors are involved in this process, including Fc receptors, complement receptors, heat shock proteins and scavenger receptors to name a few.[77] In contrast, macropinocytosis is a cytoskeleton-dependent fluid-phase process in which large amounts of the surrounding fluid (and thus antigens) are nonspecifically taken up.[77] Macropinocytosis is a constitutive process in DC, unlike in macrophages that require induction of this process by growth factors.[76] Uptake of antigen by macropinocytosis allows presentation of exogenous antigen via MHC class-II and also via MHC class-I by a process called cross-presentation.[76,78,79] Receptor-mediated endocytosis involves the engagement of surface receptors but is not dependent upon actin. Instead, this process occurs at organized domains of plasma membrane called clathrin-coated pits. In these domains, complexes of receptor proteins and proteins sensitive to the change in receptor shape are organized so that they cause an infolding of the clathrin net that facilitates the uptake of macromolecules.[77] By the use of these three pathways, DC are capable of sampling all types of tissue invading or damage antigens.

However, these three processes do not preclude the uptake and presentation of self-proteins or the vesicles generated from apoptotic cells. Consequently, the ability of DC to simultaneously promote development of immunological tolerance is critical and is based on the context (of danger) associated with antigen it presents. DC are most often located in areas where antigen is most frequently encountered. DC are at highest density on mucosal surfaces, in the skin, in organized lymphoid tissues, and in the spleen.[76,80] During infection, DC are rapidly recruited into the invaded or damaged tissue at sites of local inflammation.[76] Under homeostatic conditions, antigen capture occurs relatively efficiently by resident DC. When danger signals are perceived in the tissues environment more DC are recruited to the tissues or are differentiated from recruited monocytes within tissues. Danger in the presence of invader or damage, induces a cascade of events that leads to DC functional maturation. During this process DC are matured and transformed from primarily sampling their environment to a state of enhanced antigen processing, trafficking away from the site of invasion, and to become potent activators of naïve or memory T cells.[77]

The process of DC maturation is complex and involves coordinated modifications in DC phenotype, morphology and function. Two types of signals can induce DC maturation: 1) direct recognition of pathogens via specific pathogen recognition receptors (PRR); and 2) indirect sensing of danger signals, such as protein normally found only inside cells or inflammatory mediators.[81] In some cases, complete DC maturation only occurs as a result of cell-cell contact with T cells via CD40 – CD40 ligand (CD40L) interactions.[77] DC express a multitude of PRR that allow them to detect direct and indirect danger signals including toll-like receptors, cytokine receptors, TNF-receptor family molecules, FcR receptors and those sensing death signals.[77] Engagement and cross-linking of these surface receptors induce DC maturation.[77] Binding these receptors leads to antigen internalization and activation of intracellular signal transduction cascades culminating in the transcription of numerous genes involved in the immune response.[77] Once internalized the antigen is then processed for presentation by MHC molecules on the DC surface.

The processing of antigen for presentation is similar in DC relative to other antigen presenting cells.[77] Exogenous antigen is taken up by the mechanisms previously described and degraded into small peptides within endocytic vesicles (phagosomes, endosomes, lysosomes). These internal vesicles fuse with another vesicle containing an MHC class-II molecule and the invariant chain. The peptide is loaded into the cleft of the MHC class-II molecule displacing the invariant chain. The resulting complex is transported to the surface of the DC for recognition by $CD4^+$ T cells. In contrast, the process of endogenous antigen presentation involves degradation of antigen into peptides in the proteasomes within the cytosol. These peptides are transported to the endoplasmic reticulum where they are loaded into MHC class-I molecules in conjugation with the addition of β2 microglobulin. The MHC class-I – peptide complex is then transported through the golgi apparatus to the DC surface for recognition by $CD8^+$ T cells. Exogenous antigens are primarily presented by MHC class-II molecules but they can also be presented via MHC class-I molecules in a process called cross-presentation through the endosomal shunt. The underlying mechanism that determines which antigens are to be shuttled into the classical pathway or endosomal pathway is unclear.[77] In addition to MHC class-I and II, DC can present antigen for T cell recognition using the CD1 family of proteins.[77] These proteins are related to MHC class-I molecules and are important for the presentation of glycolipids and stimulation of a subset of $CD8^+$ T cells and γ/δ T cells.[82] The process of sampling and processing non-peptide antigens is not well established, and little is known about the details of loading antigen into these CD1 complexes (or their analogs) in the cell and its transport to the cell surface. During DC maturation, three mechanisms increase the efficacy by which antigens are presented: 1) increased peptide loading into MHC molecules; 2) increased MHC molecule half-life; and 3) decreased MHC class-II recycling with persistent accumulation on the DC surface.[77,83] Collectively, these mechanisms work in concert to ultimately enhance T cell stimulation.[77]

DC antigen internalization is dramatically truncated during the maturation process.[77] Limited new presentation of exogenous antigens following DC maturation is critical in restricting the range of antigens that DC can present to T cells. This represents a safety mechanism to prevent undesirable activation of the adaptive immune response to self-antigen.[77] Notably, presentation of endogenous antigens via MHC class-I is have not been shown to be altered with DC maturation.[77]

DC maturation also stimulates the migration of DC. DC migration to T cell-rich areas of secondary lymphoid organs involves synchronized changes in cytoskeletal organization, and expression of adhesion molecules and chemokine receptors.[77,84] In particular, the expression of the chemokine receptor CCR7 is critically important in DC trafficking following maturation[85,86] and might also provide DC with essential survival signals.[87]

After maturation and migration to local lymphoid organs, DC are the principal cell type capable of priming naïve T cells.[76,77,88] This is accomplished by dramatic up-regulation of surface molecules, principally MHC and costimulatory molecules, usually CD80 and CD86. Activation of primary T cell responses requires: 1) the recognition of the MHC-peptide complex by the TCR; 2) necessary co-stimulation provided by CD80 or CD86 interaction with CD28 receptors on the T cell; and 3) DC cytokine production to direct the developmental pathway of the effector T cell.[76] The exact driving determinants that regulate which pathway a DC will polarize the emerging T cell response is not fully defined for all types of antigens. Factors suggested to play a role in this process are the cytokines the DC encountered in the periphery from other immune cells, pathogen-derived signals, tissue-specific environmental factors, and the duration of DC stimulation.[77,89-91] In addition, the origin/subtype of DC likely also plays a role in determining the nature of the adaptive immune response generated.[77]

DC are a heterogeneous population of cells comprised of numerous subclasses. A large component of DC research is dedicated to the goal of ultimately understanding the unique biology of each subclass. This process has proven to be difficult due to the absence of distinct phenotypic markers to define each subclass. While a considerable amount of DC research has been conducted in mice and humans, differential expression of surface markers makes direct cross-species comparisons futile. For the purposes of this review, a brief overview of the three most common subclasses of DC and the current understanding of DC function in immune tolerance will be provided.

The DC lineage can be broadly divided into three principal subclasses, distinguished by their phenotypic signatures: 1) classical/conventional DC (cDC); 2) plasmacytoid (pDC), and 3) monocyte-derived DC (MoDC). Both of these subclasses originate from the common DC progenitor within the bone marrow under the control of Fms-like tyrosine kinase (Flt-3) ligand.[92,93]

Named for their round shape and resemblance to plasma cells, pDC terminally differentiate in the bone marrow[73] and primarily reside in small numbers in the blood and lymph tissue.[75,92,94] At rest, pDC express low levels of MHC class-II co-stimulatory molecules, and integrin CD11c.[75] These cells also express a limited number of PRR including the intracellular nucleic acid sensors - TLR-7 and TLR-9.[75] Upon activation, pDC efficiently prime $CD4^+$ and $CD8^+$ T cells and characteristically produce massive quantities of type I interferons that are critical in leading anti-viral immune responses.[92] Plasmcytoid DC also play a central role in the in development of $CD8^+$ T memory cells by providing the "third signal" for this process via IFN-α production.[94] The pDC in organized lymphoid tissue are also important in maintaining the population of memory lymphocytes over time.

Classical DC exit the bone marrow as preDC and terminally differentiate in the tissues.[73] These cells exhibit the traditional stellate morphology, and have superior antigen processing and presentation machinery compared to pDC.[75] High levels of MHC class-II and co-stimulatory molecule expression make cDC potent T cell stimulators.[95] Found in lymphoid and non-lymphoid tissue, cDC can comprise up to 5% of the cells of the parenchyma of an organ such as the spleen.[92] cDC can be further divided into additional categories that are differentiated by their ability to preferentially active $CD4^+$ or $CD8^+$ T cells and via their pattern of surface marker expression.[75,92]

Several studies have provided evidence that monocytes represent a precursor to a distinct population of DC that are newly generated during inflammatory damage or infection and are commonly referred to as inflammatory DC or MoDC.[93,96-99] More recently, MoDC have also been identified in healthy tissues in the skin,[100] the respiratory,[101] and intestinal tract mucosa.[102,103] While monocytes are derived primarily from the bone marrow and differentiate into DC following diapedesis into extravascular compartments, studies indicate that monocytes can also be mobilized to a lesser extent from the spleen.[94,102] Monocyte differentiation into DC is regulated by granulocyte-macrophage colony-stimulating factor (GM-CSF). Factors present in the tissue microenvironment during monocyte differentiation, such as cytokines or antigens, strongly influence the functional properties of MoDC including their ability to polarize T cell responses.[93,103,104]

The primary challenge when investigating MoDC is the ability to unambiguously identify them relative to macrophages. Both MoDC and macrophages share a common progenitor and many surface markers.[93] A critical difference is that macrophages lack the ability to migrate from inflamed tissues to local lymph nodes. Thus, the expression of CCR7 by MoDC can be useful in differentiating these two closely related cell types on a functional basis.[93] Further, studies indicate the loss of CD14 expression signifies the complete differentiation from a monocyte into MoDC.[105] While the exact mechanisms governing selection of differentiation are unknown, the cytokine milieu likely plays a critical role in regulating whether macrophages or MoDC are preferentially produced. For example, significant increases in serum concentrations of GM-CSF following its production in tissues and endothelial cells during infection provides a biomarker that favors the development of MoDC.[94]

The role of MoDC during infection continues to be an area of active research. Studies have demonstrated that in response to specific experimental conditions, MoDC are capable of polarizing $CD4^+$ T cells,[97] inducing antigen-specific activation of $CD8^+$ T cell via cross-priming,[93] promoting microbicidal action via cytokine production[106] and regulating IgA antibody production systemically and at the tissue level.[107, 103] In addition to stimulating naïve[108] and effector T cells,[109] MoDC are also capable of stimulating the development of memory T cells.[110]

The role of these different DC subclasses has been well described. Research indicates that DC subtypes are involved in activation of the immune response and the induction of immune tolerance.[95] DC play a vital role in the development of both central and peripheral tolerance by T lymphocytes. This is in addition to providing resolution of ongoing inflammation.[92] Central tolerance encompasses both positive and negative selection T lymphocytes. It initially occurs within the thymus. Positive selection ensures that T cells recognizing peptide in the context of self-MHC class-II antigens continue to develop. Negative selection is mediated by thymic DC and epithelial cell presentation of self-peptides and serves to remove auto-reactive T cells from the repertoire. In the periphery, DC again display a pivotal role in tolerance through the presentation of self-peptides to direct the development of anergic or regulatory T cells due to the lack of danger signals associated with self-antigens.[111] Self-tolerance is mediated by anergy, apoptosis or autophagy of self-reactive T cells. Initial studies suggested that presentation of antigen by immature DC lacking appropriate surface marker expression was solely responsible for T cell tolerance.[92] It has been suggested that a population of regulatory DC are activated to provide the signals for a tolerance pathway to exclude common environmental antigens from the repertoire as opposed to a distinct phenotypic signature or DC subclass.[95] A number of tolerogenic signals that promote DC regulatory functions have been proposed, including the cytokines IL-10, IL-27, and TGFβ, indoleamine 2,3-dioxygenase, and adenosine.[92,96] The ability of the DC to modulate their function in response to environmental cues highlights the unique plasticity of this cell type, which allows them to have diverse functions in all aspects of the immune response.

SECTION III: DENDRITIC CELL BIOLOGY IN ADULT HORSES AND FOALS

The Evolution of Equine DC Culture

While there has been extensive research on MoDC in humans and laboratory animals, MoDC research in horses is limited. The initial attempts at equine MoDC cultivation were described by Siedek *et al* in 1997.[112] The isolation protocol used in early studies was adopted from the human literature and involved isolation of DC from whole blood using a multi-step layering procedure. Equine whole blood was first layered over Nycoprep 1.077 to isolate PBMC, which were then incubated overnight in petri-dishes before removal of non-adherents cells the next day. These cells were then layered over a 13.7 % metrizamide gradient.[112] While this method was laborious, it was successful in generating an enriched population of cells with characteristics consistent with DC: irregular cell shapes, cytoplasmic processes, and large nuclei.[112] Surface marker expression on these cells was consistent with a MoDC phenotype, and they were also capable of effectively stimulating allogeneic T cells in a mixed lymphocyte reaction (MLR).[112] This isolation method yielded small numbers of MoDC with short viability in culture, significantly restricting its use for future experiments.[112] These limitations were overcome in later protocols when granulocyte-macrophage colony-stimulating factor (GM-CSF) was identified as an essential factor for MoDC differentiation and survival in culture in other species.[113] Studies demonstrated that when monocytes were exposed to GM-CSF in conjunction with IL-4, MoDC generation from PBMC was greatly improved.[114] While cross-reactivity of recombinant human GM-CSF (rhGM-CSF) with equine cells permitted its use in early studies, non-conserved IL-4 sequences across species required the use of equine-specific IL-4 to generate equine MoDC efficently.[114] When recombinant equine IL-4 (reIL-4) was used concurrently with rhGM-CSF, large quantities of MoDC that remained viable for up to two weeks in culture were able to be reliably generated from PBMC.[114] Equine MoDC culture conditions were further refined with the cloning of equine GM-CSF (reGM-CSF) that eliminated issues with rhGM-CSF-induced activation of equine cells.[113,115]

Recent validation of equine specific monoclonal antibodies, such as equine CD14,[116] has further enhanced our ability to accurately characterize equine MoDC. A more recent study by Dietze *et al*[117] took equine MoDC generation methodology one step further by examining plate chemistry and medium type on DC yield. In this study, MoDC were generated after separating PBMC over two density gradients (1.090 and 1.077), then culturing cells on collagen-coated culture plates (Primaria®). During the period of monocyte enrichment, cells were cultured in a serum-free (AIM-V) medium. Subsequently the cells were maintained in standard serum-supplemented medium (fetal calf serum). Using this protocol, a ~20 fold greater yield of MoDC was obtained on day three of culture as compared to culture under standard conditions (standard tissue culture treated plastic and RPMI-1640 medium). While previous protocols suggested culture durations of up to 7 days[114] were acceptable, Dietze *et al*[117] determined that a culture duration of greater than four days resulted in down-regulation of the surface markers MHC class-II and CD86. This is supported by results from Mauel *et al*[115] that also suggested a culture duration of 3-to-4 days was optimal for generation of immature MoDC. These initial studies were critical to establishing protocols to reliably isolate, generate, and characterize equine MoDC, making future studies evaluating DC biology in horses possible.

In addition to the previously described studies, three studies have described the isolation or generation of equine DC from tissue samples[118,119] or directly from the blood.[120] Lee *et al*[119] demonstrated the generation and characterization of DC from monocytes isolated directly from lung tissue of adult horses.[119] Following tissue digestion, density gradient centrifugation was used to isolate mononuclear cells from the lung tissue cell suspension with subsequent steps similar to those used for generating MoDC from peripheral blood. The DC generated from lung tissue monocytes were phenotypically distinct from traditional MoDC in their reduced expression of surface marker CD86, but comparable MHC class-II expression. Lung tissue-derived DC exhibited increased macropinocytosis of labeled albumin protein compared to peripheral blood MoDC. The authors of this study suggest that this model may be useful to evaluate the role of DC in respiratory disease. A similar, but slightly different approach was used for DC generation from equine nasal mucosa.[118] In this study, $CD172a^{+}$ cells were purified by magnetic-activated cell sorting (MACS) and after four days in culture these cells exhibited the classical DC morphology. When compared to MoDC derived from blood monocytes, $CD172a^{+}$ DC expressed less MHC class-II, but more CD1c and CD3. These cells were also less capable of internalizing antigen than MoDC. Finally, Ziegler *et al*[120] identified and characterized equine pDC from whole blood samples. In this study pDC were found to be $Flt3^{+}CD4^{low}CD13^{-}CD14^{-}CD172a^{-}CADM\text{-}1^{-}CLASS\text{-}II^{low}$ and, as expected, produced large quantities of IFN-α upon TLR-9 stimulation.

Characteristics of Adult Equine MoDC

The protocols described above generate a heterogeneous population of both mature and immature equine MoDC.[117,121] The regulation of MoDC homogeneity demonstrated by surface marker expression might be controlled in part by IL-4. It has been demonstrated that IL-4 acts in a dose-dependent fashion to produce an increasingly homogenous population of DC, likely by suppressing concurrent macrophage development.[117] Despite significant heterogeneity, equine MoDC in culture are similar in phenotype to human DC with a few exceptions. Similar to immature human MoDC, immature equine MoDC express low levels of CD14 and CD80, but increased levels of CD206 and MHC class-II.[115,122] However, human MoDC upregulate expression of CD83 as a marker of DC maturity,[123] while immature equine DC (that have not been subjected to antigenic stimulation) express both markers of DC immaturity (CD206) and markers of DC maturity (CD83) simultaneously.[115,122,124-126] Both human and equine DC express the surface marker CCR7, but increased expression of this marker has only been documented to occur during DC maturation in human cells.[122,127,128] It is plausible that equine MoDC are more sensitive to stimulation by culture conditions than human MoDC. If the equine MoDC evaluated in these experiments were partially activated by the culture conditions, the described phenotypic characteristics would reflect an intermediate differentiation state. While overall morphologic, phenotypic and functional characteristics of human and equine MoDC are similar, distinct species-specific differences remain. These include differences reported in pigs[129] and cattle.[130]

Complete evaluation of DC biology entails phenotypic and functional assessment after exposure to antigen. Thus resolution of the state of DC requires the capability to distinguish immature from mature MoDC. This task has proved to be a problem in horses due to the paucity of equine-specific antibodies and the high degree of MoDC variability that exists even after exposure to maturation stimuli (antigens) in culture. Some studies have demonstrated increased expression of surface markers MHC class-II, CD86,[115,122,131] and CD40[131] following antigenic stimulation, while others produce either inconsistent results[115] or total absence of changes in expression of these markers.[3,117,122] As previously described, the unique phenotypic signature of equine MoDC when compared to human DC limits the use of surface markers commonly employed in human systems to further delineate maturation states of equine MoDC.

While most equine studies have demonstrated appropriate maturation in response to antigenic stimulation demonstrated by increased T cell proliferation[115] and cross-priming[122] capabilities, one study reported no changes in T cell priming by LPS-stimulated MoDC.[117] Some studies have been successful in demonstrating expected reductions in endocytosis after exposure to stimuli,[122] while others observed no differences in antigen uptake between antigen-exposed cells and those not exposed to antigens.[117,131] The ability of only some studies, but not all, to distinguish immature from mature MoDC using T cell proliferation assays and endocytosis assays suggests that the effects of culture conditions may be stimulus-specific. Thus there is need to optimize and standardize culture (monocyte preparation and culture handling) and antigenic stimulation protocols.

Another indicator of DC functional maturity that is more easily demonstrated *in vitro* is altered cytokine production. Following stimulation with UV- inactivated *E. coli*, adult horse MoDC increased their expression of IL-12p40 and IL-23p19, but not IL-12p35, suggesting possible production of IL-23 in preference to IL-12.[131] While these findings are in agreement with human studies,[132] other equine studies[3,117] have reported increases in IL-12p40 or IL-12p35 after MoDC exposure to LPS or *R. equi*. While cytokine production profiles can readily be used to demonstrate effective MoDC maturation, the ensuing response remains stimulus-specific.

Characteristics of Foal MoDC

Our understanding of DC biology in equine neonatal foals is limited. Distinct differences in DC maturation and function were observed with respect to adult horse cells in several studies. One study showed that foal MoDC exhibit age-dependent differences in the expression of MHC class-II, with the density of MHC class-II expression on foal MoDC failing to reach levels comparable to adult horses until 3 months of age.[133] In contrast, another study demonstrated comparable expression of MHC class-II in MoDC generated from 2-week-old foals compared to adult horse MoDC.[134] The expression of MHC class-II was unaltered in foal MoDC following antigenic stimulation.[3] Impaired regulation of MHC class-II expression by foal MoDC following antigen exposure likely implies the need for additional signals to induce DC maturation in foals. Alternatively, it might suggest an inherent molecular limitation in the ability to up-regulate MHC class-II expression in foal cells. Reduced MHC class-II expression patterns in foals could have important consequences with respect to their ability to activate an adaptive immune response in the face of infection. In contrast, the expression of the co-stimulatory molecules CD86[3,133] and CD40[3] have been shown to be comparable between adult horse MoDC and MoDC generated from 5-day-old foals. However, the percentage of triple stained $CD86^{+}CD1w2^{+}CD14^{-}$ cells was significantly decreased in foal MoDC relative to adult horse cells. This suggests that a higher percentage of MoDC generated from foal monocytes remains quantitatively less mature at 2 weeks of age.[134]

Basal cytokine gene expression by MoDC from foals at 2 weeks of age and 3 months of age have been described Merant *et al*[134] This study showed that constitutive gene expression of IL-1, IL-6, IL-12, and IL-15 was equivalent between foal and adult horse MoDC, while IFN-γ, TNF-α, IL-10, and TGFβ genes were expressed at lower levels in foal cells compared to adult horse cells.[134] Foal MoDC displayed age-related changes in cytokine gene expression in response to various stimuli. Foal MoDC were refractory to stimulation with TLR-9 stimulating CpG-ODN during the first 3 months of life and did not show altered gene expression of IFN-α and IL-12p40 mRNA similar to adult cells.[133] This is contrast to stimulation with *R.* equi, which induced more IL-12p40 gene expression in MoDC from foals at 2 and 3 months of age than adult horse MoDC.[3] Following stimulation with LPS, increased gene expression of TNF-α, IL-6, IFN-γ, IL-12, IL-15 and IL-10 by foal MoDC paralleled the increase observed in adult horse MoDC, but IFN-γ and IL-10 expression was less in foal MoDC than in adult cells.[134] Foal MoDC also differentially expressed multiple genes involved in cell adhesion, migration, immune response, ion transport, T-cell regulation and vesicular transport compared to adult horse cells after exposure to *R. equi.*[135] The importance of these differences remains to be explored. It could be postulated that age-related limitations in surface marker expression and cytokine gene expression by foal MoDC could affect their ability to prime and polarize T cell responses, increasing the risk of infection early in life. It should be noted that the limitations in MoDC maturation and function do appear to be stimulus-specific. Studies evaluating foal MoDC maturation and function in response to various antigens are needed to provide a proper context of interpretation.

SECTION IV: THE IMMUNOMODULATORY ROLE OF SOLUBLE FACTORS

Plasma comprises the fluid portion of blood and its analog can also be found in the extravascular tissues.[136] In addition to water, these fluids contain many soluble factors, including lipids, carbohydrates, hormones, vitamins, gases, proteins and ions.[136] In addition to its role as a means of transporting oxygen-containing RBC and nutrients throughout the body, many of the soluble factors in plasma have distinct immune modulating functions that impact both the innate and adaptive immune responses.[136,137] During infection, soluble factors found in blood and lymph circulation are critical in alerting the immune system to the presence of a pathogen by rapidly disseminating signals from the site of infection to distant sites. This provides the immune system with a global picture so that the response may be tailored to the specific pathogen. Circulating concentrations of many of these plasma factors in neonates are different than those in the circulation, tissues and lymph of adults.[136] These factors are often measured in plasma for convenience, as plasma is generally reflective of the concentration and composition of the fluid in tissues and lymph. In infection, increased endothelial permeability and subsequent leaking of the vascular beds leads to transfer of plasma components into tissues and the interstitium.

The composition of plasma in neonates might play a significant role in regulating the neonatal immune response and impact the neonate's susceptibility to infection.[137] In fact, studies in humans have demonstrated the immunosuppressive function of neonatal plasma on the generation of TLR-mediated cytokine production in *ex vivo* cultures.[138,139] Such studies have triggered investigation into the various factors in plasma that might confer these suppressive effects and how these factors might impact differences between the neonatal and adult immune regulation. Some of these factors that are most likely to regulate immune function in neonatal foals are discussed in detail below.

Plasma proteins

The principal proteins found in plasma are albumin, Ig, and complement. The modulatory role of albumin in the progression of disease has been documented in mice and humans. In murine models of endotoxemia, administration of phosphate buffered saline (PBS) supplemented with 4% albumin resulted in decreased mortality compared to those receiving PBS alone.[137,140,141] Similar results were obtained in human patients with bacterial sepsis that were administered albumin-containing fluids.[137,141] An important role of albumin in mitigating fungal disease has been also proposed.[142]

It is well known that IgG aids in supporting and stimulating the immune response through the opsonization and naturalization of pathogens. Other immune regulatory roles of IgG are less well understood. Intravenous IgG is frequently used as a primary treatment modality for a number of inflammatory conditions and autoimmune diseases, as well as in adjunctive therapy for severe sepsis and septic shock.[143] Several mechanisms for the immune modulatory effects of IgG have been proposed, including: Fc receptor blockade, increased complement activity, promotion of T regulatory cell development, enhanced clearance of autoantibodies, activation of regulatory macrophages and altered cell surface adhesion molecules and receptors.[137,144]

The complement system also plays a critical role in the innate immune response and supports the actions of the innate and adaptive immune response. Composed of 20 heat-liable pro-enzymes, the proteins of the complement system are largely produced by the liver and circulate within the bloodstream and extravascular spaces. Activation of complement proteins triggers an enzymatic cascade of events that can be divided into three distinct cell killing pathways: 1) the classical pathway, 2) the mannose-binding lectin (MBL) pathway, and 3) the alternative pathway. The classical pathway involves ligation of C1q by antibody (IgM or IgG) bound to the pathogen surface. The MBL pathway is initiated by the sugar binding protein MBL, which specifically binds D-mannose and L-fucose residues in the orientation found on bacterial cell walls. The alternative pathway is activated by spontaneously cleavage of complement component C3 that is directly deposited onto the pathogen surface due to the small radius of curvature and the nature of the hydrophobic areas on the bacterial cell wall. All three of these pathways converge at the formation of C3 convertase, which facilitates the cleavage of C3 into C3b and C3a. Large amounts of C3b attach covalently to the pathogen surface and in some cases trigger further cascade of events culminating in the formation of the membrane attack complex (MAC – C5b, C6, C7, C8 and multiple copies of C9)). The MAC is microbicidal by forming pores (of C9 protein) within pathogen membranes and causing microbial lysis. If the MAC is not formed, C3 can act as an opsonin (in the form of C3i, C3dg) that coats the surface of the pathogen and enhances the ability of phagocytes to recognize and engulf the pathogen. C3a and C5a also circulate and are important in the recruitment of immune cells to the site of infection. In both human[136,145] and equine[42,51] neonates, concentrations of circulating complement components are reduced relative to adults.

Plasma Lipids

There is an increasing body of evidence that suggests that lipids both attenuate and perpetuate inflammatory immune responses.[137,146] Lipid mediators such as lipoxins, resolvins, and protectins mitigate excessive inflammation by interfering with cellular migration and cytokine production.[137,146] Feingold *et al*[147] demonstrated a marked increase in mortality in hypolipemic rats exposed to LPS compared to rats with normal circulating lipid concentrations, an effect that was reversible following infusion of exogenous lipids. The proposed anti-inflammatory effects of lipids extend beyond amelioration of endotoxin-induced inflammation. Production of phospholipids of broad chain length have been documented to occur during other types of bacterial, viral and parasitic infections.[137] However, protective effects of intravenous lipid infusion have not been appreciated in people with bacterial sepsis.[137] Infusion of lipids following LPS-administration to healthy adults did not augment the systemic effects of LPS, despite evidence that lipids with chain lengths from 6-14 bind LPS and slow the process of LBP transfer of LPS (or lipid A) to CD14 *in vitro*.[148] In horses, intravenous infusion of a lipid emulsion resulted in reduced production of inflammatory cytokines by equine monocytes *in vitro*[149] and attenuated the inflammatory effects associated with LPS administration *in vivo*.[150] Further investigation into the effects of different compositions of lipid formulations on the equine immune response in sepsis are needed.

Plasma Purines

Adenosine, a purine metabolite, minimizes inflammation by inhibiting the production of pro-inflammatory cytokines as well as inhibiting effector functions of T cells and neutrophils.[136] Adenosine receptors are also present on DC and activation of these receptors leads to differentiation of DC with distinct phenotypic signatures, decreased T cell stimulatory abilities and altered cytokine production.[151] In horses, activation of the adenosine A2A receptor results in reduced oxidative burst activity by equine neutrophils,[152] and decreased TNF-α production by equine monocytes[153] after LPS exposure *ex vivo*. Plasma concentrations of adenosine are increased in human neonates, as well as in septic adult patients.[137] The generation of adenosine from purine metabolites is favored in neonates[154] and it is found in higher concentrations in neonatal plasma compared to adult plasma.[136,155] Human neonatal mononuclear cells are profoundly more sensitive to the cellular effects of adenosine, including inhibition of TLR-2 mediated TNF-α production. Distinct features of the adenosine system in neonates could play key roles in polarization of the immune system in response to specific microbes.[155] Studies utilizing human and mouse models have shown alerted MoDC after exposure to adenosine receptor agonist. In these studies MoDC expressed increased levels of monocyte/macrophage surface markers in the presence of an adenosine agonist.[151]

Plasma Lectins

C-type lectins mediate binding of oligosaccharides to C-type lectin receptors (CLR) to conserved sugar moieties on cell surfaces or captured from tissue fluids to identify harmful pathogens, and to regulate host cell interactions. Plasma contains similar sugars (mannose and fucose) moieties that bind weakly to these receptors. Under proinflammatory conditions these complexes interact with innate cells and epithelial cells to stimulate production of the anti-inflammatory cytokine IL-10.[137] Some evidence suggests that some pathogens can evade the immune response by releasing oligosaccharides that signal through host CLR to dampen inflammatory responses and provide cover while they become established. This highlights the importance of CLR in the ecology of the immune response.[137]

Hormones

In addition to regulating physiologic processes, many different hormones also play roles in modulating the immune response.[136] Two such immunomodulatory hormones are the adipose-derived hormones adiponectin and leptin.[136] Leptin is important for energy metabolism, reproduction, endocrine and immune function.[156] The importance of leptin in regulating the immune response has been recognized in leptin-deficient human patients that exhibit reduced T cell responsiveness and impaired cytokine production. These defects were partly reversible when exogenous leptin was administered.[157] Leptin has been shown to enhance adult and cord blood T cell production of IFN-γ, IL-2, and IL-4 by T cells, and to enhance monocyte production of IL-10 in isolated cells.[136,156] The concentration of leptin initially declines after birth[158] and then increases with age, reaching its peak levels from puberty, to early adulthood.[136] Several studies have demonstrated significantly higher circulating levels of leptin in septic adult and neonatal[159] human patients. High leptin concentrations are described in surviving septic foals.[160] How leptin specifically alters the immune response is not clearly understood. Further, it is unclear how increased leptin concentrations reflect the stage of sepsis progression, and additional investigation into leptin dynamics during sepsis and associations between plasma leptin concentrations and survival are necessary.

Adiponectin also has dual roles regulating both glucose metabolism and immune system function. After birth, circulating concentrations of adiponectin in infants are increased and gradually decrease to adult levels with increasing age.[136] Some studies indicate that adiponectin suppresses the expression of co-stimulatory molecules on the cell surface (MHC class-II, CD80 and CD86) of murine DC. Further it appears to stimulate the development of regulatory T cells when co-cultured with DC and T cells.[161]

Estradiol and progesterone are two important steroid hormones that lymphocytes and monocytes carry receptors for, and that are profoundly increased in human neonatal cord blood samples.[162] Neonatal mononuclear cells are uniquely sensitive to the anti-inflammatory effects of estradiol and progesterone. The cellular density of receptors for these hormones is usually higher on neonatal than adult cells.[162] Cortisol is another circulating steroid hormone with well-defined immunomodulatory properties and is regulated differently in neonates compared to adults. The importance of cortisol on the immune response will be discussed in detail later in this chapter.

Vitamins

Vitamins function in multiple physiological processes, including the immune response.[163] Of particular importance with respect to their immune modulating properties are vitamins A and D.[136] Vitamin D is metabolized into its most biologically active form (D3) in the kidney, and by immune cells, including MoDC, macrophages and T cells.[163] Retinoic acid can be synthesized from vitamin A by DC.[163] Vitamin D has been shown to inhibit Ig production,[164] B and T cell proliferation,[164] and IL-2 and IFN-γ production.[165] Inhibition of DC maturation and enhanced production of IL-10 with concurrent reduced production of IL-12 associated with vitamin D has also been reported.[166] In contrast, vitamin A has been shown to increase T cell proliferation[136] and functions synergistically with TNF-α to induce DC maturation.[167] Vitamin A also generally inhibits B cell proliferation, but can activate B cells under certain conditions.[163] Although initially low, circulating levels of vitamin D in neonates are comparable to adults by 24 hours of age.[136,168] Vitamin A concentrations are also low in infants compared to adults.[169] Other studies suggest that anti-oxidant molecules, including vitamins E and C, modulate human and murine DC maturation and activation.[170] A recent study also proposes early infusion of vitamin C, vitamin B1, and hydrocortisone as a possible treatment for severe sepsis in people.[171]

V. CORTISOL REGULATION IN SEPSIS

Steroidogenesis

Glucocorticoids (GCC) are essential circulating steroid hormone that are instrumental in maintaining whole-body homeostasis, with critical roles in modulating all major body systems including the cardiovascular system, energy metabolism and immune responses.[172,173] As a result, the production of cortisol is tightly regulated.[173] During pathologic or physiologic stress, the hypothalamic-pituitary-adrenal (HPA) axis is activated and provides the host with the essential support needed to cope with the given stressor. Activation of the HPA axis is initiated by three main mechanisms: 1) stimulation of afferent autonomic nerve fibers resulting in release of acetylcholine; 2) secretion of inflammatory mediators by immune cells that are transported to the hypothalamus and; 3) local neuronal release of inflammatory cytokines.[174] All three mechanisms stimulate the secretion of corticotropin-releasing hormone (CRH) from the hypothalamus. CRH acts on the anterior pituitary to induce the release of adrenocorticotropic hormone (ACTH) into the systemic circulation. Finally, ACTH stimulates the *de novo* synthesis of cortisol by the adrenal gland. Cortisol provides negative feedback to the hypothalamus and pituitary gland to produce GCC concentrations in an ultradian and circadian rhythm during homeostasis or to maintain concentrations within an appropriate range for the stress incurred.

Effects of Cortisol on the Immune Response

GCC can alter the immune response in a number of different ways including modulating all aspects of protein production and secretion and direct effects on immune cell proliferation, differentiation, and function.[175] GCC have a dual role in both enhancing and suppressing the immune response.[176] GCC stimulate the generation of antibodies by B-lymphocytes.[177] They also can be critical in mitigating respiratory infection, with improved immunity documented when low-to-moderate doses of corticosteroids were used during worldwide influenza epidemics.[177-179] The immunosuppressive role of GCC is also well established, with compelling evidence detailing their function in regulating a vast range of immunological functions.[180] Using several different mechanisms, GCC are able to modulate the inflammatory response by directly decreasing gene transcription of several inflammatory cytokines, by inhibiting NF-κB and AP-1 activation, and by preventing the production of leukotrienes and prostaglandins.[175] Cortisol can also directly affect immune cell viability by decreasing lymphocyte proliferation via IL-2 inhibition coupled with increased lympholysis through apoptosis or steroid-hormone-receptor mediated death.[175] In contrast to diminishing lymphocyte numbers, neutrophil counts increase in response to GCC. This is due to decreased endothelial cell adhesion and subsequent impaired migration of neutrophils into tissues, as well as impaired neutrophil trafficking associated with diminished production of attractant factors and delayed apoptosis of neutrophils in circulation.[175] The dichotomous effect of cortisol on the immune response is an important distinction when understanding the effect of cortisol excess or deficiency and its impact on disease progression, particularly in severe infection like sepsis. Importantly, adaptation to stress requires not only the ability to respond to a given stressor but also the ability to control the response properly.[175] Alterations at any level of the HPA axis therefore could result in deleterious effects and enhanced susceptibility to or worsening of disease.[180]

HPA Axis Dysfunction and Sepsis

There are three principal systems involved in the body's response to infection: 1) the HPA axis; 2) the autonomic nervous system; and 3) the immune system.[174,181-183] Appropriate function and balance of all three of these systems is essential to ensuring maintenance of cardiovascular and metabolic functions and effectively controlling against infection.[174] However, during times of severe illness, these responses might be inadequate or counterproductive leading to detrimental effects on the host.[184] In particular, dysfunction of the HPA axis appears to play significant role in the severity of sepsis.[182,185-188]

In both human and veterinary medicine, a condition known as hypoadrenocorticism, or Addison disease, is the most common example of permanent HPA axis dysfunction.[189-191] In this syndrome, immune-mediated destruction of the adrenal cortex results in the absence of GCC and mineralocorticoid production. In response to stress this clinically manifests as hypotension, hypovolemia and shock.[191] Congenital adrenal insufficiency has also been associated with adrenal hypofunction in people.[189,192] More frequently in sepsis, though, dysfunction of the HPA axis is reversible and is induced by factors related to concurrent illness.[193]

The syndrome of HPA axis dysfunction during severe illness is termed relative adrenal insufficiency (RAI) or critical illness related cortisol insufficiency (CIRCI). Dysfunction is likely multifactorial with a combination of factors affecting multiple levels of the axis. HPA axis dysfunction can manifest as impaired GCC secretion, sensitivity, transport or activity with the exact mechanisms leading to dysfunction being complex and poorly understood.[193,194]

It has been documented that transient HPA axis hypofunction commonly occurs in critically ill people, with rates as high as 60 – 90%, and is associated with a number of clinical syndromes including sepsis, cancer, and acute respiratory distress syndrome.[193,195] During infection, suppression of CRH and ACTH synthesis has been associated with the over-expression of inducible nitric oxide (NO) triggering apoptosis of neurons and glial cells in hypothalamus and pituitary.[174] Cardiovascular comprise and coagulation abnormalities have been documented to cause ischemic necrosis of the hypothalamus and pituitary gland.[185,193] The adrenal glands are also at risk for hemorrhage and necrosis due to limited venous return.[185] While structural damage to the hypothalamus, pituitary gland and adrenal glands associated with the primary disease process are typically reversible, long-term or permanent impairment has been reported with severe hemorrhage and necrosis in these locations.[174]

Due to the limited storage capacity of the adrenal glands, the stress response during infection requires adequate *de novo* synthesis of GCC. Decreased steroidogenesis, even without structural damage to the HPA axis, has been described in critically ill people and can result from alterations at any of the steps in the cortisol synthesis pathway including competition with ACTH for adrenal receptor binding.[185,196] Pro-inflammatory cytokines can also modulate adrenal gland production of cortisol in a dose-dependent manner where low levels inhibit synthesis[197] and increased levels enhance synthesis.[185,198] HPA axis hypofunction may be further intensified by peripheral tissue resistance to GCC.[193] Factors that may affect the bioactivity of cortisol at the site of infection include, accelerated clearance due to concurrent drug interactions, GCC inactivation mediated by cytokines, and down-regulation of GCC receptors.[174,193] A reduction in the production of acute-phase transport proteins, namely cortisol-binding globulin, could prevent cortisol from effectively reaching, or working, at the site of infection.[174]

VI. CORTISOL DYSREGULATION IN FOALS

Following parturition, the foal faces a number of physiologic challenges as it adapts to life outside the uterus. While many of the foal's homeostatic functions are still developing, others must be completely or nearly functional for the foal to survive outside the uterus. As the fetus gets closer to term, GCC are critical in preparing the fetus in all species for making the transition to extrauterine life, and a surge in circulating cortisol concentrations generally precedes parturition in most mammalian species.[199] Compared to other species, the pre-partum cortisol surge occurs significantly closer to parturition in the equine fetus, with the fetal adrenal gland being unresponsive to ACTH until 2 to 3 weeks before term.[199-202] Increasing plasma ACTH and cortisol concentrations in the late term fetus indicate that cellular and/or molecular mechanisms are functional prepartum at the level of the pituitary and adrenal gland.[203] Both cortisol and ACTH are secreted in a pulsatile rhythm in the foal similar to the adult.[204] In addition the ultradian rhythm of ACTH and cortisol is fully developed at birth.[204] Dramatic increases in the weight of the adrenal gland[203,205] and changes in steroidogenic enzymes expressed in the adrenal cortices occur in the late gestation equine fetus.[206] While some enzymes are present earlier during gestation, the enzyme $P450_{C17,}$ which is essential for the production of cortisol, is not present in the equine adrenal gland until the fetus is nearly term.[203] Thus, while the complex process of development of the foal HPA axis begins *in utero*, it continues to mature during the first several weeks of extrauterine life.[199,202]

In response to parturition, foals do display a transient increase in ACTH and cortisol concentrations signifying a functional HPA axis at birth.[207] By 12 to 24 hours of age, cortisol concentrations in foals dramatically fall to levels well below those reported in adult horses.[203,207] This is a consistent finding among different horse breeds[208,209] and persists in foals at 2 weeks of age and maybe longer.[207,210] In contrast, basal concentrations of ACTH in foals at 24 hours of age are higher than those reported in adult horses, suggesting adrenocortical limitations in cortisol synthetic capacity.[207] Despite the attenuated HPA axis after birth, the foal HPA axis is capable of responding to both physiologic and supraphysiologic doses of ACTH. The degree of cortisol production is age-dependent with foals 24 hours of age producing greater concentrations of cortisol compared to older foals in response to exogenous administration of both low (physiologic) and high (supraphysiologic) doses of ACTH.[207] Premature pony foals exhibit distinct cortisol and ACTH dynamics compared to term foals, with lower cortisol concentrations and higher ACTH concentrations documented at birth.[211] A study by Ousey *et al* suggests that intrauterine growth restriction demonstrated by pony mares carrying Thoroughbred foals, resulted increased cortisol concentrations with limited response to ACTH challenge stimulation until 5 days post-partum.[212] These studies highlight the limitations of the HPA axis in neonatal foals and immaturity of axis during the first several days to weeks of life. Whether impairment is associated with reduced ACTH sensitivity or impaired cortisol synthesis in foals remains to be determined.[207] Inherent functional limitations in the HPA axis of foals might predispose them to the HPA axis dysfunction and altered cortisol availability during illness, potentially impacting disease progression.[207]

A study by Hart *et al*[209] provided compelling evidence for HPA axis dysfunction in septic foals at a prevalence of ~ 40-60%, similar to rates reported in human infants. In this study, hospitalized foals less than 4 days of age exhibited low plasma concentration of ACTH, whereas, hospitalized foals greater than 4 days, displayed significantly higher basal ACTH concentrations compared to healthy controls.[209] In addition, 52% of foals in this study had inadequate cortisol responses to exogenous ACTH.[209] The unsatisfactory cortisol response to high doses of exogenous ACTH was correlated with increased severity of disease and decreased survival.[209] These findings are in agreement with other studies that document alterations in cortisol and/or ACTH concentrations in critically ill hospitalized foals, and a similar association between HPA axis dysfunction and non-survival.[172,213-215] The increased ACTH:cortisol ratios identified in non-surviving foals suggest that dysfunction of the HPA axis in septic foals in part might be occurring at the level of the adrenal gland and is supported by a recent publication demonstrating inappropriately low aldosterone concentrations in septic foals, consistent with an overall impaired adrenal gland response in foals.[172,215]

In a study by Cudd *et al*[204] a greater fraction of free (biologically active) cortisol and a decreased circulating concentration of cortisol-binding globulin (CBG) was demonstrated in the plasma of the equine fetus compared to adult horses.[204] These differences in cortisol availability and CBG concentration persist into the neonatal period as demonstrated in study by Hart *et a.l*[216] In this study, dynamics of cortisol binding to plasma proteins in foals was investigated and revealed that the serum free fraction of cortisol in foals was significantly higher than adult horses, with 60% free cortisol in foals at birth and 30% in foals at 1 week of age compared to only 5-10% free cortisol in adults. Septic foals were noted to have even higher free cortisol concentrations compared to healthy age-matched controls, an expected response to illness.[216] Concurrent absence of changes in total cortisol concentrations suggested that decreases in CBG in foals, the primary transport protein for cortisol, were responsible for the increased free fraction of cortisol identified.[216] The increased percentage of free cortisol and probable concurrent decreased CBG in foals might put them at greater risk for CIRCI. Free cortisol is rapidly cleared by renal and hepatic mechanisms. During illness foals might have a decreased reservoir of cortisol due to decreased CBG concentrations, resulting in rapid clearance and causing them subsequently to experience a state of relative cortisol insufficiency. Studies have documented that CBG is critical not only transporting cortisol to the necessary locations, but also might be important in mediating interactions and signaling with cell surface receptors.[217,218]

VII. THE EFFECT OF CORTISOL ON DENDRITIC MATURATION AND FUNCTION

As previously discussed, during infection both DC and GCC are critical in generating and regulating an appropriate immune response. Similar to other immune cells, GCC also regulate DC responses by inhibiting their differentiation, maturation and function.[219] Studies investigating GCC exposure on human and murine DC have demonstrated deficient DC differentiation and maturation indicated by persistent expression of macrophage-like markers specifically CD16 and CD14.[219] GGC also lead to reduced expression of adhesion molecules and costimulatory molecules MHC class-II and CD86.[219-224] These changes may lead to impaired antigen presentation and have been associated with reduced ability to DC to prime naive T cells.[219,225] DC exposed to GCC increase induced IL-10 and decrease induced IL-12. GCC leads to polarization of T cells to a regulatory phenotype, inhibition of Th1 polarization, and impaired cytotoxic T cell development.[219,226-229] $CD8^+$ T cells exposed to DC treated with GCC show suppressed naïve activation, post-activation proliferation, cytokine production and migration from secondary lymphoid tissues.[230-233] GCC affect the traditional route of antigen presentation by MHC class-I and MHC class-II molecules and also affect cross-presentation of antigens from the endosomal shuttle by MHC class-I. Impaired cross-presentation in DC after GCC exposure may have important implications on antiviral T cell responses against virus with an extracellular phase in the life cycle.[230] GCC alter the composition of DC subsets reducing the number of DC that participate in cross-presentation of antigen.[230] The effects of GCC on DC maturation and function are dependent upon the precise stage of DC differentiation and maturation during cortisol exposure.[219,226] Suppressive outcomes of cortisol exposure occur prior to DC activation.[226] Moreover, the effects of GCC on DC maturation and function appear to be both dose-dependent and exposure time-dependent.[219,228] Neonatal DC appear to be more sensitive to the effects of GCC than adult DC. Mainali *et al*[228] demonstrated heightened responses of cord blood DC exposed to dexamethasone compared to the modest changes noted in adult DC. Specific effects of GCC on cord blood DC included persistent phenotypic immaturity, more robust production of IL-10, and reduced T cell stimulation capacity. It is possible that increased sensitivity of neonatal DC to GCC, and the more pronounced immunosuppressive effects of GCC in neonates, might contribute to their increased susceptibility to infection.[228] Several studies have documented DC depletion *in vivo* and *in vitro* during sepsis.[234-237] Some studies documented reduced numbers were correlated with disease outcome.[238] Other studies have not observed these differences.[226,227] These discordant results may reflect differences in experimental conditions. Collectively, these data suggest that altered DC maturation, function and turnover are modulated by GCC interaction with the GCC receptor (GR) on DC themselves.

A study by Elftman *et al*[227] demonstrated that altered DC phenotype following exposure to corticosterone was blocked by the addition of a GR antagonist. This indicates that the GR is important in mediating the effects of GCC on DC. Further, Freeman *et al*[239] demonstrated that modulation of DC maturation and function by GCC was mediated by DC directly regulating GCC bioavailability at the tissue/cellular level. In this study, immature MoDC displayed enhanced activity of the iosenzyme 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1). This enzyme promotes the conversion to inactive cortisone to active cortisol and might be important in limiting DC activation by increasing the activation threshold for DC.[239] Limiting DC activation might be important mechanism of tolerance during homeostasis. Reductase activity of the11β-HSD1 enzyme remains increased in DC that have started to mature in response to innate immune activation signals, but complete maturation of DC following T cell interaction, results in a dramatic reduction in the ability of DC to generate biologically active cortisol. This observation is in agreement with previous reports.[225,226] The underlying mechanisms that confer GCC resistance to fully mature DC have yet to be determined. Some cases appear to involve expression of differential GR isoforms. Recently, Cao *et al*[240] described the differential expression of GR isoform by MoDC. In this study it was documented that the GR-D isoform is predominately present on immature MoDC. GR-D is a functional GR that reduces of MHC class-II and CD86 surface marker expression in response to GCC exposure. It was previously shown that GR-D does not result in DC apoptosis.[240,241] Following LPS-induced DC maturation a GR isoform switch occurs from GR-D to GR-A.[240] Signaling through the GR-A isoform has been demonstrated to trigger DC apoptosis.[240] In murine models using T cells that lack the GR it has been demonstrated that DC but not T cells, are the primary targets for immunomodulation by GCC.[232] However, given the DC vital role in T cell activation this leads to impaired T cell responses. Collectively, these results reveal the complexity of important immune-endocrine interactions in regulating the immune response during infection.

SECTION VIII. RATIONALE FOR THE STUDIES PRESENTED HEREIN

As outlined above, the generation and characterization of equine MoDC has evolved over more than 20 years. Despite a considerable body of work on how to optimize MoDC culture conditions, inconsistencies among methods suggested the need for refined protocols that consistently produce reliable and reproducible outcomes. Improved protocols for generating and characterizing equine MoDC *ex vivo* was of critical goal of this research. Reproducible model systems are needed when examining the biology of DC generated from foal and horse monocytes so the impact of age on equine DC function can be assessed with confidence.

MoDC represent a cellular pathway to efficient naïve T cell stimulation and generation of an effective adaptive immune response. Thus, this model is essential for work to understand of the pathogenesis of bacterial infections initiated in mucosal tissues in neonates. There is a significant body of evidence for human infants detailing age-related changes in DC function, their contribution to increased susceptibility to neonatal infection,[242-244] and their important role in sepsis.[245] However, studies characterizing MoDC in foals and horses are limited. Despite the paucity of research in this area, initial studies have demonstrated some phenotypic and functional limitations of foal MoDC. Past studies have evaluated MoDC from foals at several different ages and in response to several antigens, but have done so in the presence of serum or plasma. As previously described, numerous immune modulating factors that may impact DC function can often be found in plasma and serum. The studies presented in Chapters 3 to 5 were performed to systematically evaluate age-related changes in equine neonatal MoDC maturation and function by defining intrinsic cellular capabilities and assessing the influence of generic and specific additives to the microenvironment on MoDC function. This was completed by addressing the following three objectives: 1) to characterize the effect of foal age on MoDC phenotype and function following exposure to bacterial antigens, 2) to determine the effect of soluble factors, as modeled by plasma, on foal MoDC functionality in response to bacterial antigens, and 3) to assess the effect of cortisol on foal MoDC maturation and function when simultaneously exposed to bacterial antigens. In total, we aimed to systematically evaluate foal MoDC maturation and function following exposure to killed bacteria as a model of DC matured from monocytes in mucosal tissues during initial bacterial invasion.

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CHAPTER 3

OPTIMIZED METHODS FOR THE EX VIVO GENERATION AND CHARACTERIZATION OF EQUINE MONOCYTE-DERIVED DENDRITIC CELLS UNDER SERUM-FREE CONDITIONS[1]

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[1] Lopez B, Giancola S, Hurley DJ, Felippe MJB, Hart KA. Optimized Methods for the *ex vivo* Generation and Characterization of Equine Monocyte-Derived Dendritic Cells Under Serum-Free Conditions. To be submitted to the *Journal of Veterinary Immunology and Immunopathology*.

ABSTRACT

Background: Protocols for generating equine monocyte-derived dendritic cells (MoDC) are described in the literature, but the impact of the choice of culture conditions, including medium utilized, plate surface chemistry, monocyte enrichment time, and duration in culture prior to assessment of MoDC characteristics, have not been fully defined. Conventional medium contains many soluble factors that are known to modulate immune cell markers and function. Generation of MoDC in chemically defined medium under serum-free conditions offers benefits for assessment of intrinsic cellular phenotype and function. The generation and maintenance of equine MoDC in serum-free medium with only recombinant equine GM-CSF and IL-4 has not been previously established.

Hypotheses: 1) Equine MoDC can successfully be generated and maintained in chemically defined medium under serum-free conditions; and 2) differential culture conditions (medium utilized, plate surface chemistry, length of monocyte enrichment, and duration of the cells in culture prior to assessment) will influence MoDC viability, yield and phenotype.

Animals: 9 healthy adult horses

Methods: MoDC were generated from 3 to 9 adult horses for a series of experiments. The effects of medium utilized (chemically defined serum-free medium vs. RPMI-1640 serum-supplemented medium), plate chemistry (standard tissue culture plates vs. collagen-coated plates), period of monocyte enrichment (2 or 4 hours), and culture duration (3, 4 or 5 days) on MoDC viability, yield and phenotype were assessed. Viability was assessed by 0.04% trypan blue exclusion. MoDC yield was determined by manual cell counting using a hemocytometer. Expression of MHC class-II, CD86 and CD14 were evaluated by flow cytometry. Propidium iodide was used to eliminate signal produced by necrotic cells. Data were analyzed using paired t-tests and repeated measures ANOVA.

Results: There was no significant difference in MoDC viability or yield between medium utilized, plate surface chemistry, or period of monocyte enrichment. MoDC yield decreased and the percentage of MoDC expressing MHC class-II increased with increased culture duration prior to assessment ($P < 0.05$). The fraction of cells expressing MHC class-II and the density of MHC class-II and CD86 expression were increased when MoDC were generated in serum-free medium ($P < 0.05$). The difference in MoDC phenotype between two distinct populations from the cultures were assessed and defined less and more differentiated MoDC, respectively.

Conclusions: A consistent population of immature MoDC (as defined by the gating strategy presented here) can be reliably generated using serum-free medium with only recombinant equine GM-CSF and IL-4 as additives. Using serum-free medium, standard tissue culture coated plates and duration of generation of 3 – 4 days prior to assessment is recommended for optimal and consistent generation of equine MoDC in culture. Two cell populations consistent with less and more mature MoDC, are produced in these cultures, which offers differential opportunities for assessment of MoDC activation and maturation.

INTRODUCTION

Dendritic cells (DC) are a heterogeneous population of immune cells and are comprised of several subtypes with vital roles in initiating and establishing an appropriate adaptive immune response.[1,2] A considerable amount of research has been focused on characterizing the precise function of DC subtypes.[2,3] During inflammation and infection, blood monocytes are recruited into the tissue where they differentiate into a distinct subpopulation of DC defined as monocyte-derived DC (MoDC).[4-6] In peripheral tissues, MoDC are constantly surveying the environment for potential danger signals. MoDC interaction with foreign antigens initiates the complex process of DC maturation. Transition into a fully mature MoDC is required for effective activation of the adaptive immune response. The stage of MoDC maturity in response to stimulation can be demonstrated *ex vivo* by assessing MoDC phenotype. Immature MoDC can be defined by moderate expression of CD14[7] and low expression of MHC class-II and CD86. The ability to consistently generate an immature and essentially pure MoDC population *ex vivo* is required for experimental evaluation of DC maturation and function following *ex vivo* antigen exposure.

Protocols for experimental generation of equine MoDC have been adapted from those described for human and murine literature. Initial methods utilized for equine MoDC generation yielded only limited numbers of MoDC from equine peripheral blood mononuclear cells (PBMC).[8] Recent studies employing granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) to generate MoDC from PBMC have greatly improved MoDC yield and provided a pathway for practical MoDC generation from horses.[9-12] The culture medium used to generate MoDC in these studies is typically supplemented with allogeneic equine or xenogeneic (fetal bovine) serum in an effort to maintain cell viability during prolonged culture duration. Serum contains many undefined factors – antibodies, hormones, growth factors, binding proteins, vitamins, and lipids – that have been shown to modify the immune response during culture.[13,14] It is possible that these factors could impact the results of studies when serum-supplemented medium is used. While there have been several advancements in the generation of equine MoDC, further optimization to yield consistently repeatable and reliable population of immature MoDC under serum-free conditions is needed.

The objective of this study was to optimize equine MoDC culture conditions using serum-free medium to improve the generation of a highly pure and reliable population MoDC under a well-defined culture system. Specifically, we assessed the effect of several culture conditions, including medium with and without serum, the surface chemistry of culture plates, the duration of monocyte enrichment, and the duration of MoDC generation, on equine MoDC viability, yield and surface marker expression.

MATERIALS AND METHODS

Animals

Nine healthy mixed breed horses (7 males, 1 female, age 2 – 15 years old) belonging to the University of Georgia were used for this study. The University's Institutional Animal Care and Use Committee approved research protocols. All animals were confirmed to be systemically healthy prior to sampling by normal physical examination.

Peripheral blood mononuclear cell isolation

Approximately 120 mL of blood was collected via direct jugular venipuncture into syringes containing EDTA (1.5 mL of 100 mM) from each adult horse. Peripheral blood mononuclear cells (PBMC) were purified from whole blood samples via a single step density centrifugation using 1.077 – 1.088 lymphocyte separation medium (LSM; Corning, Corning, NY, USA) and a centrifugation speed of 1,400 g for 30 minutes as previously described.[15] The interface of plasma and LSM containing PBMC was collected and washed twice in calcium and magnesium ion-free phosphate buffered saline (PBS, Corning, Corning, NY, USA).

Effect of serum-supplemented and serum-free medium on MoDC viability and yield

To assess the effect of serum-supplemented medium on MoDC generation, PBMC were suspended in either chemically defined serum-free media (AIM-V, Thermo Fisher, Waltham, MA, USA) supplemented with 2.5 μg/mL amphotericin B (Gibco, Thermo Fisher, Waltham, MA, USA). and 2mM L-glutamine (Corning, Corning, NY, USA) or in RPMI 1640 medium (Corning, Corning, NY USA) supplemented with 10% heat-inactivated fetal calf serum (Hyclone, Thermo Fisher, Waltham, MA, USA), 2mM L-glutamine (Corning, Corning, NY, USA), and 50 μg/mL gentamicin (Corning, Corning, NY, USA). PBMC were plated at 5 x 10^6 cells/mL in a 6-well standard tissue culture (TC) coated plate (Nunc ®, Thermo Fisher, Waltham, MA, USA) and incubated at 37°C with 5% CO_2 for 4 hours. After 4-hours, the non-adherent cells and medium were removed. Fresh medium (AIM-V or RPMI) containing recombinant equine IL-4 (reIL-4; 10 ng/mL, R & D Systems, Minneapolis, MN, USA) and recombinant equine GM-CSF (reGM-CSF; 50 ng/mL; Kingfisher Biotech, INC., Minneapolis, MN, USA) were added to each well. After 48 hours in culture, 100% of the medium was removed and fresh medium and reGM-CSF and reIL-4 were added to each well. Both adherent and non-adherent MoDC were harvested on day 4 of culture. To collect adherent cells, MoDC were gently removed using a cell scraper (VWR, Radnor, PA, USA). Yield was manually determined using a hemocytometer and viability determined using 0.04% trypan blue exclusion.

Effect of surface plate chemistry on MoDC viability and yield

To evaluate the effect of surface chemistry of the plate utilized during MoDC generation, PBMC were plated in serum-free medium at a density of 5 x 10^6 cells/mL in 6-well standard polypropylene, TC coated plates (Nunc ®, Thermo Fisher, Waltham, MA, USA) or polystyrene, collagen-coated plates (Primaria ®, Corning, Corning, NY USA). Both sets of plates were incubated at 37°C with 5% CO_2 for 4 hours. MoDC were then generated, harvested and assessed for viability and yield as described above.

Effect of the period of monocyte enrichment time on MoDC viability and yield

Assessment of the period of monocyte enrichment on MoDC yield was determined by plating PBMC at a density of 5 x 10^6 cells/mL in a 6-well standard TC coated plates with serum-free medium. The plates were incubated at 37°C with 5% CO_2 for 2 hours or 4 hours prior to removal of non-adherent cells. MoDC were then generated, harvested and assessed for viability and yield as described above.

Effect of the duration of MoDC generation on viability and yield

To assess the effect of the duration under culture conditions on MoDC yield and viability, PBMC were plated at a density of 5 x 10^6 cells/mL in a 6-well standard TC coated plate with serum-free medium and incubated for 4 hours. MoDC were then generated, harvested and assessed for viability and yield as described above after 3, 4 or 5 days in culture.

Effect of medium utilized, plate chemistry and duration of generation on MoDC phenotype

The effect of medium utilized, surface chemistry of the plate utilized, and the duration of MoDC generation on surface marker expression was assessed. Specifically, MoDC were labeled at harvest with the following monoclonal fluorophore-conjugated antibodies: 10 µL FITC-conjugated mouse anti-horse MHC class-II at a 1:160 dilution (AbD Serotec, Hercules, CA USA), 10 µL Alexa 647-conjugated mouse anti-horse CD14 at a 1:100 dilution (clone 105, Wagner Laboratory, Cornell University, Ithaca, NY, USA) and 40 µL undiluted PE-conjugated mouse anti-human CD86 (BD Bioscience, San Jose, CA, USA). All antibodies were assessed for the minimum saturation binding dilution and to minimize cross-color masking prior to this study (data not shown). To eliminate signal from necrotic cells and further assess viability, propidium iodide (5 μL at a 1:50 dilution) was added immediately prior to analysis. Cells were analyzed on Accuri C6 Flow Cytometry (BD Science, San Jose, CA, USA) with BD C6 data acquisition and analysis software. To standardize the analysis of cellular phenotype, singlet gating (forward angle height vs forward angle area) was utilized as a primary gating tool. The singlet gate map that provided the most uniform population is shown in Figure 3.1A as P1. Further, forward angle vs side scatter gating was used to delineate two distinct populations of cells (P2 and P3) within the principle (P1) population (Figure 3.1B). Phenotypic analysis of these two populations indicated that these populations differed in their level of maturity (Figure 3.2). This was demonstrated by an increased fraction MoDC within the P2 population expressing CD14 (P = 0.006), and a reduced fraction of MoDC within the P2 population expressing CD86 (P = 0.003) compared to MoDC comprising the P3 population (Figures 3.1B and 3.2). The P2 population also contained a more necrotic MoDC compared to the P3 population (P = 0.0004). These findings indicate that smaller P2 population of MoDC represents a more immature and not fully differentiated population in contrast to the larger P3 population of MoDC consisting of differentiated but not fully mature MoDC (Figure 3.1B). We wanted to generate and define a uniform population of MoDC that were fully differentiated from monocytes but were still relatively immature allowing future studies measuring maturation after exposure to stimuli to be performed. Thus, MoDC that fell within the P3 population were considered the population of interest for the experiments presented here. The gating for this population was further refined and compensation was appropriately set to limit background staining and maximize signal intensity. The percent positive cells and median fluorescence intensity (MdFI) of cells within the P3, MoDC gate are reported. For the surface markers evaluated in this study, the MdFI more accurately characterized the density of each marker because positive peaks were not resolved from background peaks.

Statistical Analysis

Analysis was conducted using commercial statistical software (Prism, version 7.0, GraphPad Software, San Diego CA, USA). Data were log transformed to achieve normality. Paired t-tests were used to identify significant effects of medium type, plate chemistry and monocyte enrichment time on MoDC yield, viability, and surface marker expression. Repeated measures analysis of variance was used to assess for differences in cell culture duration on MoDC yield, viability, and surface expression marker with Tukey correction for multiple comparisons. Statistical significance was set at $P < 0.05$.

RESULTS

Effect of serum-supplemented and serum-free medium on MoDC viability, yield and maturation

The effect of serum-supplemented and serum-free medium on MoDC viability and yield is presented in Tables 3.1 and 3.2. The effect of serum-supplemented and serum-free medium on surface marker expression (percent positive cells and MdFI) and the percentage of cells that fell within the defined P3, MoDC gate are shown in Table 3.3 and Figure 3.2. There was no difference in MoDC viability ($P = 0.9$) or yield ($P = 0.5$) for cells cultured in serum-supplemented medium compared to those cultured in serum-free medium. An increase in the number of cells expressing MHC class-II ($P = 0.003$) and an increased density of MHC class-II expression ($P = 0.03$) was observed when MoDC were generated in serum-free medium compared to those generated in serum-supplemented medium. The density of CD86 expression was greater when MoDC were generated in serum-free medium compared to those generated in serum-supplemented medium ($P = 0.005$). There was no significant difference in the percentage of MoDC expressing CD86 ($P = 0.07$) or CD14 ($P = 0.05$) when MoDC were generated in serum-free medium compared to those generated in serum-supplemented medium. The percentage of necrotic cells was low and comparable for both types of medium utilized.

Effect of surface plate chemistry on MoDC yield and viability

The effect of surface plate chemistry on MoDC viability and yield is presented in Tables 3.1 and 3.2. The effect of surface plate chemistry on surface marker expression (percent positive and MdFI) and percentage of MoDC that fall within the defined MoDC gate is presented in Table 3.3 and Figure 3.4. There was no statistically significant difference in MoDC viability ($P = 0.3$) or yield ($P = 0.9$) when MoDC were generated on standard TC coated plates compared to collagen-coated plates. A significant increase in the percentage of MoDC expressing MHC class-II was observed when MoDC were generated on collagen-coated plates compared to those cultured on standard TC plates ($P = 0.047$). There was no effect of plate surface chemistry on the percentage of MoDC expressing CD86 ($P = 0.1$) or CD14 ($P = 0.3$). An increase in the median density of CD86 expression was observed when MoDC were cultured on collagen-coated plates ($P = 0.02$). Plate chemistry did not significantly affect the density of expression of MHC class-II or CD14, or the percentage of necrotic cells ($P > 0.05$).

Effect of the period of monocyte enrichment on MoDC viability and yield

The effect of the period of monocyte enrichment on MoDC viability and yield are presented in Tables 3.1 and 3.2. No significant differences in MoDC viability ($P = 0.5$) or yield ($P = 0.6$) were observed when the monocyte enrichment time was doubled from 2 to 4 hours and MoDC were assessed after 4 days in culture.

Effect of duration of MoDC generation on viability, yield and maturation

The results for the effect of duration of MoDC generation on cell viability and yield are presented in Table 3.4. The results for the effect of duration of MoDC generation on surface marker expression (percent positive and MdFI) and the percentage of cells that fall within the MoDC gate are shown in Table 3.5 and Figure 3.5. MoDC viability was ≥ 90 % after 3, 4, or 5 of generation. A significant decrease in MoDC yield was observed when MoDC were generated for either 4 ($P = 0.04$) or 5 ($P = 0.0004$) days compared to those generated for 3 days. MoDC yield after 4 days of generation was lower than MoDC yield observed after 5 days of generation ($P = 0.002$). An increased percentage of cells expressing MHC class-II was observed when MoDC were generated for 5 days compared to 3 days of generation ($P = 0.02$). No statistically significant difference in the fraction of cells expressing MHC class-II was observed between MoDC generated for 3 days compared to 4 days ($P = 0.08$) or for between MoDC generated for 4 days compared to 5 days ($P = 0.11$). The median density of MHC class-II expression was increased when MoDC were generated for 5 days compared to 4 days. There was no effect of duration of MoDC generation on either the median density or the percentage of cells expressing CD86 or CD14.

DISCUSSION

The results of these experiments demonstrate for the first time that equine MoDC can be generated and maintained using chemically defined, serum-free medium using only reGM-CSF and reIL-4 as additives. The expression pattern of surface markers, using the specific gating strategy defined here, for MoDC generated under these conditions compared to those generated in serum-supplemented medium were different. Specifically, an increased percentage of MoDC expressing MHC class-II and CD14 and an increased median density expression of MHC class-II, CD86, and CD14 was observed when MoDC were generated in serum-free medium. The observed differences in surface marker expression pattern suggest that serum-free conditions cause partial MoDC maturation. These differences were minimal and maturation following MoDC stimulation (changes in expression of these markers) can still be assessed using this model. However, the possibility that our culture conditions generated a MoDC subtype distinct from MoDC generated in serum-supplemented medium cannot be ruled out.

We did not detect a significant difference in MoDC yield when cells were generated in serum-free medium compared to those generated in serum-supplemented medium. This is in contrast to work by Dietze *et al*[16] in which serum-free medium significantly increased equine MoDC yield (7-fold increase) after 3 days of generation. The discord between those results and the result of the study presented here may be due to other differences in MoDC generation protocols between these studies. In our study, serum-free medium was used for both monocyte enrichment and MoDC generation. In contrast, Dietze *et al*[16] cultured MoDC in serum-free medium only during the period of monocyte enrichment. In study presented here, 100% of the medium was replaced after 48 hours in culture generation. Dietze *et al*[16] did not replace the medium for the duration of culture generation; instead, culture medium was supplemented with fresh cytokines. It has been documented that when murine and human DC spontaneously mature[17,18] in culture they become non-adherent.[19,20] Thus, it is possible that a substantial proportion of MoDC were removed during medium change in our study. We felt that replenishing the medium was necessary to provide nutrients and to remove waste products produced by cellular metabolism during prolonged culture. The effect of changing medium composition and medium replacement during the generation of MoDC warrants further investigation.

In this study we also examined the effect of plate chemistry on MoDC yield and surface marker expression. Dietze *et al*[16] reported a 3-fold increase in MoDC yield when cells were plated on collagen-coated dishes compared to standard TC coated dishes. We found no difference due to surface chemistry of the plates utilized. The less than 1-$\log_{10}$ difference detected in our study using collagen-coated plates is likely irrelevant in practice. The fraction of MoDC expressing MHC class-II was significantly higher using collagen-coated plates in our study, suggesting that the plate coating might have partially matured the cells. As discussed, MoDC phenotype can be used to evaluate MoDC maturation in response to added stimuli, so using collagen-coated plates could greatly impact the ability to accurately assess this process. Thus, we recommend plates with the standard TC coating for equine MoDC generation.

In the literature, monocyte enrichment has been reported for incubation periods ranging from 45 minutes to 4 hours[10,12,16, 21-24] We assessed the impact of period of monocyte enrichment time on. MoDC yield and detected no difference in the number of MoDC generated after 4 days in culture for monocyte enrichment period of 2 or 4 hours. One possible explanation for this finding is that our plating density is optimal for the capture of monocytes, and no further enrichment occurred despite the longer culture. The broad confidence interval for MoDC yield demonstrates significant horse-to-horse variability as an important factor. Additional studies are needed to definitively determine the optimal period of monocyte enrichment. The impact of plating density on MoDC yield could also be investigated, potentially allowing comparable MoDC generation with smaller blood volumes.

Several different periods of duration for equine MoDC generation prior to stimulation with antigen to induce maturation have been utilized, ranging from 3 – 7 days.[10,11,16] The results presented here suggest that culture duration of 3 to 4 days is sufficient to reliably produce a population of relatively immature MoDC using the gating strategy defined here. This is in general agreement with some previous studies.[10,16] MoDC yield significantly decreased nearly 3-fold when MoDC were generated for 5 days compared to 3 days. The reduced number of MoDC obtained after 5 days in culture presents limitations on one's subsequent ability to conduct assays to assess MoDC maturation and function. The phenotype of MoDC after 5 days of generation was different than that of MoDC generated for 3 or 4 days, with MHC class-II near maximum expression levels. While no significant difference in the expression of CD14 and CD86 after 5 days of generation relative to shorter generation periods was observed, the ability to assess further MoDC maturation after antigen exposure using MHC class-II expression would be limited for MoDC generated longer than 4 days. Further, there was a trend toward an increased number of necrotic cells and decreased number of MoDC that fell within the defined MoDC gate after 5 days of generation ($P = 0.06$). Thus, we recommend a culture period of 3 to 4 days before equine MoDC are utilized for additional functional or phenotypic assessment.

Differential review of the P2 and P3 populations representing less and more mature MoDC, respectively, were identified in these cultures. Individual analysis of the loosely adhered and tightly adhered population indicated that the P2 and P3 populations were separated by this property. The tightly adhered less mature MoDC were essentially all isolated in P2, and the loosely adhered more mature MoDC in P3. Thus, we are able to do differential assessment in the total population of cells utilizing this FALS-90LS differential in the cells.

The primary limitations of this study are the limited sample size and the number of replicate assessments of cells from each outbred horse. Inter-horse variability of MoDC phenotype has been previously described.[10] Increasing our sample size and the capacity to run more replicate assessments could allow us to detect real differences in more of the parameters with a higher degree of confidence. Further, assessment of the effect of medium composition on MoDC function (e.g. cytokine production, antigen internalization, and T cell stimulation) is warranted.

Collectively these studies provided a well-defined model to reliably generate a uniform population of immature equine MoDC under chemically defined serum-free conditions using only reGM-CSF and reIL-4 as additives when using gating strategy described here. Specifically, we recommend that equine MoDC be generated using standard TC coated plates in serum-free medium for a culture duration of no longer than 4 days to ensure reproducible and near optimal generation of immature, but functional MoDC.

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Table 3.1. Mean (%, ± standard deviation) viability of monocyte-derived dendritic cells (MoDC) under different culture conditions. Trypan blue exclusion (0.04%) was used to quantify viable cells. There were no significant differences in viability among culture conditions.

	Mean Viability % (± SD)
Medium Utilized (n=9)	
Serum-supplemented	90 (± 7.0)
Serum-free	90 (± 5.0)
Plate Chemistry (n=8)	
Standard TC coated plate	91 (± 4.9)
Collagen-coated plate	88 (± 6.4)
Enrichment Time (n=6)	
2 hours	91 (± 5.1)
4 hours	93 (± 2.5)

Table 3.2. Mean difference and 95% confidence interval (CI) for MoDC viability (%) and yield ($x10^5$) after 4 days of generation determined by 0.04% trypan blue exclusion. ($P < 0.05$)

	Viability % Mean Difference (95% CI)	DC yield ($x10^5$) Mean Difference (95% CI)
Medium Utilized (n=9)		
Serum-supplemented vs. serum-free	0.56 % (-6.8 to 7.9)	0.4 (-6.2 to 7.1)
Plate Chemistry (n=8)		
Standard TC coated vs. Collagen-coated	2 % (-1.9 to 6.1)	- 0.9 (-6.8 to 4.9)
Enrichment Time (n=6)		
4 hours vs. 2 hours	1.3% (-3.8 to 6.4)	1.8 (-4.5 to 8.0)

Table 3.3. Percentage (mean ± standard deviation) of mononuclear-derived dendritic cells (MoDC) expressing MHC class-II, CD14, and CD86, and the percentage (mean ± standard deviation) of necrotic (propidium iodide (PI) positive) cells under different culture conditions. [a,b] Different superscript letters indicate a significant difference between culture conditions. (P< 0.05)

(%) Positive Cells	**MHC class-II Mean (%) ± SD**	**CD86 Mean (%) ± SD**	**CD14 Mean (%) ± SD**	**PI Mean (%) ± SD**
Medium Utilized (n=5)				
Serum-supplemented	40.3^{a} ± 18.8	19.9^{a} ± 18.5	4.6^{a} ± 2.3	1.7^{a} ± 0.9
Serum-free	61.7^{b} ± 21.1	38.7^{a} ± 19.2	11.6^{a} ± 3.4	1.5^{a} ± 0.6
Plate Chemistry (n=4)				
Standard TC coated	70.5^{a} ± 15.5	54.4^{a} ± 16.5	8.1^{a} ± 2.3	1.9^{a} ± 0.7
Collagen-coated	86.0^{b} ± 13.1	64.2^{a} ±12.4	9.4^{a} ± 3.5	1.6^{a} ± 0.2

Table 3.4. Mean ± standard deviation (SD) for monocyte-derived dendritic cell (MoDC) viability and yield after 3, 4 or 5 days of generation in culture as determined by 0.04% trypan blue exclusion (viability) and manual hemocytometer counting (n = 6). [a,b] Different superscript letters indicate a significant difference between days of generation in culture. (P< 0.05)

	Viability (%) (± SD)	**DC yield (x10^6) (± SD)**
Culture Duration (n=4)		
Day 3	97[a] (± 4)	3.0[a] (± 2.6)
Day 4	90[b] (± 6)	2.2[b] (± 1.2)
Day 5	91[ab] (± 8)	1.1[c] (± 1.1)

Table 3.5. Percentage (mean ± standard deviation) of monocyte-derived dendritic cells (MoDC) expressing MHC class-II, CD14, and CD86, and the percentage (mean ± standard deviation) of necrotic (propidium iodide (PI) positive) cells after 3, 4 or 5 days of generation in culture. [a,b] Different superscript letters indicate a significant difference between days of generation in culture. ($P < 0.05$)

(%) Positive Cells	**MHC class-II Mean (%) ± SD**	**CD86 Mean (%) ± SD**	**CD14 Mean (%) ± SD**	**PI Mean (%) ± SD**
Culture Duration (n=4)				
Day 3	$58.4^{a} \pm 8.5$	$31.4^{a} \pm 9.6$	$10.6^{a} \pm 6.0$	$1.2^{a} \pm 0.7$
Day 4	$74.8^{ab} \pm 11.3$	$35.6^{a} \pm 19.7$	$8.2^{a} \pm 2.4$	$1.7^{a} \pm 0.5$
Day 5	$91.4^{b} \pm 7.5$	$29.8^{a} \pm 12.3$	$9.7^{a} \pm 3.2$	$2.7^{a} \pm 1.2$

A) B)

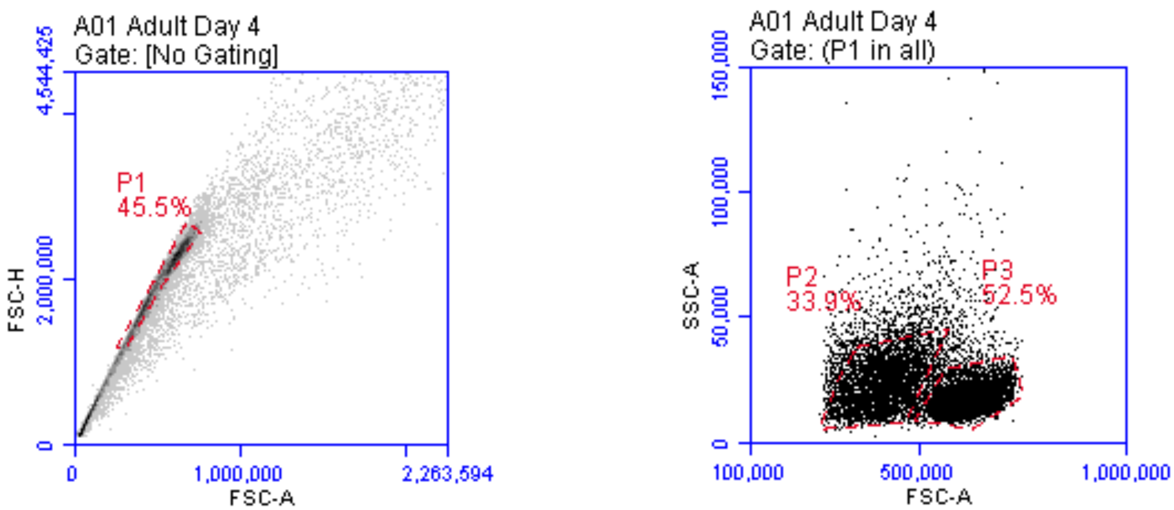

Figure 3.1. Gating of equine MoDC generated from whole blood using a representative sample from an adult horse after 4 days in culture. A) Dot plot of forward scatter-height (FSC-H) versus forward scatter-area (FSC-A) outlining the population of interest (P1). B) Dot plot depicting additional gating to delineate the P2 population comprising the most immature MoDC from the P3 population consisting of fully differentiated, but still somewhat immature MoDC using forward scatter-area (FSC-A) and side scatter-height (SSC-A). Gates were confirmed by phenotypic assessment of immature (adherent) and more mature (floating) populations from culture individually.

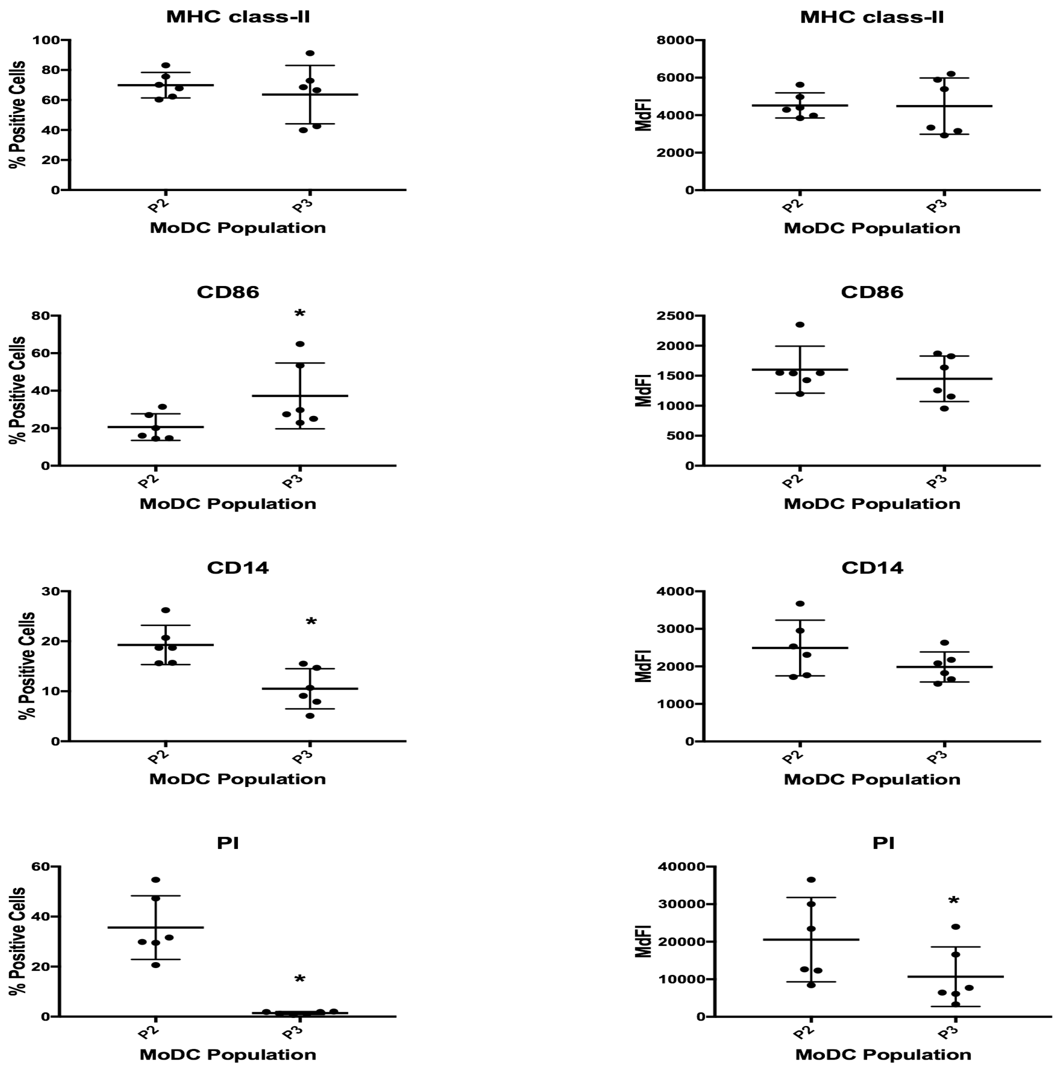

Figure 3.2. Percentage and median fluorescent intensity (MdFI) for surface marker expression (MHC class-II, CD86, CD14) and propidium iodide (PI) staining of necrotic cells by monocyte-derived dendritic cells (MoDC) derived from blood monocytes of adult horses (n=5), generated in serum-free medium for 4 days. MoDC that fell within the P2 (most immature) or P3 (more mature and well differentiated) defined MoDC gates were compared. Each solid dot represents an individual adult horse and the black horizontal line reflects the mean for those data points. *Denotes a statistically significant difference between MoDC within the P2 population compared to MoDC within the P3 population. (P< 0.05)

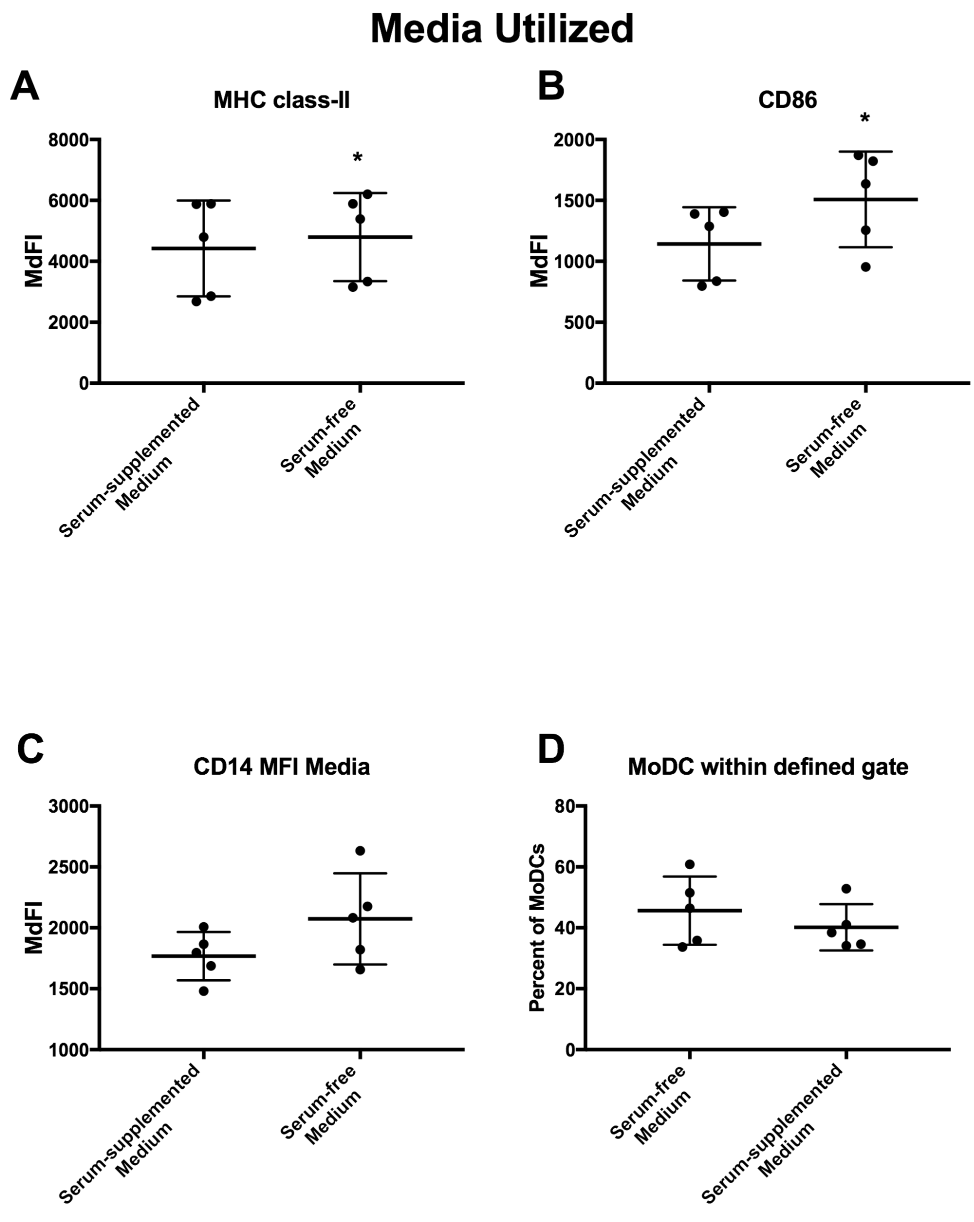

Figure 3.3. Median fluorescent intensity (MdFI) for surface marker expression [(A) MHC class-II, (B) CD86 and (C) CD14], and the percentage of monocyte-derived dendritic cells (MoDC) that fell within the defined P3, MoDC gate of interest (D) for equine MoDC generated in serum-free or serum-supplemented medium. Each solid dot represents an individual adult horse and the black horizontal line reflects the mean for those data points. *Denotes a statistically significant difference between treatment conditions. (P< 0.05)

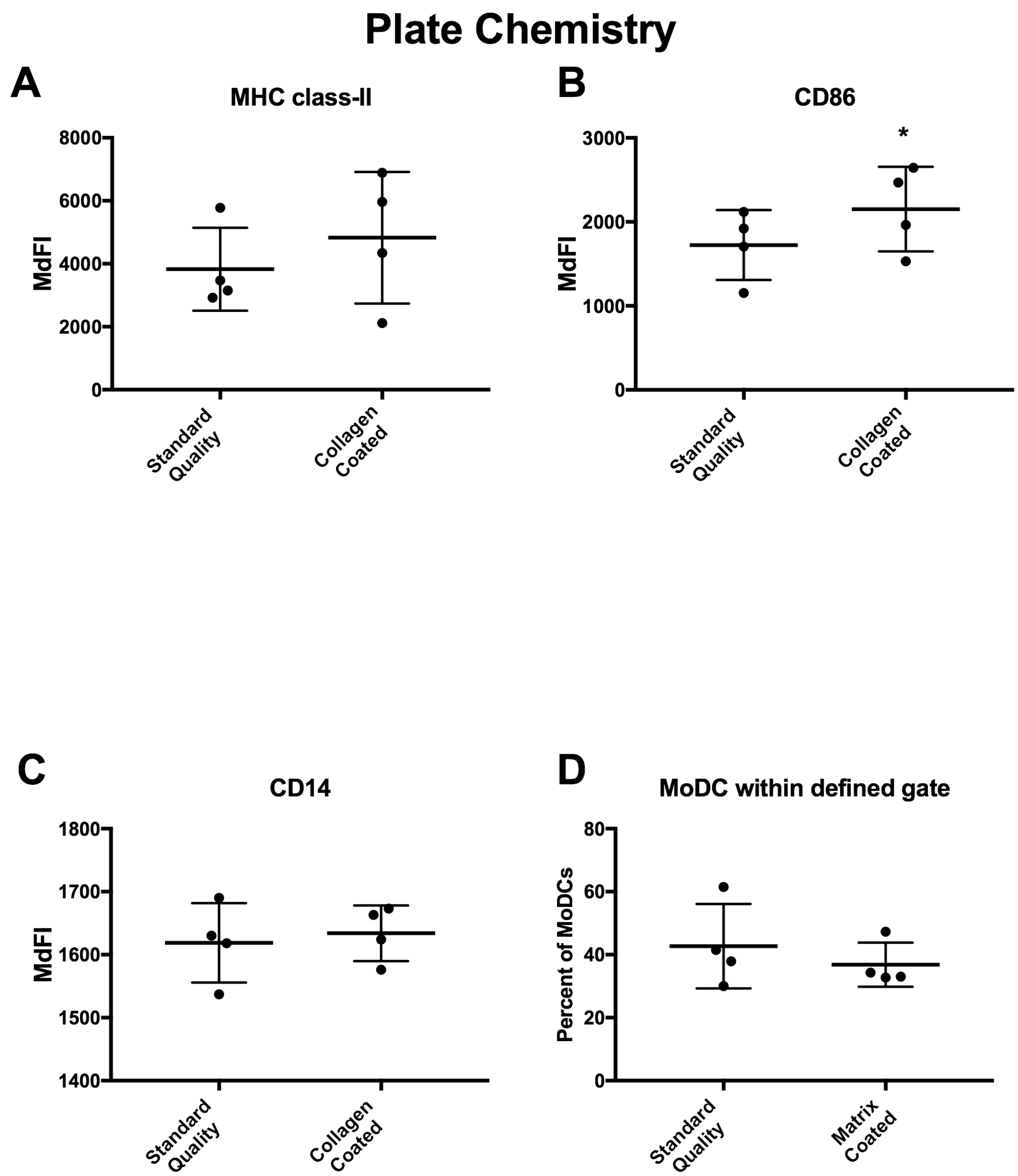

Figure 3.4. Median fluorescent intensity (MFI) for surface marker expression [(A) MHC class-II, (B) CD86, and (C) CD14], and the percentage of monocyte-derived dendritic cells (MoDC) that fell within the defined P3, MoDC gate of interest (D) for equine MoDC generated on standard tissue culture coated plates or collagen-coated plates. Each solid dot represents the results from an individual adult horse and the black horizontal line reflects the mean for those data points.

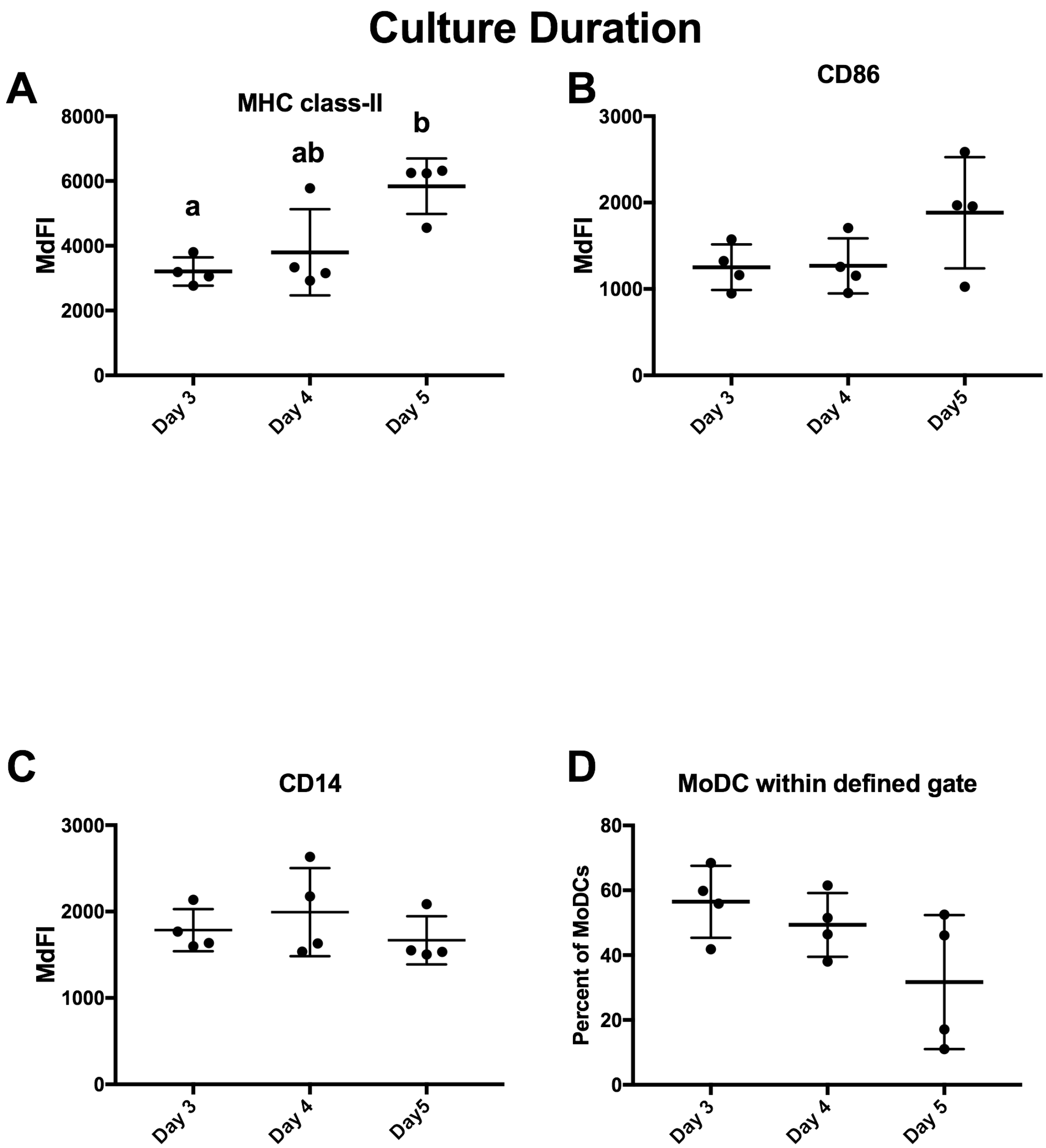

Figure 3.5. Median fluorescent intensity (MFI) for surface marker expression [(A) MHC class-II, (B) CD86 and (C) CD14], and the percentage of monocyte-derived dendritic cells (MoDC) that fell within the defined P3, MoDC gate of interest (D) for equine MoDC after 3, 4 or 5 days of generation in culture. Each solid dot represents the results from an individual adult horse and the black horizontal line reflects the mean for those data points.

CHAPTER 4

EFFECT OF AGE ON FOAL MONOCYTE-DERIVED DENDRITIC CELL MATURATION AND FUNCTION AFTER *EX VIVO* EXPOSURE TO KILLED BACTERIA[2]

[2] Lopez B, Giancola S, Hurley DJ, Giguère S, Felippe MJB, Hart KA. Effect of Age on Foal MoDC Maturation and Function After Bacterial Exposure. To be submitted to the *Journal of Veterinary Immunology and Immunopathology*.

ABSTRACT

Background: 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 (MoDC) represent a good model of DC that differentiate in the mucosal tissues during bacterial invasion from monocytes recruited from circulation.

Hypothesis: Foal monocyte-derived DC (MoDC) will exhibit age-dependent phenotypic and functional differences compared to adult horse MoDC.

Animals: 9 adult horses and 8 foals

Methods: MoDC generated from 9 horses (collected once) and 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, CD86, and CD14 were measured by flow cytometry, and supernatant cytokine concentrations of IL-4, IL-17, IFN-γ, and IL-10 were quantified using a validated equine immunoassay.

Results: The percentage of MoDC expressing MHC class-II and CD86 were 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 ($P < 0.0001$). Exposed MoDC from 1-day-old foals produced significantly less IL-4, IL-17, and IFN-γ than exposed adult MoDC ($P \leq 0.04$).

Conclusions and Clinical Importance: Following bacterial exposure foal MoDC phenotype and cytokine secretion were different than those of mature horse during the first month of life. These differences could reduce the ability of foals to generate a protective immune response against bacterial infection.

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.[1-3] 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.[4] This age-related susceptibility to infection suggests important limitations in foal host defense mechanisms.[5,6] Induction of an appropriate T lymphocyte response to a given pathogen is one critical aspect in the immune response that may limit the ability of the neonate to overcome infection.[4,7] It is the cytokine milieu generated by cells of the innate immune system, including dendritic cells, that fundamentally dictates the context of T cell response generated.[4,8]

Dendritic cells (DC) are important antigen presenting cells (APC) that play a pivotal role in activating and polarizing the adaptive immune response. Monocyte-derived DC (MoDC) represent a good model of DC that differentiate in the mucosal tissues during bacterial invasion from monocytes recruited from circulation. In peripheral tissues, immature MoDC continuously survey the environment for pathogen and damage associated molecular patterns and capture peptide antigen for presentation. Immature MoDC lack the mature complement of and density of, critical cell surface proteins to optimally induce primary T cell responses.[9,10] MoDC interaction with foreign antigen induces their differentiation and activation, and initiates the pathway of MoDC maturation. Phenotypic changes observed during the transition to fully mature MoDC include: 1) decreased CD14 expression, indicating differentiation from a monocyte into a MoDC[11-13]; 2) decreased endocytic capability[12,14-16]; 3) increased antigen presentation capacity (including enhanced expression of MHC class-II); and 4) increased expression of co-stimulatory molecules (e.g. CD86) [5,12]. Only those DC that have fully matured are capable of optimally priming naïve T cells in lymphoid tissues.[17]

Studies in human infants have documented substantial differences in the maturation and function of neonatal MoDC *ex vivo* relative to those generated from adult blood monocytes [18-21] with implications in progression of neonatal sepsis.[22] Studies investigating DC biology in foals are limited and primarily focus on induced 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 foal age on the maturation and function of equine neonatal MoDC generation in culture during *ex vivo* exposure to killed bacteria. We hypothesized that foal MoDC will exhibit phenotypic and functional differences that were related to the age of the foal, and when compared to MoDC generated from adult horses.

MATERIALS AND METHODS

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 to 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.

Monocyte-derived dendritic cell (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, Corning, NY, USA) [23]. PBMC were plated at 5 x 10^6 cells/mL in 6-well plates (Nunc ®, Thermo Fisher, Waltham, MA, USA) with serum-free medium (AIM-V, Thermo Fisher, Waltham, MA USA) supplemented with 2.5 μg/mL amphotericin B (Gibco, Thermo Fisher, Waltham, MA, USA) and 2mM L-glutamine (Corning, Corning, NY, USA). Cells were incubated at 37°C with 5% CO_2. After 4-hours, the non-adherent cells and medium were removed. Fresh serum-free medium containing recombinant equine IL-4 (10 ng/mL, R & D Systems, Minneapolis, MN, USA) and recombinant equine GM-CSF (50 ng/mL; Kingfisher Biotech, INC., Minneapolis, MN, USA) were added to the adherent cells, which were then incubated as above for 4 days to stimulate MoDC generation. After 48 hours in culture, 100% of the medium was discarded and fresh medium and cytokines were added. Control wells contained medium and cytokines only. Stimulated wells were exposed to heat-killed, briefly sonicated bacterial whole cell 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.[24,25] Preliminary studies were conducted to determine that these dilutions optimally produced DC maturation while maintaining DC viability (data not shown).

MoDC maturation assessment by flow cytometric phenotype analysis

On day 4 of culture, adherent and non-adherent MoDC were harvested. A cell scraper (VWR, Randor, PA, USA) was used to gently remove adhered cells. Viability was assessed by 0.04% trypan blue exclusion. The average viability of unstimulated and stimulated adult MoDC was 95% (± 3.5) and 94% (± 5.3), respectively. Foal MoDC viability on average was 87% (± 7.5) for unstimulated cells and 82% (± 9.1) for stimulated 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.[9] Figure 4.1 shows the morphologic difference between MoDC generated from a foal at day 1 of age (A) and an adult horse (B) after four days in culture using confocal microscopy. MoDC phenotype was used to delineate MoDC from closely related cell types including monocytes and macrophages (based on significant 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 (AbD Serotec, Hercules, CA, USA), 10 μL Alexa 647-conjugated mouse anti-horse CD14 at a 1:100 dilution (clone 105, Wagner Laboratory, Cornell University, Ithaca, NY, USA) and 40 μL undiluted PE-conjugated mouse anti-human CD86 (BD Bioscience, San Jose, CA, 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 (BD Bioscience, San Jose, CA, USA) 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 scatter) was recorded. To standardize the analysis of cellular phenotype, singlet gating (forward angle height vs forward angle area) was utilized as a primary gating tool. The singlet gate map that provided the most uniform population is shown in Figure 4.2A as P1. Further, forward angle vs side scatter signal was used to define two populations of cells from the cultures. 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 cells from culture (Figure 4.2B). These populations were confirmed by separating the basically non-adherent cells and those that needed to be scraped from the wells in several cultures. These gates served as the guide to phenotypic analysis.

Functional assays

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. 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).[26] These cytokines were selected to provide insight into the possible polarization of subsequent adaptive responses.

Macropinocytosis and Phagocytosis assays. On day 4 of culture, 1 $x10^5$ unstimulated 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, Eugene, OR, USA), bodipylated *S. aureus* (5 µg/mL; Molecular Probes, Eugene, OR, USA), or bodipylated *E. coli* (10 µg/mL; Molecular Probes, Eugene, OR, USA) for 4 hours at 37°C with 5% CO_2. After the incubation period, 40µL of 0.4% trypan blue (10% of sample volume) 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) were recorded for each sample to assess protein pinocytosis or non-opsonized bacterial phagocytosis.

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 below the limit of detection of the assay. This 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.

RESULTS

Effect of foal age and bacterial stimulation on DC maturation

Percentage and median fluorescence intensity of MoDC cells from adult horses and foals expressing surface markers MHC class-II, CD14 and CD86 are presented in Figure 4.3. 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

(Figure 4.1B, 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 medium 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 greater 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 cells 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 ($P = 0.03$). The median 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 medium density ($P \geq 0.18$) of foal or adult horse MoDC expressing the surface markers assessed, with the exception of CD86, which was on a higher fraction of MoDC after bacterial exposure, independent of age ($P = 0.0001$).

Effect of foal age on cytokine production

The effect of foal age and bacterial exposure on the production of cytokines by stimulated and unstimulated MoDC is shown in Figure 4.4. Basal production of IL-10 and IFN-γ by unstimulated foal MoDC was not significantly different to that of unstimulated adult cells. However, basal production of IL-17 and IL-4 in MoDC from foals of all ages was significantly less than adult horse MoDC ($P \leq 0.04$).

Bacterial exposure of adult MoDC resulted in significantly increased production of all the cytokines measured compared to basal conditions ($P \leq 0.01$), except for IL-4, which was not significantly increased after stimulation with *S. aureus* (P = 0.05). Similar to stimulated 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 had increased production of IL-10 compared to unstimulated MoDC from the same foal (P ≤ 0.0001). The production of IL-10 by bacterial exposed foal MoDC at all ages was comparable to that of adults. In contrast, MoDC generated from foals at 1 day of age did not have increased IFN-γ and IL-17 production above basal levels in response to exposure with *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 ≤ 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 after bacterial exposure (P ≤ 0.01).

Macropinocytosis and Phagocytosis

Macropinocytosis and nonopsonized phagocytosis activity for MoDC generated from foal and adult horse blood is presented in Figure 4.5. 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 (Figure 4.1B, P2). The percentage of MoDC taking up FITC-conjugated ovalbumin by macropinocytosis was greater in MoDC generated from foals of all ages than MoDC from to adult horses (P ≤ 0.009). The percentage of MoDC generated from foals at 1 day that phagocytized bodipylated *S. aureus* was greater than for adult horse MoDC (P = 0.0001). The percentage of MoDC phagocytizing bodipylated *E. coli* was greater for MoDC generated from foal blood collected at 30 days of age than for MoDC generated from adult horse blood (P = 0.005). There was not a significant difference in the amount (MFI) of FITC-ovalbumin, bodipylated *S. aureus*, or bodipylated *E. coli* that was taken up by adult horse or foal MoDC ($P \geq 0.36$).

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, when compared to MoDC from adult horses. Distinct phenotypic differences between immature adult MoDC and MoDC derived from foals at 1 day of age were demonstrated in our study, based on increased percentage of foal MoDC expressing a monocyte-like phenotype with a higher percentage of CD14 positive cells and a lower percentage cells expressing MHC class-II and CD86 antigen than adult MoDC. These findings are in agreement with previous work in both[5,9] human infants[27] and mice.[28] Further, they suggest that neonatal MoDC exhibit phenotypic immaturity across all mammalian species thus far 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.[29,30] Additionally, loss of MHC class-II and CD86 expression by monocytes and DC has been associated with increased severity of infection in adults,[31] children,[22] infants[32] and mice.[33] Results of 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 MoDC after exposure to bacterial antigens than MoDC from adult horses. This persisting MoDC immaturity in foals could ultimately contribute to the development, progression and severity of infection during the neonatal period.

In this study, bacterial exposure induced an increase in the percentage of MoDC expressing CD86 from 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 exposure. In addition, no difference in the density of MHC class-II, CD86, and CD14 was detected on foal MoDC or on MoDC from adult horses after bacterial exposure. The absence of robust changes in expression density of these surface proteins is in agreement with some previous studies[5,9,34] but in contrast with the findings of a number of others.[16] 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 phenotype of cell generated in response to underlying culture conditions.

In this study, we suspect that culture conditions 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 on these cells. The expression of CD14 by MoDC from adult horses was already low under our basal generation conditions, so no further decrease in expression could be measured based below our detection thresholds. Partial maturation induced by culture generation conditions observed in unstimulated MoDC from adult horses but not in MoDC from foals further demonstrates true limitations in foal MoDC maturation. It has been suggested that foal MoDC might have additional maturation threshold requirements compared to MoDC from adult horses. Specific requirements necessary to induce maturation of foal MoDC to levels observed with in adult MoDC could include (as yet undefined) environmental signals or cell-cell interaction.[5] These additional signals were not likely provided under our culture conditions. The reduced fraction of foal MoDC expressing MHC class-II and CD86 and combined with no observed increase in fraction of MoDC expressing these markers in response to bacterial exposure infer inherent 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 protection of the foal against bacterial infection.

The data presented here also revealed important differences in inflammatory cytokines production by foal and adult horse MoDC endogenously and after bacterial exposure. The overall balance of inflammatory and anti-inflammatory cytokines produced by MoDC 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 production of IL-4 and IL-17 relative to adult horse MoDC. This suggests that there may be intrinsic differences in cytokine responses in foals during homeostasis. Following bacterial exposure, MoDC derived from foals at 1 day of age demonstrated an increase in production of the anti-inflammatory cytokine IL-10 that was comparable to that induced in adult MoDC. Foal MoDC failed to increase the production of IFN-γ, IL-17, or IL-4 over basal levels after bacterial exposure. 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 the production of these cytokines foals.

The anti-inflammatory cytokine, IL-10, can suppress the production of inducible cytokines involved in inflammatory and adaptive responses[35] and is negatively associated with survival in septic foals.[36] In this study, the diminished IFN-γ and IL-17 production by MoDC generated from foals at 1 day of age could be mediated by IL-10 from foal MoDC. It is possible that foal MoDC might be biased toward production of IL-10 with a more pronounced suppressive effect of IL-10 in neonatal responses. This would explain the lower level of production of IFN-γ and IL-17 by MoDC from foals but not adult MoDC. 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. These cytokines are involved in providing context to the innate immune response against intracellular pathogens,[37] and in immune regulation and activation in adaptive responses. In contrast, IL-4 is the principle cytokine mediating a T helper type-2 (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 initial immune response.[38-40] Previous studies indicate that autocrine effects of IL-17[41] and IFN-γ[42] 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 the regulation of DC secretion of IFN-γ has also been suggested.[43] Reduced production of these cytokines by foal MoDC endogenously, and in response to bacterial exposure, might therefore reflect limitations in MoDC maturation, with potential implications in the ability of the foal to produce effective immune responses to pathogens.

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. This appears to prevent undesirable activation of the adaptive immune response against self-antigens.[44] Thus, in this study, these processes were only evaluated in unstimulated and the most immature unstimulated MoDC from our cultures. Our results revealed an increased number of immature foal MoDC at 1 day of age. These cells take up FITC-ovalbumin and *S. aureus* more avidly than MoDC from adults. As discussed previously, the pattern of surface marker expression indicates that our culture conditions induce partial maturation of the MoDC during generation. Thus, despite some degree of maturation and activation by the culture conditions in this study, foal MoDC remain in a sufficiently immature state to allow specific maturation by antigenic or inflammatory stimuli, and are less mature than MoDC from adult horses. The number of foals and horses used in these experiments was relatively small, and increasing the sample size in future experiments should allow for more confidence in defining the endocytic capacity of foals by age. Experiments evaluating endocytic functions following bacterial exposure might also provide additional insight into age-related changes in MoDC maturation related to function in foals.

Orchestrating a protective immune response requires multiple functional cellular components, in addition to a multitude of soluble factors. The factors that appear to be involved. include cytokines, hormones, antibodies, vitamins and other bioactive proteins. In this study, MoDC were investigated under isolated *ex vivo* conditions, limiting our ability to explore the role of cell-cell interactions, and cell-environmental interactions that naturally occur *in vivo* during 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 impacted by the limited numbers of contaminating cell types (monocytes, macrophages, and lymphocytes). Nevertheless, the culture protocol used here permits evaluation of inherent differences in foal MoDC maturation and function by minimizing confounding variables, e.g. endogenous plasma factors, thereby provides a platform for future studies that more closely emulate the DC microenvironment *in vivo* to be performed.

The failure to demonstrate altered surface marker expression and altered production of key cytokines, involved in both cellular and humoral immunity, by MoDC derived from foals relative to bacterial exposure suggests a state of decreased 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 be impacted by the phenotypic immaturity and altered cytokine secretion by foal MoDC described here. Additional studies further elucidating DC biology in foals will be critical to understanding the underlying mechanisms leading to infection in this highly susceptible population.

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A) B)

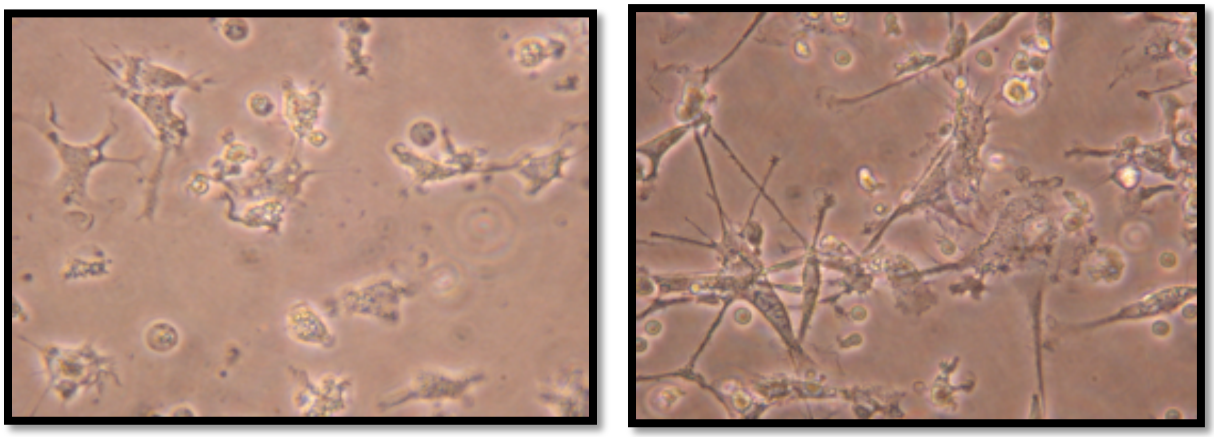
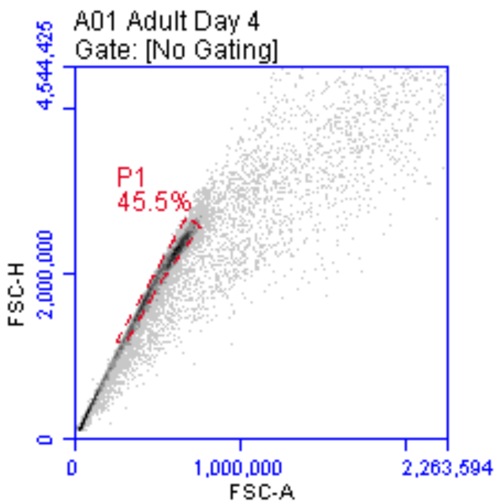


Figure 4.1. Monocyte-derived dendritic cells (MoDC) generated from a foal at day 1 of age (A) and from an adult horse (B) after four days in culture using confocal microscopy.

A)



B)

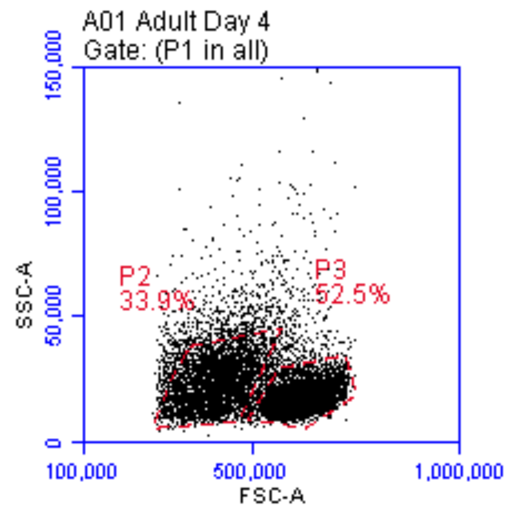


Figure 4.2. Gating of equine MoDC generated from whole blood using a representative sample from an adult horse after 4 days of generation. A) Dot plot of forward scatter-height (FSC-H) versus forward scatter-area (FSC-A) outlining the population of interest (P1). B) Dot plot depicting additional gating 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). Both gates were established using the immature (adherent) and mature (floating) population from culture individually before setting the combined population gates.

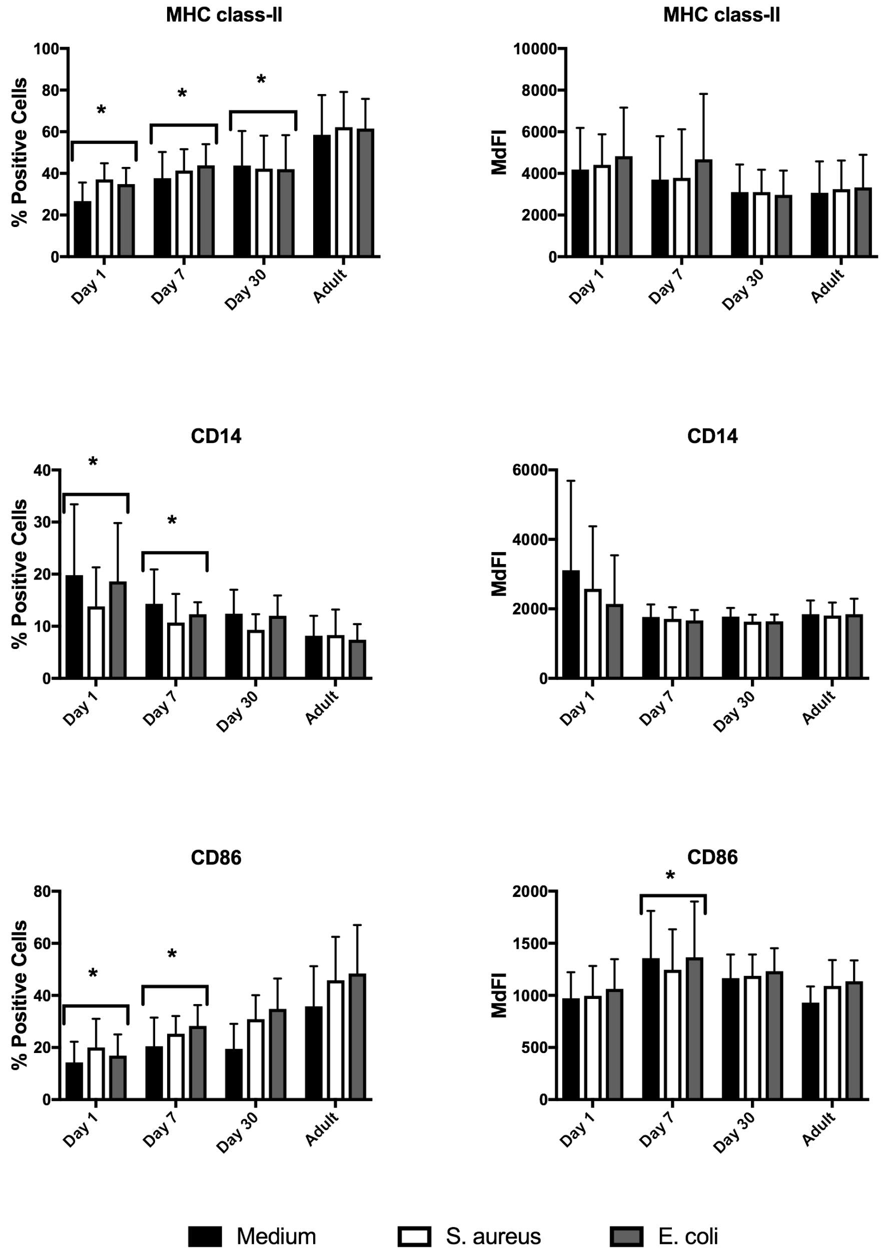


Figure 4.3. Percentage (mean ± standard deviation) and median fluorescence intensity (MdFI ± standard deviation) of MoDC expressing MHC class-II, CD14, and CD86 generated from blood monocytes from adult horses (n=9) once and foals (n=8) at 1, 7 and 30 days of age in the with and without bacterial exposure with killed whole cell preparations of *Escherichia coli or Staphylococcus aureus*. * Denotes statistically significant differences between adult horse and foal MoDC. (P< 0.05)

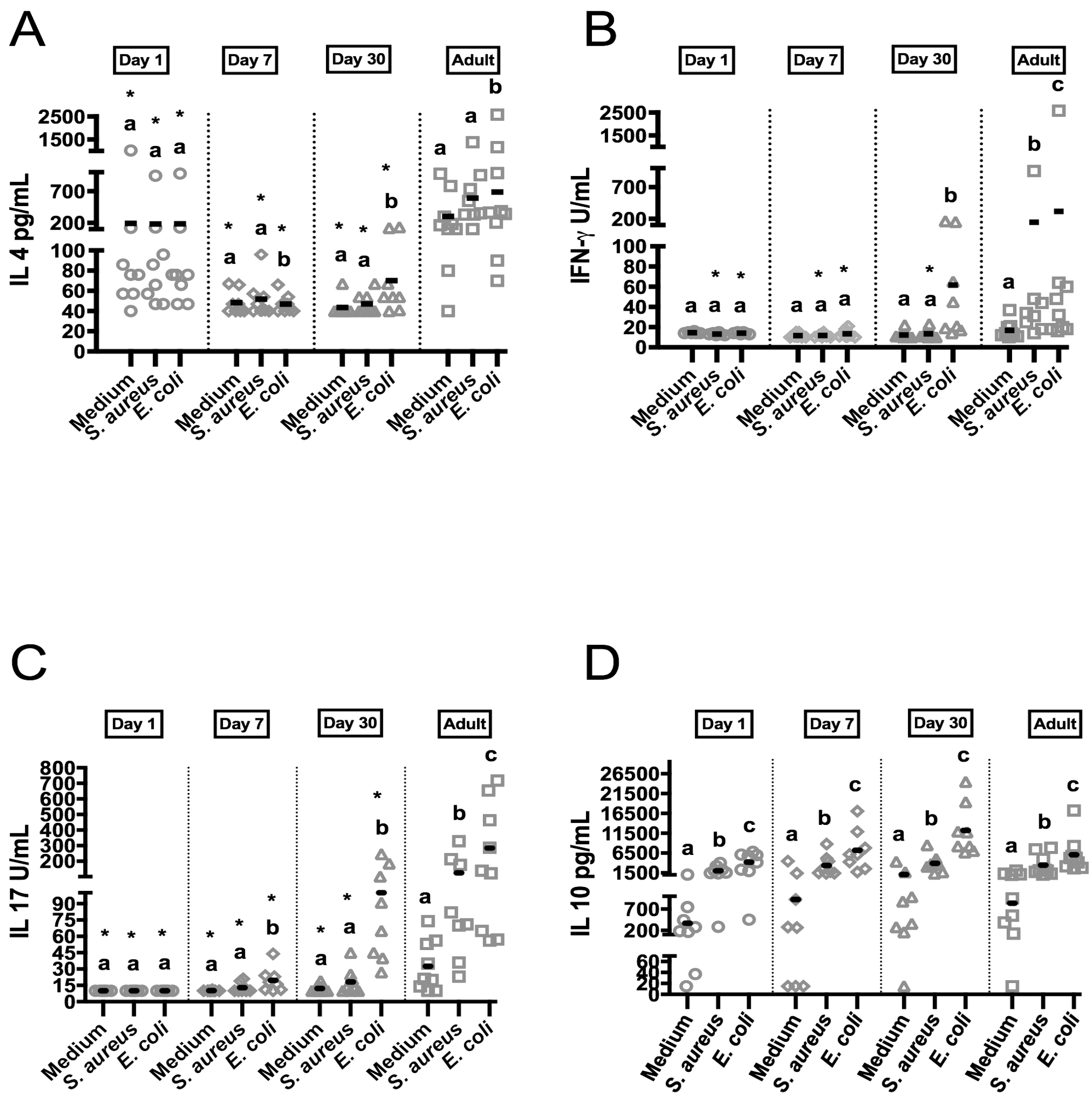


Figure 4.4. Cytokine concentration [(A) IL-4, (B) IFN-γ, (C) IL-17, and (D) IL-10] produced by isolated MoDC from adult horses (squares, n=9) and foals (n=8) at 1 (circles), 7 (diamonds) and 30 (triangles) days of age with and without bacterial exposure with killed whole cell preparations of *Escherichia coli or Staphylococcus aureus*. Each data point represents an individual animal. Horizontal lines represent the mean for that specific age and condition. * Denotes statistically significant differences between foals and adults. [a, b, c] Different letter superscripts denote statistically significant differences between treatment conditions within a given age. (P < 0.05)

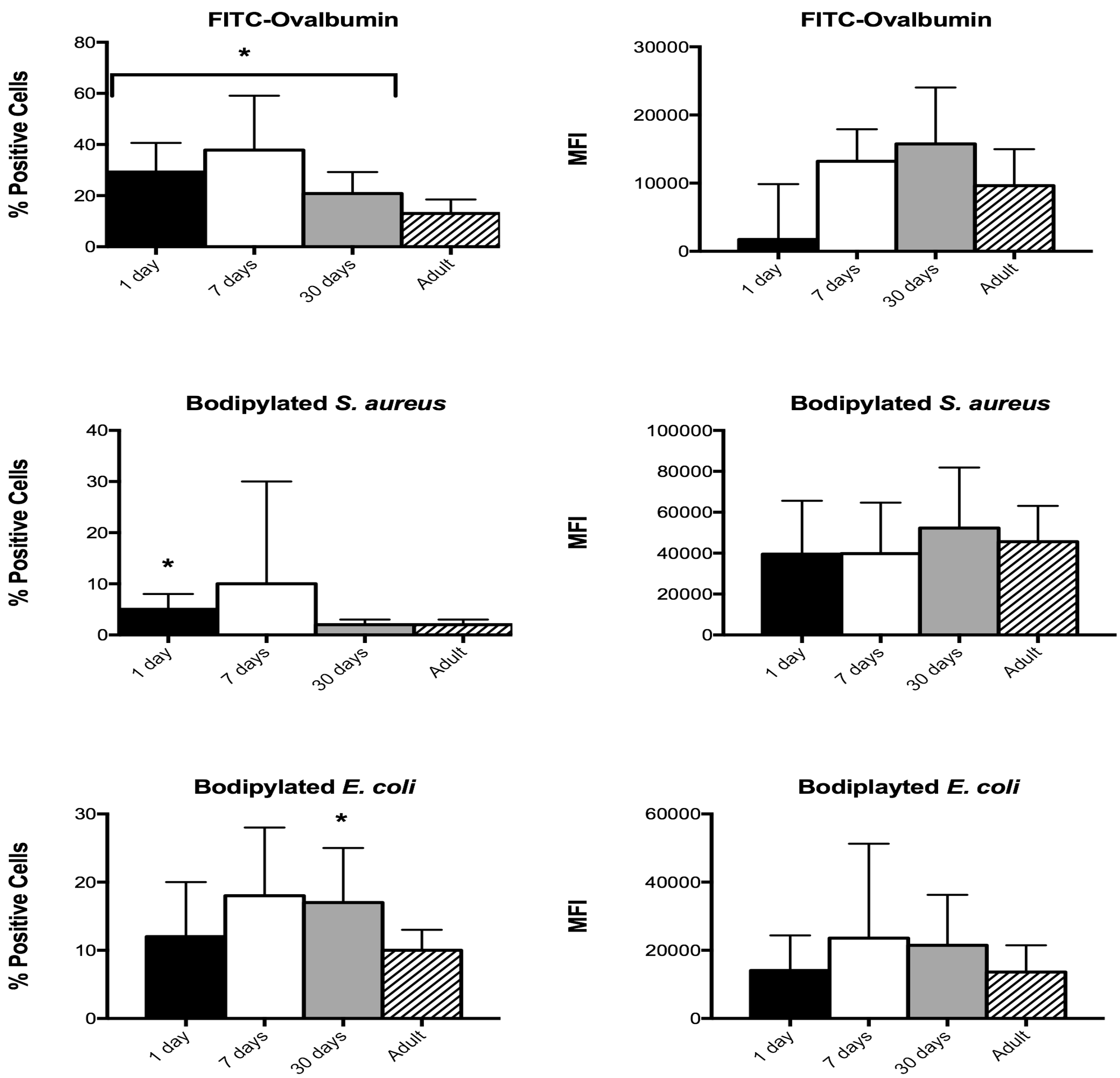


Figure 4.5. Percentage (mean ± standard deviation) and mean fluorescence intensity (MFI, mean ± standard deviation) of unstimulated, immature equine MoDC taking antigen by macropinocytosis (FITC-ovalbumin) or phagocytosis (bodipylated, killed *Staphylococcus aureus* or *Escherichia coli)*. MoDC were generated from adult horse monocytes (n=9) and foal monocytes (n=8) at 1, 7 and 30 days of age. * Denotes statistically significant differences between adult and foal MoDC. (P < 0.05)

CHAPTER 5

THE EFFECT OF SOLUBLE FACTORS IN GENERIC PLASMA AND CORTISOL ON EQUINE MONOCYTE-DERIVED DENDRITIC CELL MATURATION AND FUNCTION[3]

[3] Lopez B, Hurley DJ, Giguère S, Giancola S, Hart KA. The Effect of Generic Plasma Factors and Cortisol on Equine MoDC Maturation and Function. To be submitted to *the Journal of Veterinary Immunology and Immunopathology*.

ABSTRACT

Background: Immunologic and endocrine immaturity in foals increases foal morbidity and mortality from bacterial sepsis. Dendritic cells (DC) are critical in priming the adaptive immune response, but foal DC are phenotypically and functionally different than those of adult horses. Studies investigating the effect of undefined soluble plasma factors, and cortisol, on equine DC have not been reported.

Hypothesis: Exposure to plasma from the pairs of adult horses and foals utilized in the study, or cortisol will differentially impact monocyte-derived DC (MoDC) phenotype and function.

Animals: 9 adult horses and 9 foals

Methods: To investigate the impact of different paired sources of plasma (foals in the study, or the adult horses in the study) and cortisol, MoDC were generated from blood monocytes of healthy adult horses (n=9) and foals (n=8). MoDC were then exposed to killed whole-cell bacterial preparations and adult horse plasma, foal plasma, or cortisol (hydrocortisone). Culture medium alone was utilized as a negative control. Expression of surface markers (MHC class-II, CD86, and CD14) and uptake of labeled protein antigen and killed bacteria were measured by flow cytometry. Supernatant cytokine concentrations (IL-4, IL-17, IFN-γ, and IL-10) were quantified with a validated immunoassay.

Results: The percentage of MoDC expressing surface markers MHC class-II and CD86 was reduced in MoDC derived from foals at day 1 of age when cultured in either serum-free medium alone, and in either foal or adult horse plasma compared to MoDC from adult horses ($P = 0.0001$). Foal and adult horse MoDC exposed to plasma (from either foals or adult horses) expressed more CD86, but less CD14 than cells cultured in serum-free medium alone ($P < 0.05$). Cortisol induced a significant decrease in the percentage of MoDC expressing MHC class-II and

CD86 from both foals and adult horses ($P < 0.05$). Adult horse and foal MoDC exposed to bacteria in the presence of 1-day-old foal plasma, or cortisol produced decreased quantities of IL-10 ($P < 0.05$). Endogenous production of IL-17 by MoDC from foals at one day of age was increased compared their culture with adult plasma ($P = 0.004$). Endocytosis was not significantly affected by cortisol exposure ($P > 0.05$). Phagocytosis of labeled *Staphylococcus aureus* was reduced when MoDC generated from foals or adult horses were exposed to foal plasma from day 1 or 30 of age ($P < 0.05$). Plasma exposure increased macropinocytosis of labeled-ovalbumin by MoDC derived from both foals and adult horses ($P < 0.05$).

Conclusions: MoDC function is different for foals than adult horses when exposed to adult plasma or foal plasma collected at the time of MoDC generation. Cortisol appears to be an important factor involved in modulating foal MoDC maturation, based on altered phenotype and function. No other specific factors capable of differentially regulate MoDC phenotype or function in either foal or adult horse plasma were defined during this experiment.

INTRODUCTION

Bacterial sepsis is the most frequent cause of foal mortality within the first weeks of life and results in substantial economic losses in the equine industry.[1,2] The bacterial species most frequently implicated in neonatal sepsis are environmental, endogenous organisms,[1] This suggests that the immunologic defenses of the neonatal foal are impaired to some degree during the first few weeks of life. Predisposition to infection in neonates has been described in numerous species, including human infants,[3,4] calves,[5] and swine.[6] This state of susceptbility is attributed to deficiencies in innate and adaptive immune responses during the neonatal period.

Dendritic cells (DC) are of substantial importance in the initiation of both the innate and the adaptive immune responses. Specifically, the scope of cytokines DC produce, and by how efficiently they present antigen and shape the subsequent adaptive response. Interaction of DC with pathogens stimulates the complex process of DC maturation that is required for effective priming of naïve T lymphocytes. Thus, appropriate DC maturation and function are of particular importance in neonates.[7-9] Regulation of DC maturation and function in neonatal foals is not well understood.

The cellular microenvironment in the neonate, which include soluble factors in the tissue environment – some of which are derived from plasma, is important in regulating immune cell function. A variety of soluble components found in plasma have been implicated in governing immunological function in neonates and adults. These include: the type and quantity of cytokines transferred with colostrum or produced by innate immune cells, endogenous and induced expression of cytokine receptors, the availability and induction of specific antibody, the circulating levels and *de novo* induction of a number of hormones and lipids, and the available quantities of vitamins (primarily as co-factors).[4,10] One widely studied and important immune modulating factor found in plasma and tissue fluids is the steroid hormone, cortisol. The immune regulatory functions of cortisol have been extensively described.[11] While cortisol acts to limit damage to the host by inhibition of the production of proinflammatory cytokines, excessive cortisol production can lead to immunosuppression and perpetuation of infection.[11,12] Cortisol availability and metabolism is very different in neonates and adults.[13] Recent studies have also documented substantial differences in free and bound cortisol in the circulation of foals and adult horses. [14] Differences in cortisol availability in foals relative to adult horses parallels the timing of age-related immune impairment in foals.[14] Studies in multiple species have demonstrated impaired DC maturation and altered function of DC following exposure to corticosteroids.[15-18] To date, there are no studies that have investigated the impact of generic foal or adult horse plasma, or of cortisol, on the maturation and function of equine neonatal monocyte-derived DC (MoDC).

The objective of this study was to assess the effect of generic plasma (foal or adult horse), and cortisol on the maturation and function of MoDC derived from foals or adult horses during exposure to killed bacteria. We hypothesized: 1) exposure of MoDC to killed bacteria in the presence of generic foal plasma will impact MoDC maturation and function differently relative to the effect of generic adult horse plasma; and 2) exposure of MoDC to cortisol during bacterial exposure will impair maturation and function of MoDC from both foals and adult horses.

MATERIALS AND METHODS

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-owned animals prior to enrollment. Due to the limited quantity of blood that could be safely collected to generate MoDC from foals, the objectives of this study were investigated in two separate experiments. In part 1, the effect of plasma source (foal or adult horse) on MoDC derived from foal or adult horse blood monocytes was investigated. In part 2, the effect of cortisol on MoDC derived from foals or adult horses blood monocytes was evaluated. For part 1, 8 healthy adult mixed breed horses (4 geldings, 4 mares, age 6 to 16 years) and 8 healthy Quarter horse foals (4 colts, 4 fillies) were randomly paired using a random list generator to match each foal to an adult horse. Foal-adult horse pairs were sampled on the same day. This occurred when foals were 1 and 30 days of age. For part 2, 9 healthy adult mixed-breed horses (6 males, 3 females, 2 to 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) and born by unassisted vaginal delivery. Adequate transfer of passive immunity (IgG > 800 mg/dL) in foals at 1 day of age was confirmed prior to enrollment.

Monocyte-derived dendritic cell (MoDC) generation, culture and stimulation

Part 1: Effect of Plasma: Approximately 180 mL blood was collected via direct jugular venipuncture into syringes containing 1.5 mL of 100 mM EDTA from each adult horse and from each foal, when foals were 1 and 30 days of age. MoDC were derived peripheral blood mononuclear cells (PBMC) as previously described.[19] Briefly, PBMC were isolated from leukocyte rich plasma by single-step density centrifugation using 1.077 – 1.088 lymphocyte separation medium (LSM; Corning, Corning, NY, USA).[20] The plasma layer was collected and stored at 4°C for use later in this study. The interface layer between the plasma and LSM containing PBMC was collected for MoDC generation. PBMC were plated at 5 x 10^6 cells/mL in 6-well plates (Nunc ®, Thermo Fisher, Waltham, MA, USA) with serum-free medium (AIM-V, Thermo Fisher, Waltham, MA, USA) supplemented with 2.5 µg/mL amphotericin B (Gibco, Thermo Fisher, Waltham, MA, USA) and 2mM L-glutamine (Corning, Corning, NY, USA). Cells were incubated at 37°C with 5% CO_2. After 4 hours, the non-adherent cells and medium were removed. Fresh serum-free medium and recombinant equine IL-4 (reIL-4; 10 ng/mL, R &

D Systems, Minneapolis, MN, USA) and recombinant equine GM-CSF (reGM-CSF; 50 ng/mL; Kingfisher Biotech, Minneapolis, MN, USA) were added to the adherent monocytes. These monocytes were then incubated as above for 4 days for MoDC generation. After 48 hours in culture, 100% of the medium was removed and the wells were divided into 3 treatment conditions: 1) 100% serum-free medium (control), 2) 50% serum-free medium and 50% foal plasma, or 3) 50% serum-free medium and 50% adult horse plasma. The paired adult horse and foal plasma collected during PBMC isolation was used as the source of plasma for each foal-adult horse pair. Fresh reIL-4 (10 ng/mL, R & D Systems, Minneapolis, MN, USA) and reGM-CSF (50 ng/mL; Kingfisher Biotech, Minneapolis, MN, USA) were added to all wells to induce MoDC generation, in addition to heparin (2 U/mL) to prevent plasma coagulation. Some wells were also exposed to heat-killed and lightly sonicated bacterial whole cell preparations of *Staphylococcus aureus* (200 µL of 1:50 dilution) or *Escherichia coli* (200 µL of 1:200 dilution).[19] 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.[21,22] Negative control wells contained cells in serum-free medium alone.

Part 2: Effects of Cortisol: Approximately 120 mL blood was collected via direct jugular venipuncture into syringes containing 1.5 of 100 mM EDTA from each adult horse once, and from each foal at 1, 7, and 30 days of age. PBMC were isolated and MoDC generated as described in part 1 with the following exceptions: 1) after 48 hours in culture fresh serum-free medium and cytokines (reGM-CSF and reIL-4) were added to all wells; 2) treatment wells were exposed to cortisol (15 µg/dL) and 3) some wells were also exposed to heat-killed and briefly sonicated whole-cell preparations of *Staphylococcus aureus* or *Escherichia coli.* The concentration of cortisol used was previously determined by our laboratory to be similar to the mean plasma concentration recorded in foals with naturally-occurring sepsis.[23] Negative control wells contained cells in serum-free medium.

Part 1 and Part 2: MoDC maturation assessment via flow cytometric phenotype analysis

On day 4 of culture, adherent and non-adherent MoDC were harvested. A cell scraper (VWR, Randor, PA, USA) was used to gently remove adhered cells and viability was assessed using 0.04% trypan blue exclusion. Viability of both unstimulated MoDC and those exposed to bacterial whole cell preparations was documented. Viability for adult unstimulated and bacteria exposed MoDC from part 1 was 85% (± 7.3) and 81% (± 6.0) respectively, and 95% (± 3.5) and 94% (± 5.3) for part 2. Viability of unstimulated and bacteria exposed foal MoDC was 82% (± 7.9) and 83% (± 7.9) for part 1 and 87% (± 7.5) and 82% (± 9.1) for part 2. MoDC phenotype was used to classify the stage of MoDC maturation using a similar approach as previously described.[19] 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 (AbD Serotec, Hercules, CA USA), 10 µL Alexa 647-conjugated mouse anti-horse CD14 at a 1:100 dilution (clone 105, Wagner Laboratory, Cornell University, Ithaca, NY, USA) and 40 µL undiluted PE-conjugated mouse anti-human CD86 (BD Bioscience, San Jose, CA, USA). All antibodies were assessed for the minimum saturation binding dilution and to minimize cross-color masking prior to this study (data not shown). Cells were analyzed using an Accuri C6 Flow Cytometry (BD Bioscience, San Jose, CA, USA) with BC C6 data acquisition and analysis software. The percentage of cells positive (those showing florescent signal) for each surface marker and the median fluorescent intensity (MdFI) for each surface marker was recorded. To standardize the analysis of cellular phenotype, singlet gating (forward angle height vs forward angle area) was utilized as a primary gating tool and is shown in Figure 5.1A as P1. Further, forward angle vs side scatter signal was used to define two distinct populations of cells from the cultures and is shown in Figure 5.1B. The smaller population (P2) represents the more immature MoDC, and the larger population (P3) represents the more uniform more mature MoDC from culture (Figure 5.1B). These gates served as the guide to phenotypic analysis.

Functional assays

Cytokine quantification. Prior to harvesting MoDC for surface marker quantification, supernatants from all wells were collected and stored at -80°C. 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, Ithaca, NY, USA).[24] These specific cytokines were selected to provide insight into the possible polarization of subsequent DC-induced adaptive responses.

Macropinocytosis and Phagocytosis assays. Maturation of MoDC results in a decrease in antigen uptake processes and is a protective mechanism to prevent undesirable immune activation to self-antigens. In this study, we assessed the effect of foal plasma, adult horse plasma, or cortisol on the macropinocytic and phagocytic capacity of immature MoDC. Thus, the most immature MoDC population (P2,) as previously determined by our laboratory, (See Lopez et al., in preparation – chapter 3) was used for this assessment (Figure 5.1B). On day 4 of culture, $1x10^5$ MoDC were incubated in 96-well round bottom plates with 50 µL of either FITC-conjugated ovalbumin (5 µg/mL; Molecular Probes, Eugene, OR, USA), bodipylated *S. aureus* (5 µg/mL; Molecular Probes, Eugene, OR, USA), or bodipylated *E. coli* (10 ug/mL; Molecular Probes, Eugene, OR, USA) for 4 hours at 37°C with 5% CO_2. For part 1, MoDC generated in serum-free media or MoDC exposed to foal plasma or adult horse plasma were assessed. For part 2, MoDC generated in serum-free medium or those exposed to cortisol, but not bacteria, were assessed. After the incubation period, 40µL of 0.4% trypan blue (10% of sample volume) 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.

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 was log transformed to achieve normality. Part 1 & 2 surface marker data, endocytosis data and cytokine data for IL-10 (part 1 and 2), and IL-17 (part 2) were analyzed using linear mixed-effects models with horse modeled as a random effect to account for repeated measurements, and age, bacterial stimulation, and presence of cortisol or plasma were modeled as fixed effects. Model fit was assessed using Akaike's information criterion values. Significant portions of the cytokine data for both part 1 and part 2 were below the limit of detection for the assay. This data was analyzed using a Tobit regression to account for this. Thus, IL-17 (part 1), IL-4 (part 1 and 2) and IFN-γ (part 1 and 2) were analyzed using a Tobit regression model with random and fixed effects modeled as described above. Three-way interactions were evaluated for both part 1 and part 2. 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.

RESULTS

Part 1: Effect of plasma source on MoDC maturation

The effect of plasma source on MoDC surface marker expression is shown in Tables 5.1 and 5. 2. A significantly lower fraction of MoDC derived from foals at 1 day of age expressed the surface markers MHC class-II and CD86 compared to MoDC derived from adult horses ($P = 0.0001$). At 30 days of age, the percent of MoDC from foals expressed MHC class-II ($P = 0.12$) and CD86 ($P = 0.07$) at a similar percent of cells as MoDC derived from monocytes of adult horses collected on that same day. The median density of CD86 expression was decreased on MoDC derived from foals on both day 1 and 30 of age compared to MoDC from adult horses collected o the same age ($P = 0.005$). These differences were observed for MoDC generated in serum-free medium, and when exposed to either source of plasma collected on the same day. There was no significant effect of different sources of plasma on the fraction of MoDC expressing MHC class-II, CD86, or CD14, or on the median density of the expression of these surface markers by MoDC from foals or adult horses collected on the same day. There was a difference in the surface marker expression when MoDC were cultured in serum-free medium compared to plasma from foals or adult horses on the day of collection. The percentage of foal or adult horse MoDC expressing CD86 ($P = 0.02$), and the median density ($P = 0.006$) of its expression was increased when MoDC were exposed to plasma (foal or adult horse) compared to those cultured in serum-free medium. In addition the fraction of MoDC from foals and adult horse expressing CD14 ($P = 0.0001$) was decreased when MoDC were exposed to plasma from either foals or adult horses relative to those cultured in serum-free medium.

A difference in both foal and adult horse surface marker expression by MoDC was also observed for cells generated from blood samples collected when foals were 1 day of age compared to those generated when the foals were 30 days of age. The percentage of MoDC from adult horses expressing MHC class-II and CD86, and the median density of MHC class-II and CD86 expression was increased when MoDC from adult horses derived from blood monocytes collected when foals were 1 day of age was compared to MoDC from adult horses derived when foals were 30 days of age This was also observed with MoDC from foals generated when foals were 1 day of age compared to 30 days of age ($P < 0.05$).

Part 1: Effect of plasma source on MoDC function

The effect of the source of plasma, from either foals or adult horses, on cytokine production by foal and adult horse derived MoDC is presented in Figure 5.2. There was no significant effect of the source of plasma on the production of IFN-γ or IL-4 by MoDC derived from adults or foals. When MoDC were generated from monocytes from foals or adult horses collected when foals were 1 day of age, the endogenous (basal production by MoDC under control conditions) and induced production (after exposure to killed bacteria) of IL-10 by MoDC from both foals and adult horses was significantly reduced when exposed to foal plasma collected at day 1 of age compared to adult plasma collected on the same day ($P = 0.0008$). When MoDC were generated from monocytes from foals or adult horses collected when foals were 30 days of age, the endogenous production of IL-10 was increased by exposer to 30-day-old foal plasma relative to exposure to adult plasma collected on the same day ($P = 0.01$).

Endogenous and induced production of IFN-γ, IL-4, and IL-10 by MoDC from either foals or adult horses were not different when exposed to plasma from either adult horses or foals compared to culture in serum-free medium. Endogenous and induced production of IL-17 by MoDC derived from foals and adult horses generated in serum-free medium was significantly different than after exposure to adult horse plasma ($P = 0.004$). The endogenous production of

IL-17 was increased when MoDC derived from foals at 1 day of age, were cultured in adult horse plasma compared to MoDC from foals generated in serum-free medium. However, for 30 day old foals, endogenous production of IL-17 was reduced when MoDC were exposed to adult horse plasma compared to serum-free medium. For adult horses, the production of IL-17 by MoDC derived from blood monocytes at either sampling time points was reduced when cells were exposed to adult plasma collected at the sample time point compared to serum-free medium.

Part 1: Effect of plasma source on MoDC macropinocytosis and phagocytosis

The effects of exposure to foal or adult horse plasma on MoDC macropinocytosis and phagocytosis are presented in Figure 5.3. Cells within the immature MoDC gate (P2, Figure 5.1B) were assessed. The percentage of foal and adult horse MoDC that took up FITC-ovalbumin was increased when MoDC were exposed to either foal or adult plasma relative to serum-free medium ($P < 0.05$). The quantity (as indicated by the MFI) of FITC-ovalbumin taken up was increased for MoDC in plasma that were generated when foals were 1 day of age compared to cells generated that same day exposed only to serum-free medium ($P < 0.05$). However, MoDC that were generated from blood monocytes collected from foals at 30 days of age, and exposed to foal and adult plasma, took up less FITC-ovalbumin compared to serum-free medium ($P < 0.05$). The percentage of MoDC from foals and adult horses MoDC that took up *S. aureus,* and the quantity (MFI) of bacteria taken up was increased in cells exposed to plasma from adult horses relative to those exposed to day 1 or 30 foal plasma ,or medium alone ($P < 0.05$). No statistically significant difference of medium alone, foal plasma, or adult horse plasma on foal or adult horse MoDC phagocytosis of bodipylated *E. coli* was observed in these studies.

Part 2: Effect of cortisol on MoDC maturation

The effect of cortisol exposure on MoDC maturation is presented in Tables 5.3 and 5.4. In MoDC, derived from both foals and adult horses, exposure to cortisol significantly decreased the fraction of cells expressing MHC class-II and CD86 whether the MoDC were exposed to the killed bacteria or not ($P < 0.05$). The median density of MHC class-II and CD86 expression by MoDC from foals and adult horses was not different following exposure to cortisol ($P > 0.05$). There was no significant effect of cortisol on the percentage of cells expressing CD14 or the surface density of MHC class-II, CD86 or CD14 expression by MoDC derived from foals or adult horses.

Part 2 Effect of cortisol on MoDC function

The effect of cortisol on MoDC function is presented in Figure 5.4. Concurrent cortisol exposure induced significantly altered cytokine production by MoDC from foals and adult horses that were also exposed to bacteria. MoDC from adult horses that were exposed to *E. coli* in the presence of cortisol produced significantly less IFN-γ, IL-4 and IL-17 compared to MoDC from adult horses exposed to *E. coli* without cortisol ($P < 0.05$). Adult horse MoDC exposed to *S. aureus* in the presence of cortisol demonstrated reduced production of IFN-γ and IL-10 compared adult horse MoDC exposed to *S. aureus* without cortisol ($P < 0.05$). Similar to MoDC from adult horses, the production of IL-10 was reduced in foal MoDC exposed concurrently to *S. aureus* and cortisol ($P = 0.009$), and IL-4 production was reduced by MoDC from foals exposed to *E. coli* and cortisol ($P = 0.009$). Cortisol exposure did not significantly alter production of IFN-γ or IL-17 by MoDC from foals when collected on day 1 or 30.

Part 2: Effect of cortisol on MoDC macropinocytosis and phagocytosis

Macropinocytosis and phagocytosis activity for foal and adult horse MoDC exposed to cortisol is presented in Figure 5.5. As in part 1, cells within the immature MoDC gate (P2, Figure 5.1B) were assessed. There was no significant effect of cortisol exposure on MoDC macropinocytosis of FITC-conjugated ovalbumin, or the phagocytosis of bodipylated *S. aureus* or bodipylated *E. coli* by MoDC from foals or adult horses.

DISCUSSION

These data, generated under conditions that simulate some elements of the tissue environment, provide support for our hypotheses that exogenous factors, including those found in generic plasma, and cortisol are able to augment the maturation and function foal MoDC in culture. Our results suggest that cortisol locks equine MoDC into a state of phenotypic immaturity. This was demonstrated by a reduced fraction of both foal and adult horse MoDC that express MHC class-II and CD86 after exposure to cortisol. Similar results demonstrating glucocorticoid (GCC)-induced impairment of DC differentiation and maturation have been documented for DC from humans and mice.[15,17,25-30] In this study, plasma exposure impacted foal and adult horse MoDC phenotype. MoDC exposed to either foal or adult horse plasma expressed more CD86, but less CD14, compared to MoDC generated in serum-free medium without plasma. This finding indicates that soluble factors in plasma may play a role in supporting MoDC differentiation from monocytes to immature DC, and in enhancing MoDC maturation *ex vivo*. Culturing MoDC in serum-free medium allows for complete assessment of intrinsic cellular limitations, but immune responses do not occur *in vivo* under the isolated conditions created by this culture system. Although exposure of MoDC to plasma appears to partially mature MoDC in culture, this model it is a better representation of MoDC generation *in vivo* and is a relevant model for making general conclusions regarding the impact of soluble plasma factors on MoDC phenotype and function. No differential effect of the source of plasma utilized – plasma from foals on day 1 or 30 of age or from adult horses – on the expression of surface markers by MoDC from foals was observed in this study. However, MoDC generated from foal monocytes, in serum-free medium or exposed to any source of plasma, did express less MHC class-II and CD86 compared to MoDC from adult horses collected at the same time point. This result is in agreement with previous work by our laboratory demonstrating inherent age-related changes in foal MoDC phenotype.[19]

It must be stressed that in the study presented here, MoDC from adult horses were intended to be used as an invariant control to allow comparison with MoDC from foals with respect to phenotype and function. Our results showed a statistically significant different in adult horse MoDC phenotype when MoDC were generated from monocytes collected when foals were 1 or 30 days of age. This suggests that uncontrolled environmental or assessment factors impacted the family of measurements collected in this study. The finding of significant differences among the MoDC phenotype of adult horses was unexpected. Therefore, our ability to fully assess intrinsic differences in the maturation of MoDC phenotype as foals age, and between adult horses and foals, was limited. We were placed in a position of making comparisons between adult horse MoDC and MoDC derived from foal monocytes at 1 and 30 days of age, with the caveat that some detected differences in MoDC phenotype could have been induced by environmental and laboratory factors beyond our control rather than solely due to age differences. However, as foal and adult cells from each pair of animals were collected and processed in the same location and on the same day, time-dependent environmental and laboratory conditions likely impacted foal and adult cells similarly. The greatest disparity observed with time in our system was in phenotype, consistent with slightly different levels of MoDC maturation in culture on different sample collection days. We believe, that the observed changes in foal MoDC phenotype at the different collect time points likely reflects real age-related changes. Despite comparisons made between adult horse and foal MoDC that were generated on the same day and under the same culture conditions, absolute conclusions regarding intrinsic limitations in foal MoDC phenotype cannot be made definitively drawn from this study. While the results of this study suggest that soluble factors, including cortisol, impact MoDC phenotype *ex vivo*, additional studies are necessary to confirm our finding about inherent cellular limitations in foal MoDC phenotype.

Results from the study presented here indicate that soluble factors, and cortisol impact *ex vivo* production of cytokines by neonatal foal MoDC. This was demonstrated by increased production of IL-17 by neonatal foal MoDC that were exposed to adult plasma and decreased production IL-10 by neonatal foal MoDC that were exposed to day 1 neonatal foal plasma, or to cortisol. In contrast to our results, other studies have demonstrated increased IL-10 production by human cord blood and adult PBMC after exposure to cord blood plasma[10] and by human MoDC[14,17] and monocytes[34,35] in response to GCC. The differences observed utilizing the human models and our study might reflect species differences, difference in the assessment of cell function, or differences in the protocol utilized to test DC responses to cortisol or plasma. Dose-dependent effects of GCC on cytokine responses could account for the differences observed among these studies, and have been previously described for human whole blood culture models or human PBMC.[37] Studies assessing MoDC responses to a range of both cortisol doses and plasma concentrations are needed to more fully understand the role of soluble factors in modulating DC maturation and function. Our results suggest that the availability and/or quantity of soluble plasma factors necessary to alter IL-17 and IL-10 production by foal MoDC reflect a difference between foals and adult horses. Further, cortisol appears to impact MoDC production of IL-10. Imbalance in the production of the anti-inflammatory cytokine IL-10,[31,32] and the pro-inflammatory cytokine IL-17 by foal MoDC could impact foal immunity against bacterial infection.[33]

Our laboratory has previously detailed altered hypothalamic-pituitary-adrenal (HPA) axis responses in foals compared to adult horses.[18,38,39] Specifically, neonatal foals have significantly greater circulating free cortisol than adult horses.[18] Despite their increased free cortisol concentrations, critically ill foals frequently have clinical signs suggestive of cortisol insufficiency, including refractory hypotension.[40-42] This suggests that foal cells and tissues may exhibit some degree of tissue cortisol resistance, or impaired cortisol signaling. In our study, the production of IL-17 and IFN-γ by foal MoDC was not altered in cortisol exposure, unlike adult MoDC that demonstrated reduced production when concurrently exposed to cortisol and killed bacteria. However, endogenous production of IL-17 and IFN-γ by foal MoDC was below the limit of detection for the assay for many samples. This prevented us from assessing changes in production of these cytokines by foal MoDC in response to cortisol. Thus, the possibility that cortisol modulates cytokine production in foals differently than adult horses cannot be ruled-out. Assays of greater sensitivity or assessment of effect on additional cytokines would be needed to address this question.

In this study, we demonstrated that exposure to cortisol did not alter MoDC endocytosis. This is in disagreement with some previous evaluations of the impact of cortisol on bacterial phagocytosis in foal neutrophils.[20] This could be due to differences in the concentration or type of corticosteroid utilized, or due to inherent differences in cortisol activity on MoDC relative to neutrophils. Our results did demonstrate that soluble factors in plasma enhance *ex vivo* phagocytosis and macropinocytosis activity by equine MoDC. In agreement with previous studies showing deficient phagocytosis by equine PBMC and neutrophils in the presence of foal serum,[42-45] our studies showed significantly reduced MoDC phagocytosis of *S. aureus* when foal plasma was used. This could be explained by decreased circulating concentrations of humoral opsonins (e.g. complement C3) in foal serum, and has been demonstrated in other published work.[44,46] While no effect of foal plasma was observed with MoDC during exposure to *E. coli*, other studies have documented that foal neutrophil phagocytosis is dependent upon the optimal combination of both the quantity of serum (or components) and the number of bacteria *in vitro*. Thus, it is possible that our culture conditions, in addition to our small sample size, prevented us from detecting a relevant effect of foal plasma on MoDC phagocytosis of *E. coli.* While additional studies defining the specific factors in foal plasma involved in modulating phagocytosis are needed to fully understand the decreased antigen uptake by foal MoDC in foal plasma, we postulate that reduced antigen up take may compromise the ability of foals to stimulate an appropriate T cell response to microbial antigen. Ultimately, this could limit the ability of foal to manage and clear microbial infection during the neonatal period.

The primary limitation of this study was the use of generic foal and adult horse plasma that contained undefined factors of unknown quantity. Further, *in vivo,* MoDC are generated following recruitment of monocytes into tissues at the site of insult or invasion. The soluble factors in foal and adult horse plasma used in this model are not a complete representation of the soluble factors in the inflammatory tissue microenvironment. During inflammation and infection increases in vascular permeability do allow larger proteins within the vasculature to escape into the peripheral tissues. Thus, soluble factors represented as plasma in this study might still provide valuable information for evaluating MoDC maturation and function during infection.

Another limitation of this study was the inability to use pooled plasma to provide a uniform composition of generic plasma. Pooled plasma would have allowed a still undefined, but constant, source of factors throughout the studies. We initially intended to utilize pooled plasma to limit intra-horse variability between plasma samples, but clotting of the medium following the addition of frozen and thawed pooled plasma in developmental trials resulted in the inability to sustain MoDC cultures. This has been problematic in other similar studies using autologous plasma.[48] As result our experimental design was refined and we randomly paired adult horses with foals for sampling and generated MoDC were exposed to fresh plasma from the corresponding foal-adult horse pair. Utilizing fresh plasma and heparin, clotting of the medium was not observed. A consequence of pairing adult horses with foals was a decrease in our statistical power. Thus, we had less of a chance to detect a significant effect of different source of generic plasma (foal or adult horse) on MoDC maturation and function. Heparin was added to the fresh generic plasma utilized to prevent clot formation during culture. Heparin at concentrations of 50 U/mL has been shown to modulate human MoDC maturation and function.[49] In our study only 2 U/mL heparin was used to minimize any heparin-related effects, but no specific assessment of the impact of 2 U/mL heparin was conducted. Heparin was added to control wells (serum-free medium) during the studies that assessed the effects of generic plasma, to minimize the impact of heparin on our data of interest.

In summary, the data presented here suggest that soluble factors in foals, modeled as foal plasma, impact function of MoDC derived from adult horses and foals differently than soluble factors in adult horses, modeled as adult horse plasma. Cortisol, an important immune regulating soluble factor with different availability between adult horses and foals, modulates MoDC cytokine production *ex vivo*. Reductive studies to assess the impact of specific soluble factors involved in modulating MoDC function are needed. A greater understanding of the role of cortisol and soluble factors, currently undefined, on regulation and polarization of the adaptive immune response and their impact on the incidence of disease early in life are required.

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Table 5.1. Percentage (mean ± standard deviation) of monocyte-derived dendritic cells (MoDC) from adult horses (n=8) or foals (n=8) collected when foals were 1 and 30 days of age, expressing MHC class-II, CD86, and CD14 in the presence or absence of generic paired sources of adult or foal plasma, or during exposure to killed *Escherichia coli or Staphylococcus aureus*. *Denotes a significant difference in foal MoDC and adult horse MoDC surface marker expression that were isolated and generated the same day and under the same culture conditions. $^{+}$ Denotes a significant difference between MoDC cultured in either serum-free medium alone (negative control) or adult horse or foal plasma alone. ^{a,b}Different letter superscripts denote significant differences in surface marker expression between MoDC generated from monocytes day 1 or day 30 within each age group. ($P < 0.05$)

Cell Population (%)	**Foals Day 1**	**Adult horses Day 1**	**Foals Day 30**	**Adult horses Day 30**
	Mean (%) ± SD	Mean (%) ± SD	Mean (%) ± SD	Mean (%) ± SD
MHC class-II				
Medium alone	$32.2 \pm 16.0^{*, a}$	54.9 ± 19.6^{a}	64.5 ± 19.2^{b}	74.3 ± 10.7^{b}
Adult plasma	$27.5 \pm 17.6^{*, a}$	67.5 ± 19.4^{a}	65.9 ± 17.7^{b}	79.7 ± 9.0^{b}
Foal plasma	$31.6 \pm 20.1^{*, a}$	61.4 ± 22.3^{a}	66.6 ± 14.0^{b}	79.9 ± 8.7^{b}
Adult plasma *S. aureus*	$23.3 \pm 18.2^{*, a}$	60.6 ± 23.7^{a}	66.3 ± 20.3^{b}	72.9 ± 13.3^{b}
Foal Plasma *S. aureus*	$30.2 \pm 23.3^{*, a}$	57.5 ± 32.2^{a}	61.5 ± 11.2^{b}	72.4 ± 14.4^{b}
Adult plasma *E. coli*	$25.2 \pm 18.3^{*, a}$	58.7 ± 29.2^{a}	59.1 ± 22.9^{b}	64.7 ± 17.2^{b}
Foal Plasma *E. coli*	$28.9 \pm 19.8^{*, a}$	52.5 ± 34.2^{a}	61.9 ± 15.2^{b}	70.4 ± 16.1^{b}
CD86				
Medium alone	$16.4 \pm 9.3^{*, +, a}$	$41.2 \pm 19.9^{+, a}$	$42.5 \pm 11.4^{+, b}$	$40.8 \pm 9.6^{+, b}$
Adult plasma	$17.7 \pm 12.4^{*, a}$	50.5 ± 21.0^{a}	48.2 ± 12.5^{b}	57.4 ± 14.6^{b}
Foal plasma	$21.1 \pm 13.6^{*, a}$	51.1 ± 17.9^{a}	45.3 ± 13.9^{b}	55.3 ± 15.0^{b}

Adult plasma *S. aureus*	14.5 ± 13.3 $^{*, a}$	49.0 ± 22.0 ^{a}	44.8 ± 18.5 ^{b}	55.7 ± 16.1 ^{b}
Foal Plasma *S. aureus*	16.8 ± 11.6 $^{*, a}$	50.9 ± 17.6 ^{a}	47.6 ± 14.9 ^{b}	56.2 ± 15.3 ^{b}
Adult plasma *E. coli*	21.9 ± 12.0 $^{*, a}$	50.5 ± 21.9 ^{a}	50.7 ± 16.1 ^{b}	59.6 ± 15.5 ^{b}
Foal Plasma *E. coli*	21.1 ± 9.2 $^{*, a}$	52.2 ± 18.1 ^{a}	46.7 ± 11.5 ^{b}	57 ± 12.6 ^{b}
CD14				
Medium alone	17.2 ± 9.0 $^{+}$	8.8 ± 4.2 $^{+}$	10.5 ± 6.5 $^{+}$	8.6 ± 3.5 $^{+}$
Adult plasma	2.8 ± 1.5	3.1 ± 1.7	3.3 ± 1.7	3.1 ± 1.5
Foal plasma	2.4 ± 1.3	2.6 ± 1.2	2.7 ± 0.9	2.8 ± 1.8
Adult plasma *S. aureus*	2.3 ± 1.0	2.2 ± 1.3	2.5 ± 1.5	2.3 ± 1.3
Foal Plasma *S. aureus*	1.6 ± 0.4	2.5 ± 1.3	1.9 ± 1.3	2.8 ± 2.0
Adult plasma *E. coli*	2.2 ± 1.1	2.6 ± 1.2	2.8 ± 1.4	2.6 ± 1.9
Foal Plasma *E. coli*	2.0 ± 0.9	2.6 ± 1.0	2.6 ± 1.3	2.3 ± 1.3

Table 5.2. Median fluorescence intensity (MdFI; mean ± standard deviation) of monocyte-derived dendritic cells (MoDC) derived from adult horses (n=8) or foals (n=8) collected when foals were 1 and 30 days of age, expressing MHC class-II, CD86, and CD14 in the presence of generic paired sources of adult or foal plasma or during exposure to killed *Escherichia coli or Staphylococcus aureus*. *Denotes a significant difference in foal MoDC and adult horse MoDC surface marker expression that were isolated and generated the same day and under the same culture conditions. $^{+}$ Denotes a significant difference between MoDC cultured in either serum-free medium alone (negative control) or adult horse or foal plasma alone. ^{a,b}Different letter superscripts denote significant differences in surface marker expression between MoDC generated from monocytes collected at day 1 or day 30 within each age group. (P < 0.05)

Cell Population (MdFI)	**Foals Day 1**	**Adult horses Day 1**	**Foals Day 30**	**Adult horses Day 30**
	Mean (MdFI) ± SD	Mean (MdFI) ± SD	Mean (MdFI) ± SD	Mean (MdFI) ± SD
MHC class-II				
Medium alone	2828 ± 455^{a}	2370 ± 625^{a}	3961 ± 1884^{b}	3746 ± 1384^{b}
Adult plasma	2873 ± 935^{a}	3550 ± 1914^{a}	4146 ± 1334^{b}	4120 ± 1218^{b}
Foal plasma	3147 ± 679^{a}	3052 ± 1584^{a}	4100 ± 620^{b}	4180 ± 673^{b}
Adult plasma *S. aureus*	3067 ± 2228^{a}	2626 ± 971^{a}	3454 ± 963^{b}	3309 ± 868^{b}
Foal Plasma *S. aureus*	2960 ± 630^{a}	3284 ± 1712^{a}	3431 ± 294^{b}	3556 ± 549^{b}
Adult plasma *E. coli*	2653 ± 670^{a}	3070 ± 1501^{a}	3486 ± 1350^{b}	3099 ± 752^{b}
Foal Plasma *E. coli*	2892 ± 570^{a}	3042 ± 1519^{a}	3544 ± 787^{b}	2929 ± 1048^{b}
CD86				
Medium alone	$1164 \pm 297^{*,+,a}$	$1261 \pm 469^{+,a}$	$1572 \pm 466^{*,+,}$	$1540 \pm 497^{+,b}$
Adult plasma	$1130 \pm 282^{*,a}$	1567 ± 528^{a}	$1712 \pm 379^{*,b}$	2102 ± 458^{b}
Foal plasma	$1145 \pm 301^{*,a}$	1467 ± 482^{a}	$1735 \pm 314^{*,b}$	2140 ± 446^{b}

Adult plasma *S. aureus*	1066 ± 231 [*, a]	1397 ± 391 [a]	1680 ± 327 [*, b]	1974 ± 395 [b]
Foal Plasma *S. aureus*	1126 ± 376 *, [a]	1388 ± 483 [a]	1821 ± 373 *, [b]	2206 ± 419 [b]
Adult plasma *E. coli*	1163 ± 245 *, [a]	1649 ± 623 [a]	2052 ± 789 *, [b]	2020 ± 315 [b]
Foal Plasma *E. coli*	1143 ± 297 *, [a]	1374 ± 452 [a]	1886 ± 614 *, [b]	1997 ± 377 [b]
CD14				
Medium alone	1698 ± 412	1558 ± 334	1609 ± 500	1427 ± 542
Adult plasma	1675 ± 533	2042 ± 601	1883 ± 930	2217 ± 1039
Foal plasma	1551 ± 474	1589 ± 363	2544 ± 1831	1807 ± 560
Adult plasma *S. aureus*	1658 ± 926	1706 ± 556	1639 ± 600	1866 ± 677
Foal Plasma *S. aureus*	1553 ± 618	1610 ± 466	1898 ± 969	1827 ± 611
Adult plasma *E. coli*	1561 ± 463	1915 ± 740	1710 ± 1008	1792 ± 637
Foal Plasma *E. coli*	1479 ± 452	1579 ± 611	1676 ± 811	1705 ± 697

Table 5.3. Percentage (mean ± standard deviation) of monocyte-derived dendritic cells (MoDC) expressing MHC class-II, CD14, and CD86 generated from adult horses (n=9) once and foals (n=8) at 1, 7 and 30 days of age in the presence or absence of cortisol alone or during exposure to killed *Escherichia coli or Staphylococcus aureus*. *Denotes a significant effect of cortisol on MoDC surface marker expression.(P< 0.05)

Cell Population	1 day-old foals	7 day-old foals	30 day-old foals	Adult horses
	Mean (%) ± SD	Mean (%) ± SD	Mean (%) ± SD	Mean (%) ± SD
MHC class-II				
Medium	26.7 ± 8.9	37.7 ±12.6	43.8 ± 16.6	58.5 ± 19.1
Cortisol	19.2 ± 9.0 *	29.1 ± 10.5 *	35.3 ± 14.0 *	51.0 ± 16.9 *
S. aureus	37.1 ± 7.8	41.4 ± 10.2	42.3 ± 15.8	62.2 ± 16.9
S. aureus + Cortisol	30.3 ± 9.8 *	31.5 ± 10.3 *	35.5 ± 16.7 *	57.1 ± 15.4 *
E. coli	34.9 ± 7.7	43.9 ± 10.1	42.0 ± 16.4	61.5 ± 14.3
E. coli + Cortisol	25.8 ± 7.6 *	33.0 ± 9.0 *	38.6 ± 15.6 *	62.5 ± 16.7 *
CD86				
Medium	14.3 ± 7.9	20.5 ± 11.0	19.5 ± 9.6	35.8 ± 15.4
Cortisol	9.8 ± 6.6 *	18.6 ±10.3 *	16.4 ± 7.3 *	30.5 ± 14.4 *
S. aureus	20.0 ± 11.0	25.3 ± 6.8	30.9 ± 9.2	45.8 ± 16.7
S. aureus + Cortisol	11.2 ± 4.8 *	20.5 ± 8.3 *	23.2 ± 8.7 *	33.5 ± 16.0 *
E. coli	16.9 ± 8.1	28.3 ± 8.0	34.8 ± 11.7	48.4 ± 18.6
E. coli + Cortisol	12.8 ± 7.2 *	22.7 ± 8.9 *	27.2 ± 8.3 *	38.3 ± 16.5 *
CD14				
Medium	19.8 ± 13.6	14.3 ± 6.6	12.4 ± 4.6	8.2 ± 3.8
Cortisol	22.7 ± 12.3	16.8 ± 7.6	14.6 ± 5.6	9.5 ± 3.6
S. aureus	13.8 ± 7.5	10.7 ± 5.5	9.3 ± 3.0	8.3 ± 4.9
S. aureus + Cortisol	17.0 ± 13.1	12.9 ± 7.5	8.9 ± 3.3	8.5 ± 4.7
E. coli	18.6 ± 11.2	12.3 ± 2.3	12.0 ± 3.9	7.4 ± 3.0
E. coli + Cortisol	17.5 ± 12.7	15.4 ± 5.7	13.7 ± 4.2	8.9 ± 3.5

Table 5.4. Median fluorescence intensity (MdFI; mean ± standard deviation) of monocyte-derived dendritic cells (MoDC) expressing MHC class-II CD14, and CD86 generated from adult horses (n=9) once and foals (n=8) at 1, 7 and 30 days of age in the presence or absence of cortisol alone or during exposure to killed *Escherichia coli or Staphylococcus aureus*. (P< 0.05)

Cell Population	**1 day-old foals**	**7 day-old foals**	**30 day-old foals**	**Adult horses**
	Mean (MdFI) ± SD	Mean (MdFI) ± SD	Mean (MdFI) ± SD	Mean (MdFI) ± SD
MHC class-II				
Medium	4186 ± 1999	3706 ± 2078	3103 ± 1326	3071 ± 1508
Cortisol	4348 ± 2094	3538 ± 1933	2957 ± 1403	2875 ± 1504
S. aureus	4411 ± 1471	3784 ± 2333	3108 ± 1075	3245 ± 1377
S. aureus + Cortisol	3511 ± 1665	4197 ± 3292	3132 ± 1486	3310 ± 1603
E. coli	4827 ± 2337	4677 ± 3143	2971 ± 1163	3326 ± 1571
E. coli + Cortisol	5802 ± 5533	3888 ± 2632	3145 ± 1638	3384 ± 1724
CD86				
Medium	972 ± 251	1357 ± 453	1165 ± 227	931 ± 154
Cortisol	1009 ± 191	1380 ± 528	1130 ± 206	877 ± 119
S. aureus	995 ± 287	1245 ± 389	1188 ± 205	1090 ± 249
S. aureus + Cortisol	879 ± 220	1207 ± 361	1112 ± 245	929 ± 162
E. coli	1062 ± 286	1366 ± 534	1232 ± 220	1135 ± 201
E. coli + Cortisol	1068 ± 279	1353 ± 442	1150 ± 243	968 ± 162
CD14				
Medium	3112 ± 2573	1765 ± 363	1778 ± 247	1848 ± 392
Cortisol	2983 ± 1966	1804 ± 377	2158 ± 568	1899 ± 385
S. aureus	2575 ± 1802	1710 ± 339	1633 ± 198	1808 ± 372
S. aureus + Cortisol	2408 ± 1522	1884 ± 655	1666 ± 225	1745 ± 769
E. coli	2141 ± 1399	1668 ± 301	1641 ± 197	1849 ± 442
E. coli + Cortisol	1848 ± 761	1753 ± 306	1718 ± 222	1890 ± 382

A) B)

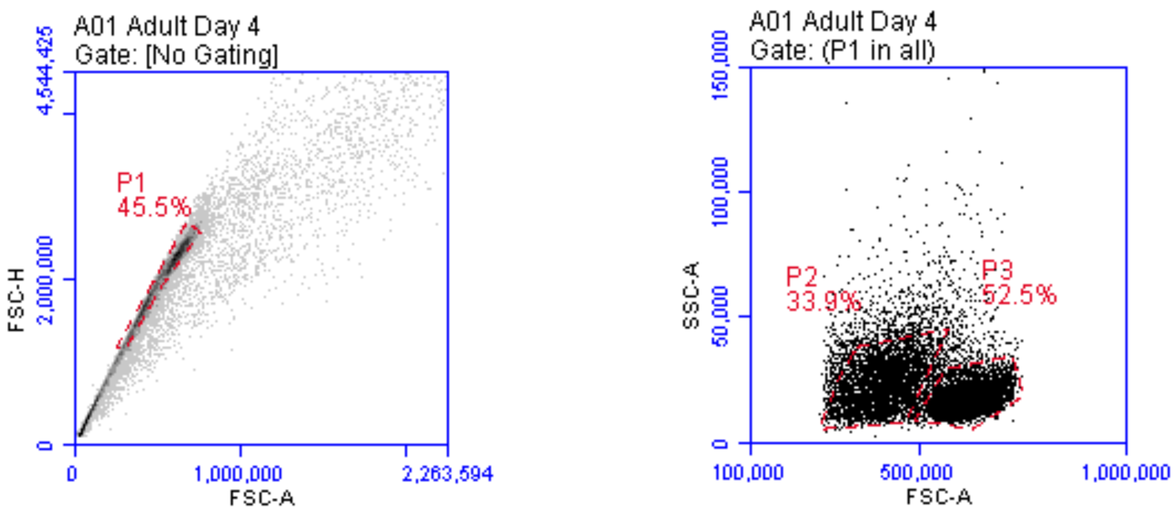

Figure 5.1. Gating of equine MoDC generated from whole blood using a representative sample from an adult horse after 4 days of generation. A) Dot plot of forward scatter-height (FSC-H) versus forward scatter-area (FSC-A) outlining the population of interest (P1). B) Dot plot depicting additional gating 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).

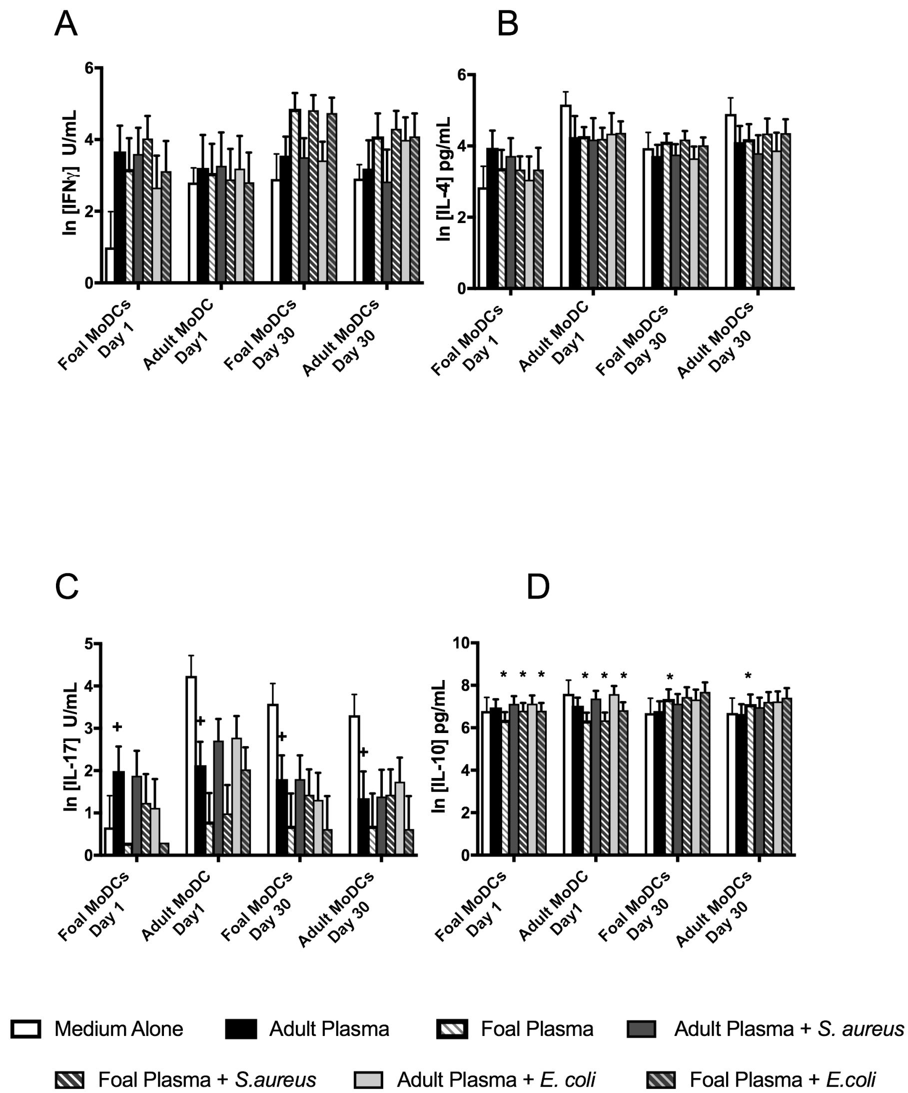

Figure 5.2. Cytokines [(A) IFN-γ, (B) IL-4, (C) IL-17, and (D) IL-10] produced by monocyte-derived dendritic cells (MoDC) generated from adult horses (n=8) and foals (n=8) when foals were 1 and 30 days of age. MoDC were generated in the presence of generic paired source of adult or foal plasma alone or during exposure to killed *Escherichia coli or Staphylococcus aureus*. Data represents least-square means with corresponding standard errors. * Denotes significant differences between plasma source (foal or adult horse) on MoDC cytokine production. +Denotes a significant differences in cytokine production by MoDC generated in serum-free medium or exposed to adult horse plasma ($P < 0.05$)

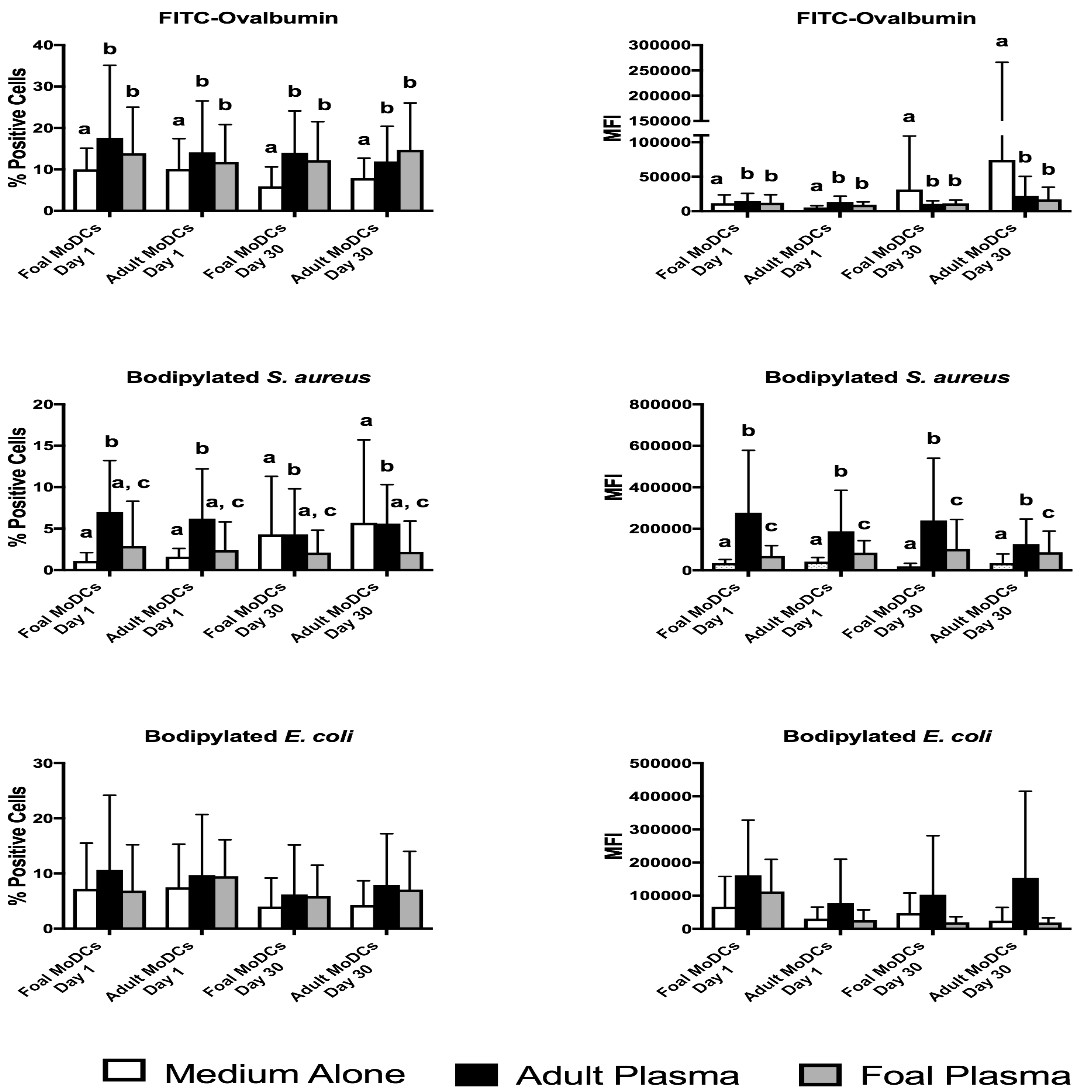

Figure 5.3. Percentage (mean ± standard deviation) and mean fluorescence intensity (MFI, mean ± standard deviation) of immature monocyte derived dendritic cells (MoDC) that were able to uptake antigen in the form of macropinocytosis (FITC-ovalbumin) and phagocytosis (bodipylated *Staphylococcus aureus* or *Escherichia coli).* MoDC were generated from adult horses (n=8) or foals (n=8) when foals were 1 and 30 days of age, and exposed to antigen and generic paired sources of adult and foal plasma or serum-free medium. [a, b] Different letter superscripts denote a statistically significant differences between treatment conditions for MoDC isolated from monocytes on the same day. ($P < 0.05$)

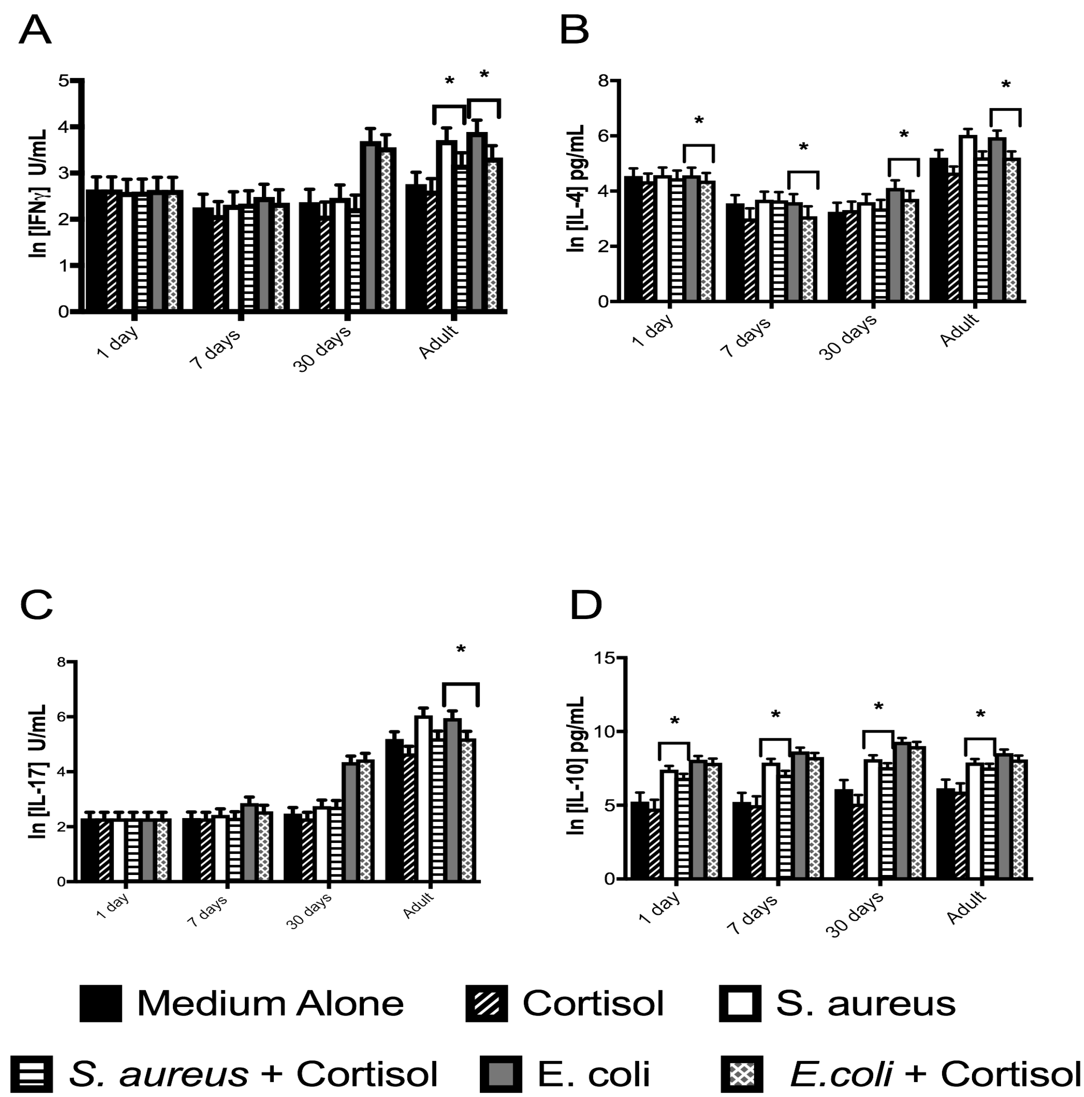

Figure 5.4. Cytokine concentration [(A) IFN-γ, (B) IL-4, (C) IL-17, and (D) IL-10] produced by MoDC generated from adult horses (n=9) once and foals at 1, 7 and 30 days of age (n=8) in the presence or absence of cortisol (15 ug/dL) alone or during exposure to killed *Escherichia coli or Staphylococcus aureus*. Data are presented as least-square means with corresponding standard errors. * Denotes significant differences between treatment conditions within that age group. (P < 0.05)

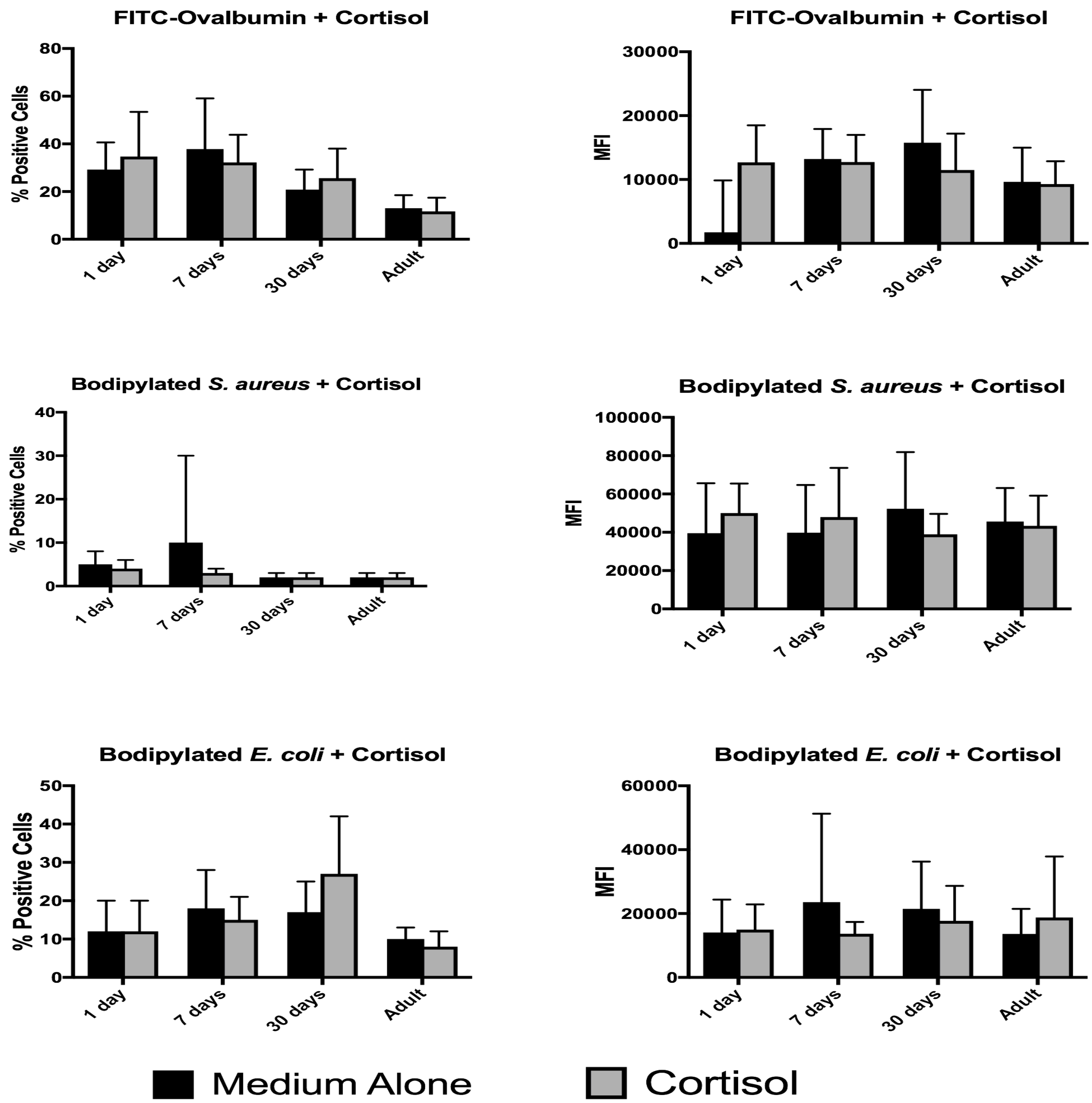

Figure 5.5. Percentage (mean ± standard deviation) and mean fluorescence intensity (MFI, mean ± standard deviation) of unstimulated, immature monocyte derived dendritic cells (MoDC) that were able to uptake antigen in the form of macropinocytosis (FITC-ovalbumin) and phagocytosis (bodipylated *Staphylococcus aureus* or *Escherichia coli)*. MoDC were generated from adult horses (n=8) once and foals (n=8) at 1, 7 and 30 days, and exposed to antigen in the presence or absence of cortisol (15 ug/dL).

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

Bacterial septicemia is the most frequent cause of foal mortality within the first few weeks of life.[1,2] There is a compelling body of evidence in the human and equine literature that demonstrate age-related alterations in both innate and adaptive immune responses mediate an increased susceptibility to infection in neonates.[3-8] Limitations of the innate immune system are of critical importance in the neonate. Innate immunity is the first line of defense against all pathogens, and is far more functional of the immune responses in neonates than adaptive immunity. Some innate immune cells, such as DC, are necessary for priming and directing the development of the adaptive immune response. DC play a vital role in the initial immune response to bacteria. They establish the context of the response at a tissue level through the production of cytokines and surface proteins. By sampling, processing, and presenting antigens to cells of the adaptive immune response, DC initiate lymphocyte participation in the fight against infection. DC are especially important in neonates as they are the most effective cell at activating naïve lymphocytes. They support the transition between the innate immune response and activation of the adaptive immune response. Many publications have reported phenotypic and functional immaturity of human and murine neonatal DC.[9-13] Collectively these previous studies suggest that immaturity in DC function might contribute to poor and inadequate immune responses in neonates. In addition to cellular limitations, studies of human and murine models also suggest that soluble factors (as represented in plasma) play a role in immunologic dysfunction in neonates.[14]

Equine *ex vivo* MoDC generation has evolved considerably over the past several years. The yield of MoDC from blood monocytes was limited using the original protocols. These utilized conventional culture methods for leukocytes, and medium that contained serum. Improved methods yielding a marked enhancement in the number of MoDC generated have been described. These provided a novel opportunity to investigate the biology of equine DC, as model by MoDC, that mimic those developed during infection and damage to mucosal tissues. The results of these studies were not concordant. This indicated the need for refinement of protocols to more consistently generate highly homogenous MoDC populations. Early studies of MoDC maturation and function from foals are sparse. However, they seem to reveal distinct differences in the maturation and function of MoDC generated from foals compared to MoDC from adult horse blood monocytes. The cell culture models utilized in these studies employed a variety of different media, most supplemented with plasma or serum that likely impacted the outcomes. This reduced the capacity of these studies to draw solid conclusions regarding true cell function. Protocols describing equine MoDC generation, in the absence of modulating factors - including those found in plasma as a model, are absent.

The overall objective of this research program was to investigate the effect of foal age on MoDC maturation and function following *ex vivo* exposure to killed whole cell bacteria. In order to examine the inherent cellular capabilities of foal MoDC, initial studies focused on optimizing a culture model that was chemically defined, free of exogenous plasma and its associated soluble factors. The first study describes in detail the methods developed to provide a matrix for this model. In this model, equine MoDC were generated and maintained in culture under serum-free conditions with the addition of only equine reGM-CSF and reIL4. This culture system was further refined in the first study to allow a consistent and reproducible population of MoDC to be produced. After establishing a well-defined culture system, this model was utilized as a baseline to characterize inherent cellular capabilities of equine MoDC in future studies. Utilizing this model, the second study determined intrinsic age-related changes in phenotype and functional indicators of MoDC generated from foals after exposure to killed bacteria. We subsequently examined the role of the extracellular microenvironment on MoDC maturation and function, using generic foal and adult horse plasma as a model for MoDC developing in mucosal tissues. Further, we investigated the effect of cortisol, a soluble factor with important physiologic and immune regulatory functions, on phenotype and function of foal MoDC.

The results of the first study highlighted the problems of inconsistencies in generating equine MoDC associated with ones choice of culture conditions. This study showed that some, but not all, culture conditions impacted the yield, viability, and state of MoDC maturation. This work provided evidence that MoDC can be generated from blood monocytes, and also maintained in serum-free medium supplemented with only defined recombinant equine growth factors. Under these conditions, comparable or enhanced numbers of MoDC, suitable for further study, can be obtained. In fact, under these culture conditions two distinct populations of MoDC that vary in their level of maturity are generated and each can be utilized assess changes in MoDC phenotype in response to stimuli. It was determined that the optimal days for MoDC harvest were after 3 or 4 days in culture. This maximized phenotype expression defining MoDC, cellular viability and cell yield from culture. In contrast, in another published study [15] no effect of the specific surface chemistry of the plate used during MoDC generation and cultivation on the overall quantity and viability of MoDC generated was observed. Plate chemistry did impact MoDC maturity in our study, as demonstrated by changes in MoDC phenotype. The ability to assess MoDC maturation after antigenic stimulation appears to be limited when collagen-coated plates are utilized. This was useful, in that standard quality TC coated plates are a fraction of the cost of collagen-coated matrix plates. Further, MoDC yield was not affected when either 2 or 4 hours of adherence culture were used for monocyte enrichment during seed stock collection. Therefore, this work validates a model for reliable, reproducible and essentially universal production of uniformly immature equine MoDC (as defined by the gating strategy described here) when cultured for 3 or 4 days in serum-free medium supplemented with defined growth factors. This model was used for the remainder of the studies in this project. We believe that it will be of benefit to future studies on equine MoDC.

The effect of foal age on MoDC maturation and function was investigated after *ex vivo* exposure to killed whole cell bacteria. Following generation of MoDC and exposure to killed bacteria, MoDC generated from foals displayed a more immature MoDC phenotype. The immaturity of foal MoDC is postulated to limit the ability of foal MoDC to effectively activate the adaptive immune response. Foal MoDC were found to produce altered quantities of several cytokines relative to MoDC from adult horses. This was most apparent following exposure to killed bacteria. Further, stimulation with *E. coli* and *S. aureus* produced different cytokines profiles. These differences in cytokine production could alter the context of the T cell response generated (Th1 vs Th2 vs Th17). The observed inherent differences in foal MoDC maturation and function have the potential to mediate an increase in foal susceptibility to infection early in life.

The effect of foal age on the function of soluble factors (as modeled by foal and adult horse plasma) and cortisol on MoDC maturation and function was assessed. Exposure of MoDC to generic plasma (foal or adult horse) results in partial MoDC maturation demonstrated by changes MoDC phenotype. Factors in neonatal foal plasma appeared to modulate MoDC cytokine production, and modulate antigen uptake differently than those in adult horse plasma. The effects observed with foal plasma from day 1 and day 30 were different, and indicated the composition of foal plasma, although not defined in these studies, may change as the foal age. Exposure of equine MoDC to cortisol concentrations simulating what is observed in septic neonatal foals, sustains MoDC immaturity. This was indicated by a phenotype that was more monocyte-like, in altered cytokine production, and in enhanced endocytic capabilities. Cortisol-induced MoDC phenotypic and functional immaturity could impact antigen presentation, and alter the nature of the T cell response generated during infection. It must be stressed that the soluble factors in the adult horse and the foal plasma utilized for this study were not characterized relative to their use. While some is known about the differences in composition in foal and adult horse plasma, the results presented here must be interpreted with caution. *In vivo,* MoDC develop from blood monocytes in mucosal tissues, and never in the peripheral circulation. Thus, these results are not directly applicable to what is occurring *in vivo* in the tissues. Small lipid-soluble factors, including cortisol, diffuse freely into tissues from the plasma during homeostasis. Increases in vascular permeability that occur with infection allow filtration of larger plasma proteins into the interstitium. While plasma is not an absolute representation of factors in mucosal tissue modulating MoDC generation and maturation, it is a convenient model for studies, such as the ones presented here, that focus on investigating MoDC biology during infection. The differences in MoDC biology observed using this model open the door for additional studies. Many factors found in equine plasma are available as purified products or as recombinant proteins. These factors could easily be studied using the well-defined model refined in these studies.

In summary, the results of this research provide a standardized protocol for isolating, generating, and maintaining equine MoDC in serum-free medium with only defined growth factors added. Using this model, future studies assessing the maturation and function of equine MoDC following exposure to various antigens or compounds to be easily performed against a common background and under equivalent conditions. The studies presented here indicate that inherent age-related limitations in foal MoDC maturation and function exist at a cellular level in this *ex vivo* model. Beyond the observed cellular impairment in phenotype and function, foal MoDC function were also modulated by soluble factors, specifically cortisol. It appears that these factors contributed to MoDC functional impairment in foals. Collectively, the documented cellular limitations, and those inferred to occur in the microenvironment (as represented by the use of generic foal and adult horse plasma), likely help to explain the altered effectiveness in stimulating naïve T cell responses to pathogens during early neonatal life in the foal.

While these studies developed tools to support future studies investigating immune-endocrine mechanisms in foals, further investigation of age-related differences in the capability of foal MoDC to prime naïve T cell responses to specific antigens and induce lymphocyte clonal expansion are needed. *Ex vivo* studies investigating the translation of innate immune activation into the development of an effective and appropriate adaptive immune response are critical to more completely understanding DC biology in foals, and to our understanding of the limitations of the foal immune response during infection. Only then can we being to develop novel preventative and therapeutic strategies to decrease disease susceptibility and severity in neonatal foals.

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APPENDIX A

MONOCYTE-DERIVED DENDRITIC CELL STIMULATION OF ALLOGENEIC T LYMPHOCYTE PROLIFERATION IN MIXED LEUKOCYTE REACTION (MLR) CULTURES

Objectives

1) To compare the ability of foal and adult horse MoDC, after exposure to cortisol, to stimulate proliferation of lymphocytes from an MHC class-II mismatched adult horse in an *ex vivo* mixed leukocyte reaction (MLR).

Methods

Reagents for mixed leukocyte reaction (MLR) cultures:

- RPMI medium: supplemented with 10% heat-inactivated fetal calf serum, 2mM L-glutamine, and 50 µg/mL gentamicin
- Staphylococcal enterotoxin B (SEB) – 5 µg/mL
- Hydrocortisone sodium succinate – 15 µg/dL

MoDC Generation:

1. Isolate and generate MoDC as previously described in Chapter 3. For this experiment MoDC are exposed to cortisol (15 µg/dL) on day 2 of MoDC generation after medium is changed.

Harvesting MoDC for MLR:

1. After 3 days of MoDC generation, MoDC are harvested in a sterile tissue culture laminar flow hood.
2. Collect adherent cells and non-adherent cells. Adherent cells can be gently removed using a cell scraper.
3. Transfer supernatant containing MoDC into a conical tube.
4. Centrifuge conical tube at 1,400 g for 5 minutes at room temperature.
5. Decant supernatant.
6. Resuspend MoDC pellet in 5 mL warmed RPMI medium.

7. Transfer 50 µL of this cell suspension to a bullet tube containing 450 µL of 0.04% trypan blue. Vortex cell/trypan suspension.
8. Add 10 µL of the cell/trypan blue suspension to a hemocytometer and manually count live and dead cells.
9. Adjust the concentration of your cell suspension to achieve a final concentration of 1.5 x 10^6 cells/mL.
10. Transfers 100 µL of this suspension to wells of a 96 well round-bottom plate. If using SEB as a mitogen, add 10 µL to corresponding wells of the 96 well plate.

Lymphocyte Isolation:

1. Isolate peripheral blood mononuclear cells (PBMC) as previously described in Chapter 3.
2. After final wash step, suspend cell pellet in 10 mL RPMI medium and add to a T175 flask containing 10 mL RPMI medium.
3. Incubate flask at 37°C with 5% CO_2 for 4 hours.
4. Gently swirl the contents of the flask and collect entire medium volume from the flask into a 50 mL conical tube.
5. Centrifuge at 1,300 g for 5 minutes at room temperature.
6. Decant supernatant.
7. Resuspend lymphocytes in 10 mL RPMI medium.
8. Remove a 50 µL sample of the cell suspension and add to a bullet tube containing 450 µL of 0.04% trypan blue, vortex to mix.
9. Add 10 µL of the cell/trypan blue suspension to a hemocytometer and manually count live and dead cells.
10. Adjust cell concentration by adding RPMI medium to reach a final concentration of 2 x 10^6 cells/mL.
11. Aliquot 100 µL of the 2 x 10^6 cells/mL suspension into wells of the same 96-well plate containing MoDC with and without SEB. Final concentration of lymphocytes per well is 200,000 cells/well.
12. Incubate plate at 37°C with 5% CO_2 for 5 days.

Lymphocyte Proliferation - [^{3}H]-thymidine:

1. After 5 days of co-incubation, expose cells in the 96-well plate to 10 µl of 0.2 uCi [^{3}H]-thymidine.
2. Incubate plate at 37°C with 5% CO_2 for 6 hours.
3. After 6 hours, harvest cells on glass fiber mats, dry, and count using a beta-scintillation counter to quantify lymphocyte proliferation.

Results

The ability of MoDC derived from foal at day 1 to stimulate T lymphocyte proliferation during co-incubation was less than MoDC derived from adult horses independent of cortisol exposure during MoDC generation (Figure A1).

Preliminary Conclusions

Preliminary results suggest impaired ability of foal MoDC to stimulate sufficient T cell proliferation, which could increase the foal's susceptibility to infection early in life. Additional replicates are needed to fully assess differences in MoDC-stimulated lymphocyte proliferation between foal and adult horse cells and to determine the impact of cortisol exposure during MoDC generation on MoDC ability to stimulate lymphocyte proliferation.

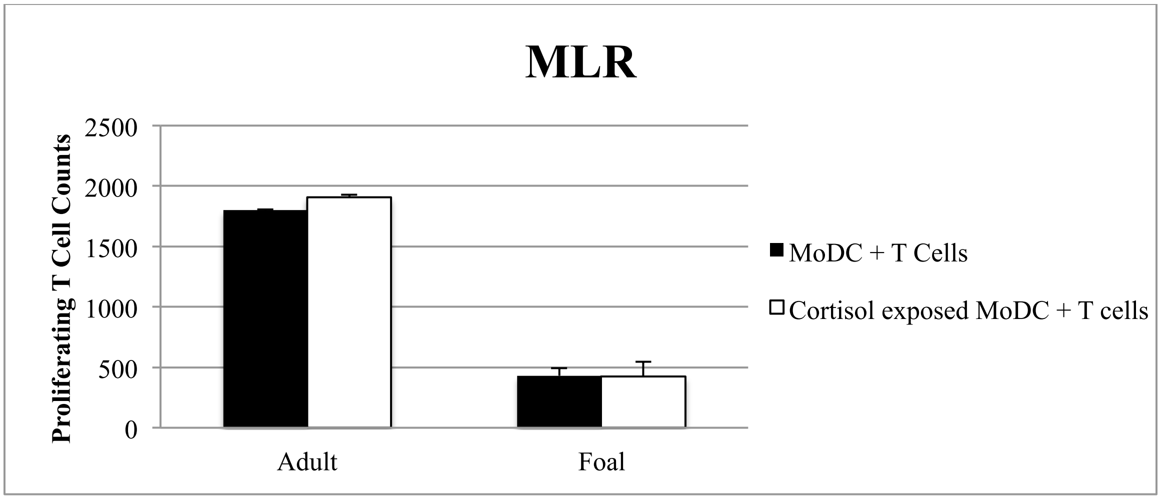

Figure A1. T-lymphocyte proliferation counts (mean ± SD) for co-incubated allogeneic lymphocytes from a MHC class-II mismatched adult horse and monocyte-derived dendritic cells (MoDC) generated from 1 day old foals (n=2) or adult horses (n=2). MoDC were generated in the presence or absence of cortisol, and lymphocyte proliferation assessed using 0.2 uCi [^{3}H]-thymidine incorporation as previously described (Tourkova IL *et al Immunol Lett* 2001).

APPENDIX B

ASSESSMENT OF CYTOKINE PRODUCTION OF ALLOGENEIC T LYMPHOCYTES CO-INCUBATED WITH MONOCYTE-DERIVED DENDRITIC CELLS

Objective

1) To assess cytokine production from monocyte-derived dendritic cells (MoDC) exposed to cortisol during generation and then co-incubated with lymphocytes.

Methods

Reagents:

- RPMI medium: supplemented with 10% heat-inactivated fetal calf serum, 2mM L-glutamine, and 50 µg/mL gentamicin
- Staphylococcal enterotoxin B (SEB) – 5 µg/mL

MoDC Generation:

Isolate and generate MoDC as previously described in Chapter 3 and expose to cortisol during generation as in Appendix A.

Harvesting MoDC for Co-Culture:

1. After 3 days of MoDC generation, MoDC are harvested in a sterile tissue culture laminar flow hood.
2. Collect adherent cells and non-adherent cells. Adherent cells can be gently removed using a cell scraper.
3. Transfer supernatant containing MoDC into a conical tube.
4. Centrifuge conical tube at 1,400 g for 5 minutes at room temperature.
5. Decant supernatant.
6. Resuspend MoDC pellet in 5 mL warmed RPMI medium.
7. Transfer 50 µL of this cell suspension to a bullet tube containing 450 µL of 0.04% trypan blue. Vortex cell/trypan suspension.
8. Add 10 µL of the cell/trypan blue suspension to a hemocytometer and manually count live and dead cells.

9. Adjust the concentration of your cell suspension to achieve a final concentration of 1.5 x 10^6 cells/mL.
10. Transfers 1 mL of this suspension to wells of a 12-well standard tissue culture coated plate.
11. If using SEB as a mitogen, add 50 μL to corresponding wells of the 12 well plate.

Lymphocyte Isolation:

1. Isolate peripheral blood mononuclear cells (PBMC) as previously described in Chapter 3.
2. After final wash step, suspend cell pellet in 10 mL RPMI medium and add to a T175 flask containing 10 mL RPMI medium.
3. Incubate flask at 37°C with 5% CO_2 for 4 hours.
4. Gently swirl the contents of the flask and collect entire medium volume from the flask into a 50 mL conical tube.
5. Centrifuge at 1,300 g for 5 minutes at room temperature.
6. Decant supernatant.
7. Resuspend lymphocytes in 10 mL RPMI medium.
8. Remove a 50 μL sample of the cell suspension and add to a bullet tube containing 450 μL of 0.04% trypan blue, vortex to mix.
9. Add 10 μL of the cell/trypan blue suspension to a hemocytometer and manually count live and dead cells.
10. Adjust cell concentration by adding RPMI medium to reach a final concentration of 2 x 10^6 cells/mL.
11. Add 500 μL of the 2 x 10^6 cells/mL suspension to corresponding wells of a 12 well plate containing MoDC.
12. Incubate plates at 37°C with 5% CO_2 for 24 hours.

Cytokine Production of Co-cultured MoDC and Lymphocytes:

1. After 24 hours of co-incubation, harvest cells from the 12 well plate by gently scraping the wells and aspirate medium and transfer to conical tube.
2. Centrifuge the conical tube at 1,300 g for 5 minutes at room temperature.
3. Collect supernatant in to bullet tubes and store at -80°C for cytokine quantification (Horse 5-plex, Cornell University, Ithaca, NY, USA).

Results

Reduced production of all cytokines (IL-4, IFN-γ, IL-17, and IL-10) by MoDC co-incubated with lymphocytes was observed for MoDC derived from foals at day 1 of compared to adult horse MoDC. Exposure of MoDC to cortisol prior to T cell co-incubation appears to increase the production of IL-17 and IL-10 by foal MoDC-T cell co-cultures (Figure B1).

Preliminary Conclusions

Exposure of foal MoDC to cortisol alters the quantity of cytokines produced during MoDC-T lymphocyte co-incubation. Additional replicates are needed to fully assess differences in cytokine production of co-incubated MoDC and lymphocytes, and to determine the impact of cortisol exposure during MoDC generation on cytokine production during co-culture.

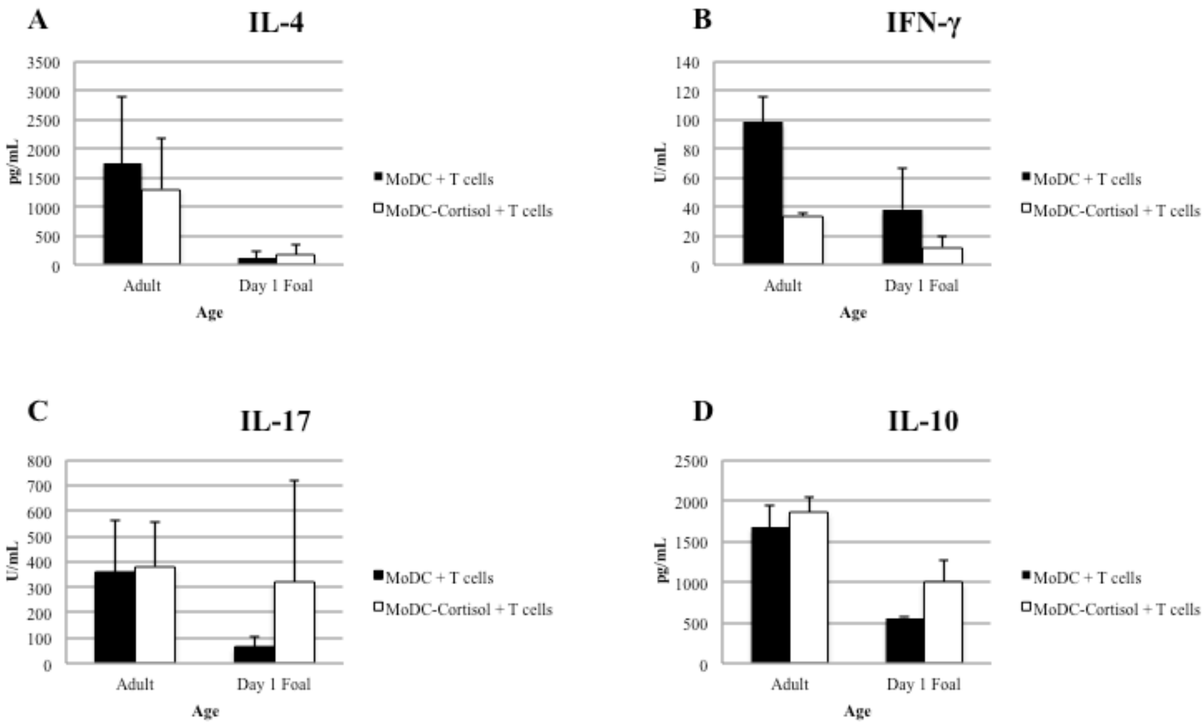

Figure B1. Cytokine production (mean ± SD) [(A) IL-4, (B) IFN-γ, (C) IL-17, and (D) IL-10] by monocyte-derived dendritic cells (MoDC) generated from 1 day old foals (n=2) or adult horses (n=2) in the presence or absence of cortisol and then co-incubated with allogeneic lymphocytes from a MHC class-II mismatched adult horse.

APPENDIX C

PHENOTYPIC ASSESSMENT OF ALLOGENEIC T LYMPHOCYTES CO-INCUBATED WITH MONOCYTE-DERIVED DENDRITIC CELLS

Reagents

- RPMI medium: supplemented with 10% heat-inactivated fetal calf serum, 2mM L-glutamine, and 50 μg/mL gentamicin
- Staphylococcal enterotoxin B (SEB) – 5 μg/mL
- Primary Monoclonal antibodies: CD25 at a 1:100 dilution, CD4 at a 1:50 dilution, andCD8 at a 1:50 dilution with FACS buffer
- Secondary fluorophore-conjugated antibody: Alexa 647-conjugated anti-goat IgG at a 1:200 dilution with FACS buffer.

MoDC Generation:

Isolate and generate MoDC as previously described in Chapter 3.

Harvesting MoDC for Co-cultures:

1. After 3 days of MoDC generation, MoDC are harvested in a sterile tissue culture laminar flow hood.
2. Collect adherent cells and non-adherent cells. Adherent cells can be gently removed using a cell scraper.
3. Transfer supernatant containing MoDC into a conical tube.
4. Centrifuge conical tube at 1,400 g for 5 minutes at room temperature.
5. Decant supernatant.
6. Resuspend MoDC pellet in 5 mL warmed RPMI medium.
7. Transfer 50 μL of this cell suspension to a bullet tube containing 450 μL of 0.04% trypan blue. Vortex cell/trypan suspension.
8. Add 10 μL of the cell/trypan blue suspension to a hemocytometer and manually count live and dead cells.
9. Adjust the concentration of your cell suspension to achieve a final concentration of 1.5×10^6 cells/mL.
10. Transfers 1 mL of this suspension to wells of a 12-well standard tissue culture coated plate.
11. If using SEB as a mitogen, add 50 μL to corresponding wells of the 12 well plate.

Lymphocyte Isolation:

1. Isolate peripheral blood mononuclear cells (PBMC) as previously described in Chapter 3.
2. After final wash step, suspend cell pellet in 10 mL RPMI medium and add to a T175 flask containing 10 mL RPMI medium.
3. Incubate flask at 37°C with 5% CO_2 for 4 hours.
4. Gently swirl the contents of the flask and collect entire medium volume from the flask into a 50 mL conical tube.
5. Centrifuge at 1,300 g for 5 minutes at room temperature.
6. Decant supernatant.
7. Resuspend lymphocytes in 10 ml RPMI medium
8. Remove a 50 μL sample of the cell suspension and add to a bullet tube containing 450 μL of 0.04% trypan blue, vortex to mix.
9. Add 10 μL of the cell/trypan blue suspension to a hemocytometer and manually count live and dead cells.
10. Adjust cell concentration by adding RPMI medium to reach a final concentration of 2 x 10^6 cells/mL.
11. Add 500 μL of the 2 x 10^6 cells/mL suspension to corresponding wells of a 12 well plate.
12. Incubate plates at 37°C with 5% CO_2 for 24 hours.

Lymphocyte Surface Marker Staining After Co-incubation with MoDC:

1. After 24 hours of co-incubation, harvest cells from the 12 well plate by gently scraping the wells and aspirate medium and transfer to conical tube.
2. Centrifuge the conical tube at 1,300 g for 5 minutes at room temperature.
3. If assessing cytokine production - collect supernatant in to bullet tubes and store at -80°C for cytokine quantification (Horse 5-plex, Cornell University, Ithaca, NY USA).
4. Resuspend cell pellet in 1 mL FACS buffer.
5. Transfer 50 μl to a bullet tube containing 450 μl 0.04% trypan blue, vortex to mix
6. Add 10 μL of the cell/trypan blue suspension to a hemocytometer and manual count live and dead cells.
7. Plate cells at a concentration of 1-2x10^5 cells/well in a 96 well round-bottom plate.
8. Centrifuge plate at 200 g for 5 minutes.
9. Snap the plate to remove buffer, vortex to suspend cells.
10. Add 100 μL of 10% normal goat serum to all wells.
11. Incubate at room temperature for 30 minutes at room temperature.
12. Centrifuge plate at 200 g for 5 minutes at room temperature.
13. Snap the plate to remove buffer, vortex to suspend cells.
14. Add 20 μl of diluted antibodies (CD25, CD8, and CD4) to corresponding wells.
15. Incubate at 4°C for 1 hour.
16. Add 100 μl FACS buffer to each well.
17. Centrifuge plate at 200 g for 5 minutes at room temperature.
18. Snap and vortex plate.
19. Repeat steps 16 -18 twice more (total of 3 washes with FACS buffer).

20. Add 100 μl of diluted anti-goat AF647 to corresponding wells including all wells containing primary antibodies.
21. Incubate plate at 4°C for 30 minutes.
22. Add 100 μl of FACS buffer to each well.
23. Centrifuge at 200 g for 5 minutes.
24. Wash with 200 μl FACS buffer 3 more times.
25. After the final wash step, add 100μl of FACS Fix to each well.
26. Incubate overnight at 4°C.
27. The next day, centrifuge the plate at 200 g for 5 minutes at room temperature.
28. Snap plate and vortex.
29. Add 200 μl of FACS buffer to each well and transfer to 1.5 ml bullet tubes containing 200 μl of FACS buffer and store at 4°C until analysis.
30. Analyze cells within 7 days of transferring to FACS buffer.

APPENDIX D

PURIFICATION OF MONOCYTES USING MACS SEPARATION

Objective: To determine if MoDC can be derived from peripheral blood mononuclear cells (PBMC) using MACS separation of monocytes before MoDC generation to eliminate residual lymphocytes.

Methods

Reagents:

- MACS Buffer: add 5.9 g of bovine serum albumin, and 819 mg of EDTA to 1.1 L phosphate buffered saline (PBS)
- Anti-equine CD2 monoclonal antibody at a 1:20 diluted in FACS buffer
- Rat anti-mouse IgG1 microbeads
- Secondary fluorophore-conjugated antibody: FITC-conjugated anti-mouse IgG at a 1:400 dilution with FACS buffer

MACS isolation of Monocytes for MoDC Generation

1. Isolate peripheral blood mononuclear cells (PBMC) from EDTA-anti-coagulated whole blood as previously in Chapter 3.
2. Suspend PBMC in 50 mL cold MACS buffer.
3. Centrifuge at 200 g for 15 minutes at room temperature.
4. Decant supernatant and suspend cell pellet in 10 mL cold MACS buffer.
5. Transfer 50 µL of the cell suspension to a bullet tube containing 450 µL of 0.04% trypan blue
6. Vortex cell/trypan suspension.
7. Add 10 µL of the cell/trypan blue suspension to a hemocytometer and manually count live and dead cells.
8. Add 40 mL MACS buffer to the cell suspension.
9. Centrifuge cell suspension at 300 g for 15 minutes at room temperature.
10. Discard supernatant.
11. Resuspend pellet in 80 µL MACS buffer per $1x10^7$ cells.
12. Add 20 µL anti-equine CD2 monoclonal antibody.
13. Incubate at 4°C for 30 minutes. Gently mixing by inverting the tube every 10 minutes.
14. Centrifuge at 200 g for 15 minutes at room temperature.
15. Discard supernatant.

16. Wash pellet in 5 mL cold MACS buffer and centrifuge at 200 g for 15 minutes at room temperature.
17. Repeat for a total of 3 washes, discard supernatant after each wash.
18. Suspend pellet in 80 μL MACS buffer and pass through a 30 μm nylon mesh filter moistened with MACS buffer.
19. Add 20 μL of rat anti-mouse IgG1 microbeads per $1x10^7$ cells.
20. Mix and incubate at 4°C for 15 minutes.
21. Prepare the LS column when 5 minutes of the incubation time remain by attaching the column to magnetic field and running 3mL of MACS buffer through the column.
22. After the incubation, wash cells using 1-2mL of MACS buffer per $1x10^7$ cells.
23. Centrifuge at 300 g for 15 minutes at room temperature.
24. Discard supernatant.
25. Suspend cells in 500 μL of MACS buffer per $1x10^8$ cells.
26. Apply cell suspension to column and collect flow-through containing unlabeled cells (monocytes) in to a conical tube.
27. Wash the column with 1 mL of cold MACS buffer 2X and collect into same conical tube containing unlabeled monocytes. This lymphocyte-depleted fraction will be used for MoDC generation as previously described in Chapter 3.
28. Remove the column from the magnet. Add 3 mL of MACS buffer and gently attach the column plunger and slowly push the plunger down, collecting the flow through into a conical tube. Repeat 2 more times. This is the CD2 positive cell fraction (lymphocyte-enriched).
29. Lymphocytes are stained for CD2 immediately after MACS separation and MoDC were assessed after 4 days of generation.

Expression of CD2 after MACS Separation:

1. Lymphocytes are stained for CD2 immediately after MACS separation and MoDC are assessed after 4 days of generation.
2. MoDC are harvested by gently scraping the wells and then transferring medium and loose cells to a conical tube.
3. Centrifuge the conical tubes containing MoDC and lymphocytes at 1,300 g for 5 minutes at room temperature.
4. Discard supernatant.
5. Suspend cell pellets in 1 mL FACS buffer.
6. Transfer 50 μl to a bullet tube containing 450 μl 0.04% trypan blue, vortex to mix.
7. Add 10 μL of the cell/trypan blue suspension to a hemocytometer and manually count live and dead cells.
8. Plate cells at a concentration of $1\text{-}2x10^5$ cells/well in a 96 well round-bottom plate.
9. Centrifuge plate at 200 g for 5 minutes at room temperature.
10. Snap the plate to remove buffer, vortex to suspend cells.
11. Add 20 μl of diluted CD2 to corresponding wells.
12. Incubate at 4°C for 1 hour.
13. Add 100 μl FACS buffer to each well.
14. Centrifuge plate at 200 g for 5 minutes at room temperature.

15. Snap and vortex plate.
16. Repeat steps 16 -18 twice more (total of 3 washes with FACS buffer).
17. Add 100 μl of diluted FITC-conjugated anti-mouse IgG to corresponding wells.
18. Incubate plate at 4°C for 30 minutes.
19. Add 100 μl of FACS buffer to each well.
20. Centrifuge at 200 g for 5 minutes at room temperature.
21. Wash with 200 μl FACS buffer 3 more times.
22. After the final wash step, add 100μl of FACS Fix to each well.
23. Incubate overnight at 4°C.
24. The next day, centrifuge the plate at 200 g for 5 minutes at room temperature.
25. Snap plate and vortex.
26. Add 200 μl of FACS buffer to each well and transfer to 1.5 ml bullet tubes containing 200 μl of FACS buffer and store at 4°C until analysis.
27. Analyze cells within 7 days of transferring to FACS buffer.

Results

Equine MoDC can successfully be generated from purified monocytes using the lymphocyte-depleted fraction after MACS separation of PBMC using anti-equine CD2 monoclonal antibody (Figure D1) to deplete lymphocytes. Purity of the MoDC population was confirmed by a small percentage of cells expressing the surface marker CD2 in contrast to the positive fraction of lymphocytes after MACS separation expressing near maximum levels of CD2.

Preliminary Conclusions

Accurate assessment of MoDC-induced lymphocyte proliferation requires highly pure MoDC cultures that can be attained utilizing MACS separation of PBMC with equine CD2 monoclonal antibodies.

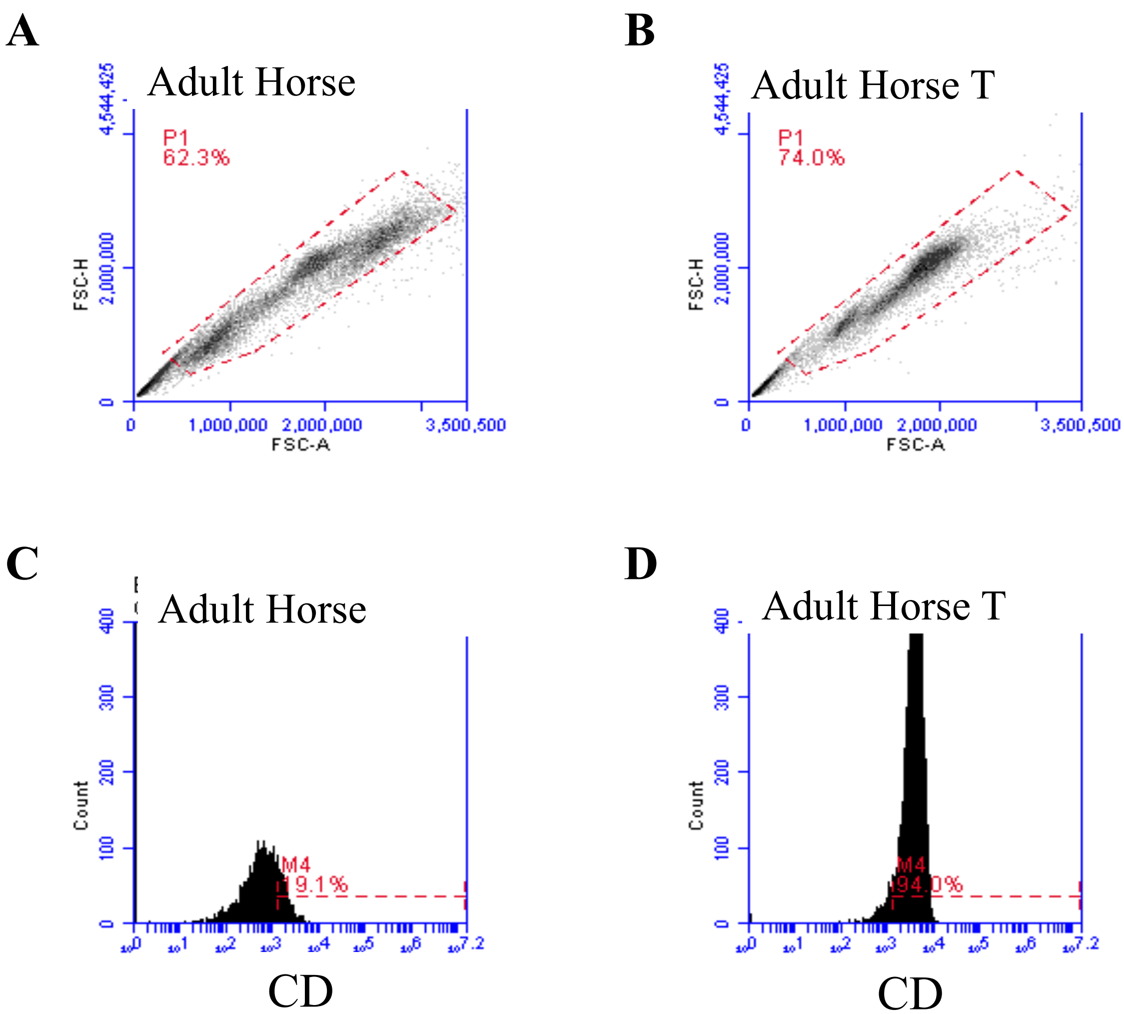

Figure D1. Dot plots of forward scatter-height (FSC-H) versus forward scatter-area (FSC-A) outlining the population of interest containing monocyte-derived dendritic cells (MoDC) generated from an adult horse after 4 days of generation or (B) adult horse lymphocytes immediately after isolation, both derived from MACS separation using anti-equine CD2 antibody. Histogram plots showing the percent of (C) MoDC derived from an adult horse and (D) lymphocytes purified from the same adult horse, that stained positive for surface marker CD2. MoDC were generated from the CD2-negative fraction after MACS separation of PBMC using anti-equine CD2 antibody, while lymphocytes were generated from the CD2-positive fraction.