The influence of equine bone marrow derived stem cells on the response of cultured peripheral blood mononuclear cells to endotoxin

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ABSTRACT

Endotoxemia is a major cause of morbidity and mortality in horses. The presence of large amounts of circulating endotoxin inititates a number of cell signaling pathways leading to a systemic inflammatory response. Activation of these pathways causes the release of a number of pro- and anti-inflammatory mediators. An overwhelming release of these mediators leads to the development of clinical signs associated with endotoxemia. Treatment options are limited mostly to supportive care at this time. Mesenchymal stem cells (MSCs) have been shown to have anti-inflamamtory and immune modulatory effects that may have some benefit for the treatment of horses with endotoxemia.

To evaluate the effect of equine MSCs on the response to endotoxin challenge, the study was performed on two different stem cell lines with peripheral blood mononuclear cells (PBMCs) used as controls. After stimulation with endotoxin, secretion of tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), interleukin-10 (IL-10), and interferon gamma (IFN-γ) were determined by ELISA. The immunogenic properties of MSCs were assessed with a one-way mixed lymphocyte reaction. In addition, the ability of MSCs to alter production of cytokines from stimulated PBMCs was assessed.

TNF-α was not produced by MSCs when compared to PBMCs ($p = < 0.001$). There was no significant difference between MSCs and PBMCs in the production of IL-6. IL-10 production was significantly different ($p = <0.001$) at 6 and 12 hours with MSCs producing more than PBMCs in one stem cell line only. MSCs did not stimulate proliferation of PBMCs. Co-incubation of MSCs with PBMCs decreased the production of TNF-α in both stem cell lines although it was not statistically significant ($p = 0.4$ and 0.9) at either time point. IL-6 secretion was suppressed at twelve hours with co-incubation. IL-10 production was increased with co-incubation in one stem cell line. MSCs secrete soluble factors that can alter PBMC cytokine production and they do not appear to be immunostimulatory. These findings have potential implication for treatment of equine inflammatory conditions.

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List of Abbreviations

AA	Arachidonic acid
ALI	Acute lung injury
APC	Antigen presenting cell
ARDS	Acute respiratory distress syndrome
CD105	Cluster of differentiation antigen 105
CD11b	Cluster of differentiation antigen 11b
CD14	Cluster of differentiation antigen 14
CD19	Cluster of differentiation antigen 19
CD34	Cluster of differentiation antigen 34
CD45	Cluster of differentiation antigen 45
CD73	Cluster of differentiation antigen 73
CD79α	Cluster of differentiation antigen 79α
CD90	Cluster of differentiation antigen 90
CLP	Cecal ligation and puncture
COX-2	Cycoloxygenase-2
CT	Computed tomography
CV	Cardiovascular
DMSO	Dimethyl Sulfoxide
ERU	Equine recurrent uveitis
EGF	Epidermal growth factor
eMSCs	Equine mesenchymal stem cells
FGF	Fibroblast growth factor
GI	Gastrointestinal
HLA	Human leukocyte antigen
IBD	Inflammatory bowel disease
ICAM-1	Intracellular adhesion molecule 1
IDO	Indoleamine 2,3-dioxygenase
IFN γ	Interferon gamma
IL-1	Interleukin-1
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-18	Interleukin-18
iNOS	Inducible nitric oxide synthase
IRAK	IL-1 receptor-associated kinases
kDa	KilaDalton
LBP	Lipopolysaccharide -binding protein
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MHC-I	Major histocompatibility complex-I
MHC-II	Major histocompatibility complex-II
ml	Milliliters
MODS	Multiple organ dysfunction syndrome
MSCs	Mesenchymal stem cells

MyD88	Myeloid differentiation factor 88
NF- κB	Nuclear factor κB
ng	Nanogram
NK	Natural killer
NO	Nitric oxide
PAF	Platelet activating factor
PAMP	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PDGF	Platelet derived growth factor
pg	Picogram
PGE_2	Prostaglandin E_2
$PGF_2\alpha$	Prostaglandin $F_2\alpha$
PGI_2	Prostaglandin I_2
PRR	Pattern recognition receptor
RAO	Recurrent airway obstruction
SC	Stem cells
SDF-1	Stromal cell-derived factor 1
SIRS	Systemic inflammatory response syndrome
TGF- β	Transforming growth factor – β
TLR-4	Toll-like receptor 4
TNBS	Trinitrobenzene
TNF –α	Tumor necrosis factor alpha
TRAF	TNF receptor-associated factors
TXA_2	Thromboxane A_2
VCAM-1	Vascular cell adhesion molecule -1
VEGF	Vascular endothelial growth factor
µl	Microliter

Chapter 1. Introduction and Review of the Literature

Endotoxemia

Endotoxemia refers to the presence of circulating endotoxin, which causes detrimental clinical manifestations by stimulating the host's cells to release a large number of soluble products, and membrane-bound cellular receptors that lead to an overwhelming inflammatory response. Endotoxin is the lipopolysaccharide (LPS) portion of the outer cell wall of gram-negative bacteria, and it is composed of three structural components. There is a hydrophilic polysaccharide portion that consists of the core polysaccharide and the O-antigenic region. Second, a core region consisting of mostly monosaccharides and finally, the hydrophobic lipid A component. The lipid A component is highly conserved among bacterial species and is responsible for most of the toxic effects seen following LPS absorption (Barton, 2003). LPS is released from gram-negative bacteria in large amounts during bacterial multiplication or during bacterial death when cells lyse.

The largest reservoir of gram-negative bacteria in the horse is within the gastrointestinal (GI) tract. In a healthy horse, endotoxin is sequestered within the intestinal lumen and only minute amounts of LPS may enter the portal circulation. Local defense mechanisms which limit LPS translocation into the circulation include tight mucosal junctions between epithelial cells and secretions from epithelial cells (e.g., the mucosal barrier) (van Deventer et al. 1988) (Moore, 1988). Mucus secreted from the epithelial cells provides a continuous protective layer over the mucosa to prevent organisms from adhering (White, 2009). If LPS enters the circulation from the GI tract, most of it is rapidly neutralized by anti-LPS antibodies, and the remainder is transported bound to specific proteins (LPS-binding protein) and cleared by the Kuppfer cells of the liver (Barton, 2003). Through these mechanisms, exposure of the horse to LPS and the systemic effects of LPS are limited. There are, however, a number of clinical situations that result in clinically relevant endotoxemia.

If the amount of LPS entering the portal circulation is large enough, the clearance capacity of the liver can be overwhelmed, allowing LPS to enter the systemic circulation. This occurs most commonly with conditions that compromise blood flow to the bowel or severe mucosal inflammation (such as in colitis) that compromises the GI mucosal barrier. Infection with gram-negative bacteria in body cavities or tissues (such as the pleural or peritoneal cavity, lungs or uterus) provides another common source for endotoxemia. LPS that originates from

these other sites in the body bypass the portal circulation and may be directly absorbed into the systemic circulation (Moore et al. 1981).

LPS results in activation of mononuclear phagocytes and systemic inflammation by interacting with cell surface receptors with secondary intracellular signaling. LPS has a very high affinity for the LPS-binding protein (LBP) as well as receptors that are found on the surface of mononuclear phagocytes. LBP is an acute-phase serum protein synthesized by the liver, and is present at baseline levels in normal animals (Tobias et al. 1989). Increased concentrations of LBP are seen following a response to insult (systemic inflammatory response syndrome or sepsis) (Schumann and Latz, 2000) (Schumann et al., 1990). Once bound to LPS, the LPS-LBP complex binds to the cluster of differentiation antigen 14 (CD14) on the surface of phagocytes. CD14 is a pattern recognition receptor (PRR). PRRs sense the presence of microorganisms by recognizing structures conserved among microbial species called pathogen associated molecular patterns (PAMPs). PRRs recognize endogenous molecules released from damaged cells (Takeuchi and Akira, 2010). The important role CD14 plays in recognition and activation of the downstream signaling has been shown by a number of studies (Ferrero et al. 1993; Haziot et al. 1996; Wright et al. 1990). CD14 lacks an intracellular domain, so it is not capable of activating cytokine production alone; cellular activation requires the action of additional proteins. The LPS-LBP-CD14 interaction results in phosphorylation of Toll-like receptor 4 (TLR-4) (da Silva Correia et al. 2001). TLR-4 signals the activation of Myeloid differentiation factor 88 (MyD88), IL-1 receptor-associated kinases (IRAK), and TNF receptor-associated factors (TRAF) (Muzio et al. 1998). The end result is activation of the intracellular signaling molecule, nuclear factor κB (NF-κB). Upregulation of NF-κB results in production of multiple pro-inflammatory and anti-inflammatory cytokines, chemokines, adhesion molecules, enzymes and acute phase proteins. The pro-inflammatory cytokines include tumor necrosis factor alpha (TNF-α), interleukin 1 (IL-1), interleukin-6 (IL-6), interleukin-12 (IL-12) and interleukin-18 (IL-18). Release of these pro-inflammatory cytokines further amplifies NF-κB activity by paracrine effects (Wang et al. 2003). This interaction of LPS, LBP, and CD14 increases the sensitivity of cells to endotoxin (Moore and Barton, 2003) further amplifying the response.

There are a number of acute phase proteins and anti-inflammatory cytokines that are activated by upregulation of the NF-κB pathway. Acute phase proteins have a number of functions including facilitating the local inflammatory process and aiding in elimination of the

pathogen. Complement proteins C3 and C4 are important in the elimination of bacteria (Furr, 2003). The concentration of anti-inflammatory cytokines, specifically IL-10, also increases. IL-10 acts to directly inhibit the release of pro-inflammatory cytokines from monocytes and macrophages. It also induces production of IL-1 receptor antagonist protein and soluble TNF receptor to reduce the circulating concentrations of IL-1 and TNF (Oberholzer et al. 2002). When this process occurs at the local level, it is a protective response, but at the systemic level it can lead to the development of systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS).

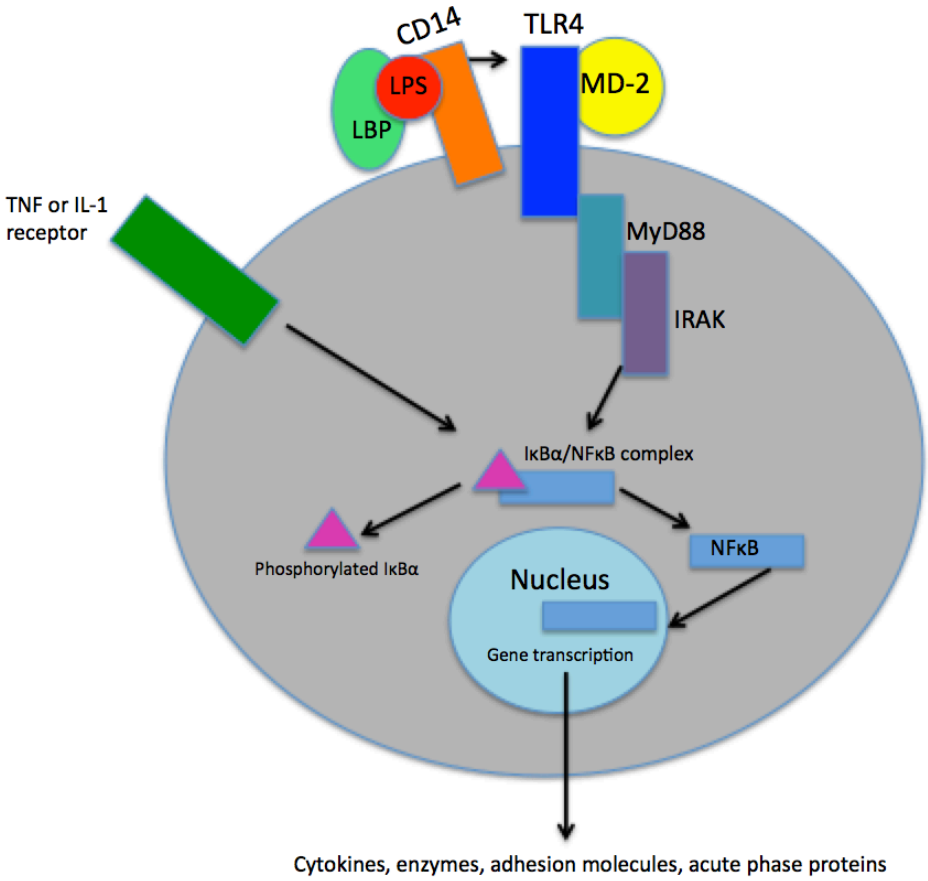


Figure 1. Biochemical cascade initiated by LPS and other pro-inflammatory agents leading to the production of cytokines, enzymes, adhesion molecules, and acute phase proteins.

SIRS is a clinical syndrome that occurs as a result of malignant activation of multiple pro-inflammatory pathways. It is defined by the presence of two or more of the following abnormalities: fever or hypothermia (rectal temperature greater than 39.2°C or less than 37.2°C), tachycardia (heart rate greater than 60 beats per minute), tachypnea (respiratory rate greater than

30 breaths per minute) or hypocapnia (partial pressure of arterial carbon dioxide less than 32 mmHg), leukocytosis or leukopenia (leukocyte greater than 12,500 or less than 4,000 cells/ul), or increased numbers of immature forms of granulocytes (greater than 10% band neutrophils) (McKenzie and Furr, 2001). The changes associated with SIRS can lead to shock and progress to MODS. MODS is defined as the presence of altered organ function in an acutely ill patient such that homeostasis cannot be maintained without intervention (Osterbur et al. 2014). The release of inflammatory mediators, chemokines, and adhesion molecules and further cellular activation are critical to the development of SIRS.

Inflammatory Mediators of Endotoxemia

Tumor Necrosis Factor- alpha

TNF-α is one of the earliest mediators to be produced following exposure to endotoxin and it has a central role in the early response to endotoxin. It has been described as the principle mediator in endotoxic shock (Männel and Echtenacher, 2000). The synthesis of TNF-α is tightly regulated to ensure that production of this inflammatory mediator is minimal. Following stimulation there is a rapid (15-30 minutes) increase in the production of TNF (Wang et al. 2003), which usually peaks at 90-120 minutes after LPS exposure. TNF-α is synthesized as a 26kDa type II transmemembrane precursor that is displayed on the plasma membrane. The precursor is proteolytically cleaved to yield a biologically active molecule that forms a trimer. Membrane bound pro-TNF-α in the effector cells bind to TNF-α receptors on the target cells and induces TNF-α responses via receptor aggregation (Kriegler et al. 1988). TNF-α is produced by a number of immune cells in response to many different stimuli including LPS, hypoxia, IL-1, leukotrienes, parasites, nitric oxide and viruses (Wang et al. 2003). The response to a wide variety of stimuli indicates the essential role TNF-α plays as a mediator of the inflammatory response.

Increases in serum TNF-α concentration are associated with hypotension, hemoconcentration, acidosis and disseminated intravascular coagulation. It also initiates formation of more pro-inflammatory cytokines. The fact that TNF-α has a central role in the development of endotoxemia is demonstrated by the observation that administration of recombinant TNF-α causes the development of clinical signs that are associated with endotoxemia (Morris, 1991). In addition it also induces the synthesis of interleukins,

prostaglandins, tissue factor and initiates the acute phase response (Moore and Barton, 1998). Further evidence of its central role is shown by the fact that administration of TNF-α antibodies prevents the development of septic shock (Barton et al. 1998a).

TNF-α produces fever and anorexia via the hypothalamic centers. TNF-α has direct effects on hypothalamic neurons and through indirect effects by the induction of IL-1 release to induce a fever (Plata-Salamán, 1991). It also appears to suppress the glucose-responsive neurons in the hypothalamic ventromedial nucleus and glucose-sensitive neurons in the lateral hypothalamic area leading to a suppression in feed intake (Plata-Salamán, 1998). TNF-α also causes rearrangement of the actin filaments leading to endothelial cell damage and loss of tight junctions; this is a key factor in the development of the capillary leakage syndrome, which characterizes severe endotoxemia, and SIRS. TNF-α also has a negative inotropic effect on myocardial contractility by decreasing the extent of shortening of the myocyte (Kumar et al. 2007). Production of nitric oxide (NO) by the endothelium activated by TNF-α has been implicated for the decreased peripheral vascular resistance (Wang et al. 2003). Capillary leakage syndrome, negative inotropy, and decreased peripheral resistance contribute to hypotension, decreased circulating blood volume and poor peripheral perfusion of the cardiovascular system (CV).

TNF results in a number of additional clinical signs, in addition to effects upon the CV system. In experimentally induced endotoxemia, TNF-α activity increased within one to two hours and pyrexia was associated with the appearance of circulating endotoxin (MacKay et al. 1991). TNF-α activity decreased rapidly after the first two hours but other studies have shown it to persist longer and still be detectable in the system for longer periods of time after LPS infusion. Increased serum TNF-α activity can also be directly associated with the onset of abdominal pain or depression and leucopenia (Morris et al. 1990). High levels of serum TNF-α in foals with presumed septicemia is associated with a poor outcome (Morris and Moore, 1991). Pretreatment with TNF-α antibodies has been shown to block the development of shock after experimental endotoxemia and to markedly decrease the serum concentrations of IL-1 and IL-6 (Beutler and Cerami, 1989). TNF-α plays a role in activating further essential mediators.

Interleukin-6

The main sources of IL-6 are monocytes and macrophages, but it is also produced by fibroblasts, endothelial cells, T and B lymphocytes, mesangial cells and keratinocytes. (Robinson et al. 1993). IL-6 is a glycoprotein cytokine with a molecular mass between 21-28kDa (Kishimoto, 2003). IL-6 stimulates target cells via a membrane bound interleukin-6 receptor, which upon ligand binding associates with the signaling receptor protein gp130 and ultimately the activation of the mitogen-activated protein kinase (MAPK) pathway (Scheller et al. 2011). Production of IL-6 is induced by T-cell mitogens or antigenic stimulation. LPS enhances IL-6 production in monocytes and fibroblasts and in the monocytic cell lines. NF-κB is essential for LPS induced IL-6 gene expression (Kishimoto, 2003). The exact role that IL-6 plays in endotoxemia is still not fully understood, but production by numerous cells after exposure to LPS and TNF-α *in vitro* has been documented. It is reported to stimulate hemopoeitic precursor cell growth and differentiation, induction of fever, inhibition of TNF-α production, and stimulation of antibacterial mechanisms (Kimura and Kishimoto, 2010).

Experimental induction of endotoxemia by LPS administration resulted in an increase in IL-6 activity within one hour of the onset of endotoxemia. Peak IL-6 activities were seen between three to four hours and were also associated with an increasing rectal temperature (Morris et al.1992). In horses presenting for acute abdominal disease, IL-6 concentration was significantly correlated with serum LPS concentrations, and increasing IL-6 concentrations were correlated with higher risk of mortality (Steverink et al. 1995).

Interleukin-10

IL-10 is a key regulator of the immune response. Activated T cells, B cells, monocytes/macrophages, mast cells, and keratinocytes produce IL-10. IL-10 is an 18-21 kDa polypeptide produced in response to many pathogens. Antigen presenting cells and lymphocytes are the primary target of IL-10 (Asadullah et al. 2003). IL-10 has been shown to inhibit monocyte and macrophage synthesis of IL-1, IL-6, IL-8, IL-12, TNF-α, reactive oxygen and nitrogen intermediates, and dendritic cell production of IFN-γ. Monocytes activated by LPS produce high levels of IL-10 in a dose-dependent fashion (Moore et al. 1993). It suppresses multiple immune responses through actions on T cells, B cells, and APCs and shifts the immune response from Th1 to Th2 (Ding et al. 2003). Mice deficient in IL-10 developed lethal

inflammation of the intestine that was then prevented by the administration of IL-10 (Ding et al. 2003). In a murine model of cecal ligation and puncture, administration of IL-10 six hours after induction of sepsis suppressed the levels of circulating TNF-α and decreased lethality (Kato et al., 1995). IL-10 can induce the expression of E selectin on vascular endothelium (Vora et al. 1996) which could sustain inflammatory responses that could become detrimental. In an *in vitro* model of LPS stimulated equine peritoneal macrophages that were treated with recombinant human IL-10 showed a dose dependent suppressive effect on TNF, IL-6, and PGE_2 production (Hawkins et al. 1998). It is generally considered an immunosuppressive cytokine, but it can have immunostimulatory effects depending on the cell type involved or the other immune events that are occurring at the time (Ding et al. 2003). Some reports show that IL-10 has stimulatory effects on CD4+, CD8+ T cells, and NK cells which may result in increased IFN-γ production. IL-10 injections in mice accelerated graft-vs-host disease and graft rejection in bone marrow recipients (Blazar et al. 1995). In an *in vivo* model of human endotoxemia, higher doses of IL-10 administered shortly after an inflammatory stimulus had pro-inflammatory effects (Lauw et al. 2000).

Interferon gamma

IFN-γ is produced primarily by natural killer cells and T lymphocytes following exposure to immune and inflammatory stimuli. Natural killer cells are an important source of IFN-γ during the innate phase of a developing immune response. They are also influenced by exposure to cytokines, specifically IL-12 and TNF-α. In T cells, once activated, IFN-γ production reaches peak extracellular levels by 18-24 hours (Schreiber and Schreiber, 2003). IFN-γ production is inhibited by the presence of IL-10. IFN-γ plays an important role in coordinating the transition from innate to adaptive immunity by encouraging the development of a Th1- type response, promoting B cell isotype switching, and regulating local leukocyte-endothelial interactions. IFN-γ knockout mice demonstrate its essential role. They have no overt developmental defects but exhibit deficiencies in resistance to bacterial, parasitic, and viral infections. IFN-γ plays a role in amplifying LPS induced responses by priming macrophages for a more rapid and heightened response to LPS. IFN-γ receptor knockout mice are highly resistant to LPS induced toxicity (Schroder et al. 2004). IFN-γ promotes both innate and adaptive protective immune response in

the host against infectious and inflammatory stimuli, but it also plays a central role in the development of immunopathologic conditions.

Other mediators

Other mediators play a role in endotoxemia/SIRS. Important pro-inflammatory cytokines include IL-1, IL-2, IL-8 and IL-12. IL-1 initiates the COX-2 pathways, type 2 phospholipase A, and inducible NO synthase. Its actions are similar to TNF-α, but less pronounced (De Backer et al. 2013). It also induces fever, has chemotactic effects on neutrophils, increases synthesis of acute phase proteins, and activates lymphocyte (Dinarello, 1984). Following stimulation by TNF-α and IL-1, IL-8 is released. It plays a role in recruitment and activation of neutrophils (Taylor, 2015). IL-12 enhances cell-mediated immunity, Th-1 type responses to CD4+ cells and the function of B cells, APCs, vascular and stromal cells.

Arachidonic acid (AA) is released from the phospholipid of cell membranes and is metabolized by cyclooxygenase to thromboxane A_2, prostaglandin $F_2\alpha$, prostaglandin I_2, and prostaglandin E_2 (Moore and Barton, 1998; Moore and Morris, 1992). In the initial stages of endotoxemia, thromboxane A_2 and prostaglandin $F_2\alpha$ cause vasoconstriction. The initial hypertension, dyspnea, and hypoxemia seen in endotoxemia have been attributed to the rapid increase in thromboxane A_2 (Moore and Barton, 1998). Prostaglandin I_2 and prostaglandin E_2 cause vasodilation and are released later in the disease process. Increased concentrations of prostaglandin I_2 have been associated with the clinical signs of abdominal pain, discoloration of the mucous membranes, prolongation of the capillary refill time, and development of systemic hypotension (Moore and Barton, 1998). Prostaglandin E_2 has been associated with the development of fever. AA is also metabolized by lipooxygenase to produce leukotrienes, which contribute to the development of endotoxemia.

Platelet activating factor (PAF) is a potent vasodilator in most tissues except in pulmonary, renal, and coronary vasculature where it causes vasoconstriction. It is responsible for platelet aggregation (Jarvis and Evans, 1996). ICAM-1 and VCAM-1 play a role in the movement of neutrophils. Other important factors include inducible nitric oxide synthase, tissue factor and plasminogen activator type-1.

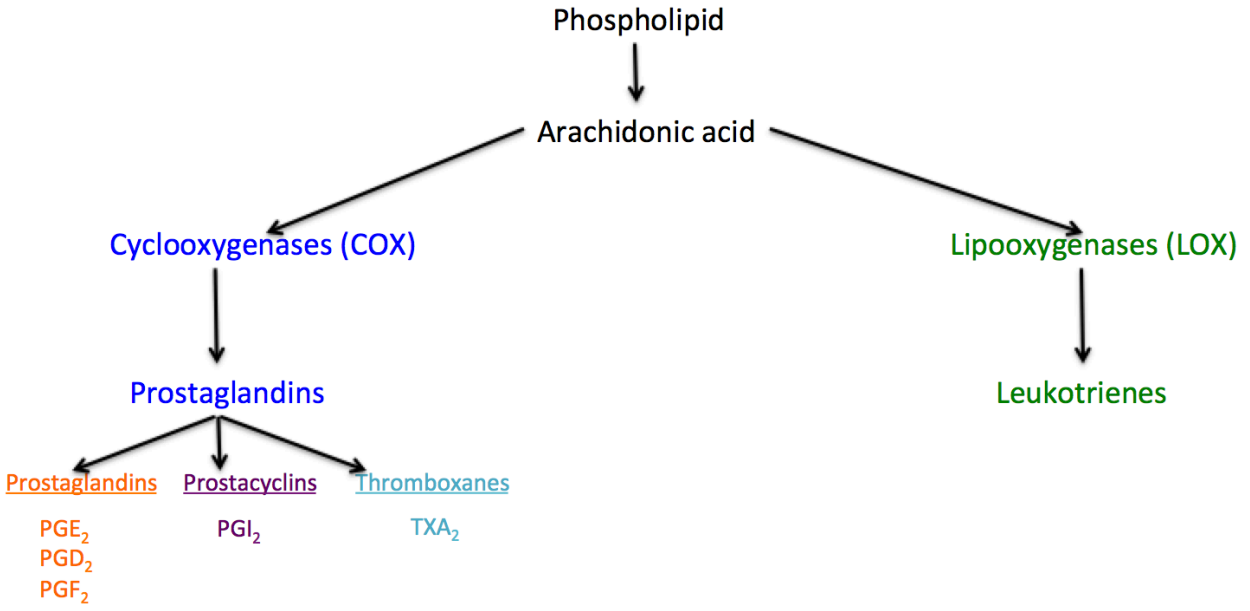


Figure 2: Major mediators and metabolites derived from the metabolism of arachidonic acid that contribute to the development of endotoxemia.

Endotoxemia in the horse

Endotoxemia affects 20-30% of human patients in intensive care units and accounts for more than 200,000 human deaths per year in the United States (Andreasen et al. 2008). In humans elevated concentrations of circulating endotoxin have been correlated with the development of multiple organ failure and death (Danner, 1991). Colic remains the leading cause of death in the adult horse, and reports show that mortality is very closely related to the degree of endotoxemia (Thoefner et al. 2001; Tinker et al. 1997). Of the adult horses that present to referral hospitals for colic, 30-40% have detectable endotoxin in their circulation (Senior et al. 2011). Higher concentrations of endotoxin at admission and intra-operatively have been associated with increased mortality (Steverink et al. 1994). While the gastrointestinal tract is the most common source of endotoxin, and strangulating or obstructive lesions are the most common cause, endotoxemia can be caused by any gram-negative infection that may occur in colitis, peritonitis, pleuropneumonia and metritis. Of the foals that present for septicemia, up to 50% have detectable circulating endotoxin (Barton, 2003).

The clinical signs due to endotoxemia in the horse are non-specific, dose dependent, and include fever, depression, tachycardia, tachypnea, hyperemic mucous membranes, prolonged capillary refill time, abdominal discomfort, dehydration, and diarrhea (Moore and Barton, 1998). Leukopenia is usually observed early in the disease process (MacKay, 1991). Thirty minutes

after exposure, a period of tachypnea occurs and generally resolves within two hours. Ninety minutes after exposure, depression, restlessness and inappetence along with an increased rectal temperature are present (Jones, 2009). These changes are mediated by the initial cytokine release. Two hours after exposure, intermittent signs of colic, loose feces, and an elevated heart rate are generally seen. Mucous membranes become congested and capillary refill time is prolonged. This will be associated with the release of iNOS causing local release of NO, production of thromboxane A_2 and prostaglandin $F_2\alpha$ leading to the development of hypotension.

Around four to six hours after exposure, a second wave of tachypnea and tachycardia occurs that is associated with fever and the development of hypotension (Jones, 2009) and is related to the production of prostaglandin I_2 and prostaglandin E_2. Prostaglandin E_2 causes vasodilation except in renal tissue where it increases renal blood flow. Affected horses can initially have increased urine production despite dehydration (Carr, 2015). In this time period, the activation of ICAM-1 and VCAM-1 along with β2 integrins and other chemokines activates neutrophil binding to the endothelial wall. The neutrophils then usually follow the chemotactic gradient and extravasation into the affected tissue occurs. In the patient with endotoxemia, the neutrophils adhere to the activated endothelium, but are less responsive to the underlying chemotactic factors therefore do not move into the affected tissue. This contributes to the initial leucopenia observed. Continued neutrophil activation and release of reactive oxygen species cause further endothelial injury and permeability. The overall response is tissue hypoperfusion and hypoxia leading to tissue injury. AA is also metabolized to leukotrienes that promote fluid leakage across capillaries, and act as potent chemoattractants for neutrophils and potent broncho- and vasoconstrictors (Morris, 1991). The presence of circulating endotoxin can be correlated with alterations in hemostatic and fibrinolytic indices (Barton et al. 1998). The severity of clinical signs is related to the levels of circulating endotoxin. These systemic changes seen can lead to a number of other complications including thrombophlebitis and laminitis (Hunt et al. 1986).

Clinical signs
Fever
Depression
Tachycardia
Tachypnea
Hyperemic mucous membranes
Abdominal discomfort
Diarrhea

Figure 3: Common presenting clinical signs of endotoxemia

Treatment

To date, there is no single effective treatment for endotoxemia. The current goals of therapy are to reduce or prevent the movement of endotoxin into circulation, neutralize circulating endotoxin, prevent or reduce interaction with inflammatory cells, prevent the synthesis of proinflammatory mediators, and supportive care.

Nonsteroidal anti-inflammatory drugs (NSAIDs). Nonsteroidal anti-inflammatory inhibit cyclooxygenase and reduce production of prostaglandins and thromboxanes, and are a critical component in the treatment of endoxemia. Flunixin meglumine is most commonly used NSAID for endotoxemia (Moore and Barton, 2003) and has been extensively studies in many models of equine endotoxemia. Low doses of flunixin (0.25mg/kg bwt) inhibit eicosanoid production without masking the physical manifestations of endotoxemia (Semrad et al. 1987). Flunixin also decreases the time of inappetence and blocks the signs of abdominal pain (Semrad and Moore, 1987). Treatment of horses with flunixin meglumine in experimental studies blunts the pyrexic effect of LPS infusion but has no significant effect on circulating endotoxin (MacKay et al. 1991). The use of flunixin at 1.1mg/kg has been shown to maintain normal cardiovascular function in experimental endotoxemia and to eliminate some of the physical signs of endotoxemia.

Intravenous fluids. Intravenous fluids are now recognized as an essential part of the management of endotoxemia, maintaining circulating blood volume to optimize oxygen delivery to tissues (Sykes and Furr, 2005).

Hyperimmune plasma. Hyperimmune plasma is harvested from horses that have been immunized against endotoxins. The belief is that anti-lipid A antibodies in the plasma bind LPS to minimize interaction with macrophages and reduce the production of proinflammatory mediators. Hyperimmune plasma may also contain proteins such as the soluble TNF-α receptor,

which can bind to TNF-α and inactivate it. Some studies have shown that plasma has beneficial results and improves prognosis (Peek et al. 2006; Spier et al. 1989) while others have shown no beneficial effects (Morris and Whitlock, 1987). Pretreatment of foals with plasma prior to administration of endotoxin showed worsening of the clinical signs when compared to foals that were not pretreated (Durando et al. 1994). A further study has shown that pretreatment with hyperimmune plasma in adult horses did not significantly change the clinical signs associated with endotoxemia, but it did appear to reduce the TNF bioactivity (Forbes et al. 2012). Whether hyperimmune plasma has a beneficial effect of binding LPS or not, it is a colloid and also contains other nonspecific proteins that may be beneficial for the treatment of endotoxemia.

Polymyxin B. Polymyxin B is a cationic cyclic polypeptide antimicrobial. It has a broad spectrum of activity against gram-negative bacteria at high doses, and at lower doses it binds and neutralizes the lipid A component of LPS. This prevents the interaction of bound LPS with receptors and prevents it from initiating the inflammatory cascade. It has been shown to have a significant dose and time dependent decrease in endotoxin-induced TNF activity (Parviainen et al. 2001). At the recommended doses for treatment of endotoxemia (6,000 U/kg administered every eight hours for five treatments), Polymyxin B was considered safe, and no side effects were observed (Morresey and MacKay, 2006).

Dimethyl Sulfoxide (DMSO). DMSO is a reactive oxygen species scavenger and reduces platelet aggregation (Sykes and Furr, 2005). In mice, DMSO inhibited LPS induced TNF-α production and formation of intracellular adhesion molecules (Kelmer, 2009). One study has shown that a high dose (1mg/kg) of DMSO ameliorated the LPS-induced fever but had minimal effects on other clinical signs (Kelmer et al. 2008). Despite the lack of evidence supporting its use for the treatment of equine endotoxemia, it is widely used.

Additional Treatments. Many other treatment options have been discussed in the literature. The use of antimicrobials to treat endotoxemia is controversial (Sykes and Furr, 2005) but indicated if an infection is present. Pentoxifylline was frequently used at one time. It was reported to improve cardiac output, oxygen delivery, and tissue oxygen uptake in experimental models of sepsis in laboratory animals. In the equine patient, it has been shown to inhibit *ex vivo* endotoxin induced tumor necrosis factor activity (Barton et al. 1997). The true benefit of pentoxifylline for treatment of endotoxemia is unclear. Other treatments that have been investigated include Tyloxapol, Naloxone, anti-TNF antibodies, PAF inhibitors, activated protein

C, insulin and ketamine (Jones, 2009). Some of these treatments show promise in other species but have not been proven efficacious in the equine. The long list of potential treatment options shows that there remains an unmet need for a safe and effective treatment for endotoxemia in the horse.

Mesenchymal Stem Cells

Mesenchymal stem cells are multipotent cells with unique characteristics that distinguish them from other cell types. They are unspecialized and self-renewing cells that can be induced to differentiate into various specialized cell types and they can be expanded *ex vivo*. Embryonic stem cells are derived from the blastocyst and can differentiate into the cells of all three germ layers. Adult stem cells have a more limited differentiation potential. Although MSCs reside in almost all post-natal tissues of the body, including bone marrow, adipose tissue, brain, lung and the liver (Wang and Zhao, 2013) their number within the system declines with age (Borjesson and Peroni, 2011). Donor variation was seen in the rate of proliferation, ability for cell passaging and trilineage differentiation in a small group of gender and age-matched horses (Carter-Arnold et al. 2013).

There are a number of different methods for isolating MSCs, and the Mesenchymal and Tissue Stem Cell committee has proposed a set of standards to define human MSCs. MSCs must be plastic adherent when maintained in standard culture conditions. Greater than 95% of the MSC population must express CD105, CD73, and CD90. The cells must lack expression of CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA class II. Finally, the cells must be able to differentiate to osteoblasts, adipocytes, and chondroblasts under standard *in vitro* differentiating conditions (Dominici et al. 2012).

No standards have been established for equine MSCs, although equine MSCs derived from bone marrow are adherent to plastic, exhibit the ability to differentiate into osteoblasts, adipocytes and chondroblasts and are CD 90 positive (Arnhold et al. 2007). The most common sources of equine stem cells are bone marrow, adipose tissue, and umbilical cord. These stem cells all exhibit the ability to differentiate into osteogenic, adipogenic and chondrogenic cells. More importantly, they exhibit high expression of CD105, CD44, and CD90 with low or negative expression of CD34 and MHC-II (Barberini et al. 2014). One study of adipose tissue produced mixed results, showing an increased expression of CD44 with increased number of

passages in a small number of samples (de Mattos Carvalho et al. 2009). These differences demonstrate that despite similarities to the human definition of stem cells, making uniform conclusions about the true definition of an equine stem cell is difficult. Based on the research performed to this point, De Schauwer et al propose that the definition of an equine stem cell should be that the cell is plastic adherent, exhibits the ability for trilineage differentiation and expresses CD29, CD44 and CD90 while lacking expression of CD14, CD79α and MHC-II (De Schauwer et al. 2011).

The mechanism of action through which stem cells work has not been fully characterized. It is controversial which is more important: their role in primary tissue regeneration by differentiating into the injured tissue or their paracrine effects (Borjesson and Peroni, 2011). In the equine patient, the focus has been on the use of MSCs for tissue regeneration and healing. This is based on MSCs ability to differentiate *in vitro* to the desired tissue type, but this may not reflect what occurs *in vivo*. In fact, labeling studies have shown that MSCs injected into a lesion are lost from the injection site over time (Stewart and Stewart, 2011). There are a few situations in which MSCs appear to differentiate into the required tissue for healing. In models of spinal cord injury, implanted MSCs differentiate into various neural cell types to participate in cord regeneration (Sadan et al. 2009). In cardiac muscle following infarction, MSCs differentiate into endothelial cells, undergo cardiomyogenic differentiation, and can fuse with existing muscle cells to help prolong the survival of the intrinsic cells (Kuraitis et al. 2011)**.** Besides their ability to differentiate, MSCS are known also to have anti-inflammatory and immune-enhancing response (Peroni and Borjesson, 2011).

Immune modulation and Anti-inflammatory Role

MSCs express low concentrations of major histocompatablity complex-I (MHC-I) and do not express major histocompatablity complex-II (MHC-II) (Wang and Zhao, 2013), which contributes to their lack of immunogenicity. In addition, MSCs produce a large number of cytokines, growth factors, chemokines, and immunomodulatory proteins that influence the cells around them. They play a role in increasing angiogenesis, stimulating intrinsic cells to regenerate function, inhibiting apoptosis, and inhibiting scar formation. MSCs are able to induce apoptosis of activated T cells, decrease T cell proliferation, and alter T cell phenotype (Peroni and Borjesson, 2011). MSCs also alter lymphocyte proliferation by inducing the expansion of

regulatory T cells (Siegel et al. 2009). MSCs exhibit these functions even when there is not direct cell contact (Lavoie and Rosu-Myles, 2013), suggesting that they secrete soluble factors as well.

The exact mechanism of MSC modulation is not known, but in the correct environment MSCs are activated to express a number of inhibitory factors including nitric oxide, IDO, IL-10, TGF-β, TSG6, and PGE_2 as well as surface molecules ICAM-1 and VCAM and a number of growth factors including EGF, FGF, PDGF, VEGF, and SDF-1 (Shi et al. 2012). Exposure to pro-inflammatory molecules such as IFN-γ, TNF-α, IL-1 and LPS helps hone the MSCs to the sight of injury and activate them to start secreting their bioactive markers (Shi et al. 2012). MSC honing to the sight of injury is aided by VCAM-1 and E selectin activated by injured endothelial cells (da Silva Meirelles et al. 2009). Phenotype, differentiation potential, and gene expression are altered by *in vitro* passage which may influence the response that MSCs have in their environment (Wagner et al. 2008). There is strong evidence that stem cells on their own do not release these inhibitory factors.

In humans and rodents, MSCs act through secretion of soluble factors or direct cell-to-cell contact to affect T cells, NK cells, B cells, and dendritic cells. MSCs induce apoptosis of activated T cells, induce cell cycle arrest, decrease T cell proliferation, and alter T cell phenotype. They target all T cell subsets (CD4+, CD8+) equally (Najar et al. 2010). MSCs alter NK cell phenotype and suppress cytokine induced proliferation of NK cells (Rasmusson et al. 2007). MSCs also promote the survival and inhibit the proliferation and maturation of B cells by arresting them in the G_0/G_1 phase of the cell cycle (Tabera et al. 2008). MSCs can also modulate dendritic cell maturation, differentiation, and function (Zhang et al. 2009). Currently, the interaction of equine MSCs with B cells and dendritic cells has not been described (Peroni and Borjesson, 2011).

Quiescent equine MSCs derived from multiple sources (bone marrow, adipose tissue, and umbilical cord tissue) do not alter lymphocyte proliferation or secrete mediators except for transforming growth factor–β. The immunosuppressive properties are exhibited when the MSCs are stimulated. Equine MSCs decrease production of TNF-α and IFN-γ while showing increased production of PGE_2 and IL-6 (Carrade et al. 2012). They must be exposed to the inflammatory mediators to exhibit their immunosuppressive abilities.

The sources of MSCs produce slightly different responses. MSCs derived from bone marrow and umbilical cord blood produce nitric oxide, and MSCs derived from adipose tissue do

not (Carrade et al. 2012). The pathways through which MSCs inhibit T cell proliferation are also different between tissue sources. MSCs from bone marrow and umbilical cord blood inhibit T cell proliferation through cell cycle arrest while MSCs from adipose tissue and umbilical cord tissue inhibit T cell proliferation by apoptosis (Carrade Holt et al. 2014).

Role for Stem Cells in Veterinary Medicine

Based on our current understanding of the biology of stem cells, there is substantial potential for the use of stem cells in equine medicine. The role of stem cells to assist with tissue healing has been documented. While stem cells reside in all parts of the body, damage to certain tissues can result in functional impairment during healing when compared to the original tissue type. Equine MSCs are widely used clinically to improve the healing of musculoskeletal disorders, specifically of the tendon and cartilage (Fortier and Travis, 2011).

In human medicine, the focus for use of MSCs has been inflammatory, immune-mediated, and ischemic diseases (Borjesson and Peroni, 2011). It is well recognized now that MSCs play both an anti-inflammatory and immune modulating roles. Their anti-inflammatory attributes could contribute to the treatment of osteoarthritis as well as other systemic inflammatory diseases in veterinary medicine, while their role in altering the immune response may be important in treating autoimmune diseases. Autoimmune diseases are more common in small animals, but there is potential for use of MSCs in disease processes such as inflammatory bowel disease or recurrent airway obstruction in horses.

Endotoxemia and Mesenchymal Stem Cells

The effects of endotoxin are due to its ability to cause an overwhelming inflammatory response; hence treatments that could alter the immune response would be beneficial. Work investigating the use of anti- TNF-α antibodies and IL-10 has produced disappointing results. A number of immunosuppressive properties of stem cells have been recognized to date. They strongly inhibit the proliferation of T cells and also B cells while inducing expansion of the regulatory T cells. Stem cells can inhibit the differentiation of monocytes to immature dendritic cells and prevent the production of TNF-α by dendritic cells. Stem cells can inhibit the proliferation of resting NK cells and therefore reduce cytokine release (Peroni and Borjesson, 2011).

Multiple studies support the positive role that stem cells play in reducing the effects of endotoxin in mice. Endotoxin injection in mice causes a systemic inflammatory response and alterations in lung structure and function. Endotoxemia results in increased numbers of inflammatory cells in the lung associated with pulmonary edema. Untreated mice that received endotoxin exhibited vascular congestion and an increase in neutrophils within the lungs. The mice that received MSCs after endotoxin were no different from the controls. They showed no evidence of vascular congestion or increase in the number of neutrophils within the lungs (Xu et al. 2007).

Cecal ligation and puncture (CLP) is a widely used method of inducing endotoxemia in laboratory rodents. After undergoing the CLP procedure, mice develop clinical signs associated with sepsis from the infection that is established within the peritoneal cavity. The initial inflammatory response is characterized by the release of pro-inflammatory cytokines and results in a hemodynamic response that mimics the course of sepsis in humans (Buras et al. 2005). Multiple studies have shown that an alteration in cytokines (TNF-α, macrophage inflammatory protein-2) within the plasma and bronchoalveolar lavage fluid of septic mice plays a role in altering the immune response to sepsis (Gupta et al. 2007). When mice are subjected to CLP and then treated with MSCs six hours after the procedure, they exhibit decreased production of plasma IL-6, IL-1β and IL-10 (Mei et al. 2010). In addition, administration of MSCs following CLP has demonstrated a reduction in systemic levels of creatinine and reduced the number of apoptotic cells in the kidney (Mei et al. 2010) as well as a reduction in the number of apoptotic and necrotic cells in the spleen (Németh et al. 2009). The kidney is often one of the first affected organs during sepsis. These findings suggest that the stems cells may serve in a protective role to the organs.

MSCs appear to modulate the host's abilities to clear bacterial infection. Significantly fewer colony forming units were present in the spleen of septic mice compared to controls after undergoing CLP (Mei et al. 2010). Mice treated with MSCs following CLP exhibited a mortality of 24% at twenty-eight hours after the procedure while the controls receiving sham treatment exhibited a mortality of 45%. Treatment with MSCs alone appears to improve mortality when compared to controls. Septic mice treated with antimicrobial therapy in addition to treatment with MSCs exhibited significant improvement in survival when compared to the sham treated (Mei et al. 2010). In this study, investigators did not compare survival after the use of

antimicrobials or MSCs individually. Labeling studies also show that the MSCs appear to attract macrophages as they are often surrounded by macrophages (Németh et al. 2009). These mice models provide strong evidence that MSCs may be beneficial in the treatment of endotoxemia.

Respiratory System and Mesenchymal Stem Cells

A number of acute and chronic respiratory diseases affect humans and equines. Horses are not that prone to the high morbidity and mortality from chronic respiratory diseases, but respiratory disease can affect their athletic performance. The lung is also very sensitive to endotoxin, and acute lung injury is often caused by sepsis in very ill patients.

Acute lung injury (ALI) is caused by an acute uncontrolled inflammatory process that disrupts the lung endothelial and epithelial barrier. This disruption leads to pulmonary edema and respiratory failure often referred to as acute respiratory distress syndrome (ARDS). ALI is characterized by the loss of alveolar-capillary membrane integrity, excessive neutrophil migration, and release of pro-inflammatory mediators (Johnson and Matthay, 2010) that result in respiratory failure if not controlled. This can be caused by primary lung injury or can be secondary to other problems such as trauma, sepsis, or endotoxemia. Despite supportive care, it can carry up to a 50% mortality rate in humans. In foals, there is a 60-70% survival rate from ALI reported (Lakritz et al. 1993), and it is often secondary to systemic inflammatory response syndrome or pulmonary infection. Following the initiating event, there is an uncontrolled release of inflammatory mediators. Activated neutrophils and alveolar macrophages move into the pulmonary tissue where they release oxidants, reactive oxygen species, and more cytokines to perpetuate the damage. The alveolar endothelial and epithelial barrier breaks down, leading to pulmonary edema and the initial onset of respiratory distress. Within twenty-four hours, there is a fibroproliferative response characterized by necrosis of type I pneumocyte and proliferation of type II pneumocytes to try to restore the epithelial barrier. Deposition of collagen and fibrosis cause reduced pulmonary compliance. The overall effect is severe hypoxemia with hypo- or hypercapnia due to a ventilation perfusion mismatch (Dunkel and Wilkins, 2009). Treatment is often limited to supportive care and anti-inflammatory drugs. The mainstay of treatment in humans is ventilation, but this is usually not possible in the equine population.

Intrapulmonary administration of bone marrow-derived MSCs showed a promising decrease in the severity of injury to the lung in a murine model of endotoxin-induced ALI (Gupta

et al. 2007). At 24 hours after administration, decreased lung edema was observed. At 48 hours, further decreased lung edema, decreased protein infiltration into the lung, and a reduction in the presence of TNF-α in the bronchoalveolar lavage fluid were observed. At 72 hours, survival in the MSCs group was 64% compared to18% in the control (saline) group. The mice did not receive any other supportive treatment. Intravenous injection of stem cells demonstrated similar results with a decrease in the neutrophil accumulation within the lung and a reduction in the presence of TNF-α (Yang et al. 2011). It further demonstrated a reduction in the neutrophil accumulation within the lung and a decrease in the presence of TNF-α. Intratracheal human stem cells also demonstrated similar results in a sheep model of endotoxin-induced ARDS (Rojas et al. 2014). The treated group showed significant improvement in lung function two hours after treatment when compared to the control group that never returned to baseline values in the six-hour monitoring period. The positive improvements were also confirmed by histopathology. These models provide evidence that the SCs shift the response after injury from a proinflammatory response to an anti-inflammatory response. Further research is needed, but stem cells show promise in the therapy of ALI or ARDS. While ALI has not been well described in adult horses, there are similar conditions, such as recurrent airway obstruction (RAO) that may benefit from stem cell therapy.

RAO in horses is an inflammatory disease process characterized by excessive mucus production, neutrophil accumulation, bronchial hyperreactivity and reversible bronchospasm. Hypersensitivity to inhaled molds and other organic dusts is believed to be the initiating cause with a prevalence from 2% to 80% depending on the inclusion criteria of the study (Léguillette, 2003). The underlying immunologic mechanisms that lead to pulmonary inflammation have not been fully described at this time. In an unaffected horse, any possible stimuli (molds or dust) that enters the airway is cleared by the immune system. In an affected horses, this stimulus activates the lymphocytes, leading to neutrophil recruitment and inflammation within the airway. There is an influx of neutrophils into the airways with an increase in CD4+ T cells in bronchial alveolar lavage fluid (Pirie, 2014). Different studies show inconsistent results whether a T helper 1 or 2 response predominates, but the cytokines involved in the pathogenesis include IL-4, IL-5. IL-8. IL-13 and IFN-γ (Pirie, 2014). Increased concentrations of IL-4 are believed to play a role as well (Horohov, 2000). Treatment for all species is limited to environmental management, anti-inflammatory therapy, and bronchodilator therapy. For horses, the use of corticosteroids, the

mainstay of treatment, may be contraindicated in some patients. Inhaled corticosteroids, which reduce the risk of complications, are now available but may be cost prohibitive for some clients.

RAO is similar to asthma seen in humans and cats, but the disease process varies in that asthma is dominated by an influx of eosinophils rather than of neutrophils. Experimental studies using murine models of ovalbumin-induced asthma have shown that stem cells may be beneficial for managing the disease process. After one injection of human stem cells, there was a significant decrease in the presence of eosinophils in bronchial alveolar lavage fluid, a significant reduction in IL-5, IL-13 and IFN-γ, and a decrease in circulating IgE concentrations (Bonfield et al. 2010). An improvement was shown histologically as well with the treated mice exhibiting a decrease in airway inflammation, goblet cell hyperplasia, epithelial cell lining thickening, and collagen deposition. Similar results were shown in a ragweed-induced murine model of asthma (Nemeth et al. 2010).

In the murine models, sensitized animals are challenged for a variable period of time, and treatment often occurs shortly after initial sensitization. This does not reflect what is seen in the clinical patient. Often the clinical patient has been showing signs of disease for longer periods of time, and the severity of changes at presentation may be more than that induced in the experimental models. In a feline model of chronic allergic asthma, there was no difference in bronchial alveolar lavage cytology between stem cell treated and control groups (Trzil et al. 2014). Computed tomography (CT) was used to assess airway remodeling, and reductions in the scores for remodeling were seen in the treated group at month eight, but this effect was not sustained. Repeated CT at month 12 showed no difference between the treated and control groups (Trzil et al. 2014).

To date, there are no published reports on the use of stem cells to treat RAO in horses. While SCs may be beneficial in the treatment of RAO based on the murine models, the timing of their administration may play a role in response to treatment. The number of doses and follow-up treatment need to be investigated.

Gastrointestinal system and Mesenchymal Stem Cells

Inflammatory bowel disease (IBD) is a broad term used to describe several small and large intestinal disorders in animals. In humans, IBD represents a specific disease process such as ulcerative colitis (the colon only is affected) or Crohn's disease (any part of the

gastrointestinal tract is affected). The clinical signs include diarrhea, weight loss, dependent edema, and lethargy and are often associated with protein-losing enteropathy and malabsorption. The pathogenesis of IBD was believed to be inflammation mediated by the acquired immune system with an imbalance between the Th1 cells and proinflammatory cytokines overcoming the control mechanisms. An alternative theory proposes that it could be a primary failure of regulatory lymphocytes and cytokines to control inflammation. New evidence also suggests that for Crohn's disease, there is resistance to T cells undergoing apoptosis after activation (Bamias et al. 2005), leading to perpetuation of the inflammation.

In small animals and horses, infiltration of the GI submucosa and mucosa with eosinophils, plasma cells, lymphocytes, basophils or macrophages causes IBD (Schumacher et al. 2000). The etiology is often unknown and the type of cell that infiltrates the mucosa and submucosa can affect prognosis.

The current goals of treatment for humans are directed at relieving inflammation and treating signs and symptoms (Panés et al. 2010). Remission is hard to maintain, and patients often suffer from the side effects of drugs and surgeries (Duijvestein et al. 2010). The only treatment available for horses at this time is corticosteroid therapy and the response is often poor (Barr, 2006). Stem cells could prove to be a good alternative with their ability to alter the inflammatory response and to immunomodulate.

In a murine model, colitis was induced by injection of trinitrobenzene (TNBS) in ethanol. Treatment with human adipose derived stem cells showed a dose-dependent improvement in survival of the mice. The mice rapidly recovered body weight and regained a healthy appearance. Down-regulation of the Th1 cytokine response also occurred with induction of regulatory T cell responses (González et al. 2009).

Initial phase one trials of bone marrow derived stem cells for the treatment of refractory Crohn's disease in humans have produced mixed results. Some patients have shown improvement by reduction in their clinical assessment score, improvement in the mucosa on endoscopy, and reduction of inflammation and presence of cytokines on biopsy (Duijvestein et al. 2010), but the beneficial effects may be improved if the stem cells are given earlier in the course of the disease.

IBD is influenced by interactions between genetics, environment and microbial factors. If MSCs are used for the treatment of this inflammatory disease, it is presumed that they will

engraft in the mucosa of the gastrointestinal tract and be exposed to bacteria. Interaction between stem cells and bacteria could alter the immune response. An *in vitro* canine model has shown that MSC behavior is altered by exposure to microbes, and the change in behavior is microbe dependent (Kol et al. 2014).

Murine models have also shown other benefits of stem cells in the gastrointestinal system. Stem cells can accelerate gastric ulcer healing by honing to the site of injury. Labeled stem cells have been found only in the injured gastric mucosa and not in the normal mucosa (Chang et al. 2012). The route of administration for treatment of gastrointestinal disease is important. Mice that received intravenous stem cells showed a significant reduction in clinical and histopathologic severity when compared to mice that received stems cells intraperitoneal stem cells (Gonçalves, 2014). Stem cells have also shown benefits when injected intravenously to attenuate peritoneal adhesions in experimentally induced lesions (Wang et al. 2012). The use of stem cells for preventing peritoneal adhesions may become a further area of interest in the equine field.

Equine Recurrent Uveitis and Mesenchymal Stem Cells

Equine recurrent uveitis (ERU) has often been cited as the most common cause of blindness in horses. In the United States, prevalence has been reported between 8% to 25% (Dwyer and Gilger, 2005). The true etiology and risk factors are not clear, but it is well established that ERU is an immune-mediated disorder characterized by recurrent episodes of inflammation. Immunohistochemistry has shown that the infiltrating cells in the ciliary body are lymphocytes and predominately T-cells with an increased transcription of IL-2 and IFN-γ (Gilger et al. 1999).

Current treatment goals are to improve comfort and to reduce inflammation (Gilger and Michau, 2004). Subconjunctival cyclosporine implants have been shown to reduce the duration and severity of inflammation, cellular infiltration and decrease the production of proinflammatory cytokines (Gilger and Michau, 2004). Not all horses are good candidates for this procedure and it also has possible complications. Topical corticosteroids are the most common anti-inflammatory medication, but their long-term use has side effects that include potentiation of infections, delayed epithelialization of corneal ulcers, and possible potentiation of calcific band keratopathy. A study of recurrent autoimmune uveitis in rats examined long-term

effects of different treatment regimes and compared the efficacy of MSCs to that of dexamethasone. Administration of MSCs at the onset of the disease, reduced the inflammation during the peak of the attack and in the recovery phases. MSC treatments significantly reduced retinal damage and photoreceptor loss (Zhang et al. 2014). They may prove to be a better alternative to the use of corticosteroids in the management of this disease in horses.

This is only a small selection of the equine diseases may further benefit from the use of stem cells. Advances in stem cell biology and therapeutics have been substantial and continue to accelerate. The use of stem cells to enhance wound healing, and for treatment of ischemic, cardiac, renal and neurologic diseases are being investigated and may find application in equine medicine.

Limitations

A number of obstacles must be overcome before stem cells are more widely used. Stem cells are only beneficial for use in acute situations (such as endotoxemia) if they can be administered immediately. The delay necessary for expansion of autologous stem cells may render their usefulness minimal. Allogeneic stem cells may be more useful as they can be administered almost immediately. Human and mice stem cells are immune privileged and poorly immunogenic, but complications have occurred following allogeneic use.

The use of allogeneic stem cells has been examined in horses to a limited degree. In a preliminary study of two horses, allogeneic eMSCs were injected into lesions created in the superficial digital flexor tendon, and no signs of inflammatory reaction or immune rejection were seen (Guest et al. 2008). In another study, a single intra-articular injection of equine umbilical cord-derived stem cells showed no significant difference in response between the joints injected with autologous or allogeneic stem cells (Carrade et al. 2011b). Allogeneic equine umbilical stem cells did not stimulate immediate or delayed hypersensitivity reactions following repeated intradermal injection (Carrade et al. 2011a). Intravenous injection of allogeneic MSCs (0.2×10^6) showed no clinical adverse reactions in 291 horses. A small number of these horses received a second injection six weeks after the first with no reported complications (Broeckx et al. 2014). Three intravenous doses of allogeneic MSCs (25×10^6) cells administered two weeks apart showed no adverse effects in healthy horses (Kol et al. 2015).

Intravenous injection of allogeneic MSCs in cats with chronic kidney disease has produced complications that include vomiting and increased respiratory rate and effort (Quimby et al. 2013). These studies of different animal species suggest that the safety of SC remains unclear and there may be a difference between the healthy and diseased patient.

Determining the appropriate dose necessary for different applications is a challenge. A wide range of doses has been reported, from $10x10^6$ MSCs for tendon lesions (Schnabel et al. 2013) up to 80 $x10^6$ cells systemically for immunomodulation. In humans doses of $1\text{-}2x10^6$ cells/kilogram of body weight are being used in studies for immunomodulation in unhealthy patients (Duijvestein et al. 2010) suggesting that the doses tested in horses may not be sufficient for the treatment of systemic inflammatory disease. The optimum timing of injection remains unclear. If the local tissue environment into which they are injected influences MSCs, then the timing of the stem cell treatment during the acute or chronic phase of the disease may be important.

There is a huge potential for stem cells to treat a wide range of conditions in the horse. The therapeutic potential is attributed to the unique properties of the MSC that target damaged tissues, inhibit the immune and inflammatory response and facilitate repair. A large amount of research is needed to test the efficacy and safety of these novel treatments in equines. This will require properly constructed clinical trials that are often challenging to perform, and are currently lacking.

Chapter 2. The influence of equine bone marrow derived stem cells on the response of cultured peripheral blood mononuclear cells to endotoxin.

Introduction

Endotoxemia is a major cause of morbidity and mortality in horses. It is reported that 30-40% of adult horses presented to a referral hospital for colic have detectable endotoxin in their circulation (Senior, 2011) and up to 50% of foals presenting with septicemia (Barton, 2003). In horses that present for colic mortality is closely linked to the degree of endotoxemia (Thoefner, 2001). The gastrointestinal tract is the most common source of endotoxin, but endotoxemia can develop due to a number of other gram-negative infections such as pleuropneumonia and metritis.

The presence of a large volume of circulating endotoxin initiates a number of cell signaling pathways that can lead to the development of systemic inflammatory response syndrome. There are reported to be over 200 mediators that play a role in the development of SIRS. While there is a large amount of interaction between all of these mediators there are some that play a very important role. TNF-α is one of the earliest mediators to be produced in response to endotoxin and is responsible for producing some of the first clinical signs observed. High levels of circulating TNF-α are associated with mortality. It also plays an essential role in activating further pro-inflammatory mediators. IL-6 is also produced early on in the disease process and contributes to development of the initial clinical signs. High levels of circulating IL-6 have also been associated with increased risk of mortality. IFN-γ is an important immunoregulator with both immunostimulatory and immunosupporessive effects. It plays a role in coordinating the transition from innate to adaptive immunity. IL-10 is generally considered an anti-inflammatory cytokine and has been shown to suppress TNF-α and IL-6 production. Administration of IL-10 has been shown to improve mortality in experimental models of sepsis. However, it has also been reported to have immunostimulatory effects.

There has not been one effective treatment option for endotoxemia. The current goals of therapy are to reduce or prevent the movement of endotoxin into circulation, to neutralize the circulating endotoxin, to prevent or reduce interaction with inflammatory cells and to prevent the synthesis of proinflammatory mediators. NSAIDs are a mainstay of treatment to try and inhibit the metabolites of the arachidonic cascade along with intravenous fluids. Products such as Polymyxin B to bind LPS are frequently used along with DMSO for the reactive oxygen species

scavenging abilities and hyperimmune plasma. Others treatments have been proposed but have shown poor results *in vivo* or lack conclusive evidence on their efficacy to treat endotoxemia.

MSCs have been shown to have anti-inflammatory and immune modulatory effects that may be beneficial for the treatment of endotoxemia. MSCs are multipotent cells with unique characteristics that distinguish them from other cell types. They are unspecialized and self-renewing cells. They can also be induced to differentiate into various specialized cell types and they have the ability to be expanded *ex vivo*. The exact mechanism of MSC modulation is not known, but in the correct environment they have shown to express a number of inhibitory factors including nitric oxide, IDO, IL-10, TGF-β, TSG6 and PGE_2 as well as surface molecules ICAM-1 and VCAM and a number of growth factors including EGF, FGF, PDGF, VEGF and SDF-1. Activation occurs by exposure to pro-inflammatory molecules such as IFN-γ, TNF-α, IL-1 and LPS. These immunomodulatory effects of MSCs may be beneficial for the treatment of endotoxemia.

There were three objectives of this study. The first was to characterize the response of equine MSCs cultured in monolayer to endotoxin exposure. The second was to evaluate the immunogenic properties of equine MSCs. The third objective was to evaluate the ability of equine MSCs to alter the production of inflammatory cytokines by PBMCs exposed to endotoxin. The hypothesis was that equine MSCs will not produce pro-inflammatory cytokines in response to endotoxin exposure and will produce anti-inflammatory cytokines, that MSCs will not elicit an immunogenic response and will block production of pro-inflammatory cytokines when co-incubated with PBMCs.

Materials and Methods

Stem Cell Preparation

Bone marrow aspirates were collected aseptically from two 2-4 year old horses euthanatized for reasons unrelated to musculoskeletal disease. Samples were obtained in accordance with the guidelines reviewed and approved by the Institutional Animal Care and Use Committee of the Virginia Polytechnic Institute and State University. The left tuber coxae of the eMSC donor horses was clipped, aseptically prepared and a bone marrow biopsy needle was used to aspirate a total of 60 milliliters (mls) of bone marrow into 2 syringes each containing 5,000 units of heparin diluted to a volume of 10 mls with phosphate buffered saline (PBS). Bone marrow aspirate was

then transferred to centrifugation tubes, diluted with PBS solution (2:1) and centrifuged at 300 x g for 15 minutes at 4ºC. The cell pellets were resuspended in PBS solution, and centrifugation repeated. Pelleted cells were resuspended in bone marrow MSC medium. The cells were counted and then split into 5 million cells/vial. They were suspended in 0.5mls of media before being added to the freezing medium. The cells were then frozen down in cryovials and stored in liquid nitrogen cylinders.

Bone Marrow Media Preparation

Bone marrow media was prepared aseptically in hood using 500mls of low glucose DMEM + GlutaMAX-1[a] with 10% (50ml) MSC qualified fetal bovine serum[b] and 1% (5ml) penicillin streptomycin[c] after they were warmed to room temperature. The fluid was filtered through a Nalgene™ Rapid-flow ™ sterile 0.2µm SCFA membrane [d]. For the actual experiment the fetal bovine serum concentration was decreased to 5% (25ml). Bone marrow media was stored in the refrigerator until it was used. It was warmed to room temperature before use each time.

Thawing Stem Cells

The cap was removed from the vial of stem cells and placed in a sterilized box with metal beads and allowed to thaw. Once thawed the contents of the vial were added to 25mls of bone marrow media with a 2ml pipette and then resuspended. The cells were spun at 800g for 10 minutes at 4 degrees Celsius. The supernatant was discarded and the cell pellet is resuspended in 10mls of fresh media. 100µl sample was removed for cell counting.

Stem Cell Count

A 1:10 dilution of the stem cells was made using 400µl PBS, 50µl Tryphan blue and 50µl cells. The solution was mixed well and then 10µl of the solution was placed onto the Neubaur chamber. The live and dead cells were counted in all four big corner cells on 10x power. The total cell count, percent dead and percent live cells was calculated. Total cell count was obtained by taking the average live cell count (x) times the area (10^4) times the dilution factor (10) times the volume of cell suspension (10ml).

Plating Thawed Cells

Once thawed and counted the stem cells were then placed into 175cm^2 Nunc^{TM} Cell Culture treated flasks [e]. The ideal cell concentration is 0.0066x10^6 /cm^2. The total cell count obtained is divided by the ideal cell concentration and then divided by the flask volume (175) to obtain the number of flasks to divide the cells among. The flasks are then filled with 60ml of bone marrow media and then incubated at 37 degrees Celsius in a 5% carbon dioxide atmosphere with 90% humidity. Media supplementation was performed every 48 hours by removing half of the media and replacing it with fresh media. The cells were examined every 48 hours to monitor confluence.

Trypsinizing cells

Once approaching 70-80% confluence the adherent cells were trypsinized. All media was aspirated from the flasks and 10mls of phosphate buffered saline was added. The fluid was gently swirled around the flask to remove any residual debris/media. The fluid was then aspirated from the flask by using a fluid trap. 10mls of 0.05% Trypsin –EDTA[f] was added to each 175cm^2 tissue culture flask (5mls for 75cm^2 flask). The flask was incubated for 5 minutes at 37 degrees Celsius. It was gently mixed half way through. An equal volume of bone marrow media was added to the flask to stop the trypsin reaction. The entire contents of the flask were transferred to a 50ml conical centrifuge tube. The cells were centrifuged at 800g for 10 minutes at 4 degrees. The supernatant was carefully aspirated as to not disturb the cell pellet. The pellet was then resuspended in 10mls of fresh bone marrow media and mixed well with a 10ml pipette. A 50µl volume was then removed into a small eppendorf for cell counting.

Peripheral blood mononuclear cell collection

Four healthy mature horses were used for blood collection. All horses were determined to be healthy by physical examination and complete blood count prior to inclusion in this study. None of the horses had been experimentally exposed to endotoxin previously, however the possibility of natural exposure to endotoxin could not be excluded. Complete blood counts were performed using an automated cell counter. Peripheral blood mononuclear cell collection occurred the day prior to the experiment.

Blood was collected aseptically into lithium heparin vacutainer tubes[h] and gently mixed (10 tubes per horse). Blood was centrifuged for 10 minutes at 600g. Six milliliters of media was removed from a 100ml bottle of RPMI-1640 medium with L-glutamine and $NaHCO_3$[i]. 10% (10ml) of heat intactivated fetal bovine serum (Sigma-Aldrich), 1ml Penicillin-Streptomycin[j], and 10µl Gentamicin solution[k] were added to make a complete media. The buffy coat was aspirated from the lithium heparin tubes after centrifuging and resuspended in 6 ml of the complete RPMI 1640 media. Four ml of the RPMI-1640 media and buffy coat suspension was gently layered onto 8 ml of lymphoprep[l] in a 15ml conical, sterile, screw-top vial. The vial was centrifuged at 350g at 4°C for 30 minutes. Cells at the lymphoprep-cell interface were aspirated and resuspended in 1 ml PBS. The cells were then washed 3 times following these steps:

a)Dilute cell suspension with 1ml PBS

b)Centrifuge cell suspension 5 minutes at 3500 RPM,

c)Decant supernatant

d) Resuspend in 1ml PBS and mix

After the final wash the supernatant was decanted and the cells were then suspended in 1ml of PBS. Cell count was obtained by using an automatic blood cell counter. Manual differential cell count was performed to ensure greater than 90% lymphocyte recovery using Dif Quick[m]. The cells were stored overnight in an incubator. The morning of experimentation the cells were centrifuged for 5 minutes at 3500 RPM. The supernatant was discarded and then cells were resuspended in bone marrow to obtain the required concentration (200,000 cells/ml or 2,000,000 cells/ml)

Lipopolysaccharide preparation

Lipopolysaccharide (O55:B5)[n] was prepared by reconstituting with 1ml of DMEM to produce a 1mg/ml solution. The LPS was then aliquoted out into 100µl volumes. The samples were frozen immediately or used. When used the sample was allowed to return to room temperature and mixed well prior to use. The LPS was then diluted out to the required concentration.

Mitomycin C preparation and cell treatment

2mg of powdered Mitomycin C[o] was reconstituted with 5 ml of sterile PBS (Lonza) to create a 400µg/ml solution. After collection of the PBMCs as described above or trypsinization of the

stem cells they were adjusted to 1 x 10^6 cells/ml in DMEM. 25 µg (62.5 µl) of Mitomycin C was then added to the centrifuge tubes with the cells. The cells were incubated at 37 degrees Celsius with 5% CO_2 in 90% humidity for 30 minutes. After 30 minutes the cells were spun down at 800g for 5 minutes at 4 degrees Celsius. The supernatant was then poured off and then cells resuspended in 1ml of DMEM. The cells were washed three times as described before use.

Objective 1: Response of MSCs to LPS

The stem cells were trypsinized as previously described and then diluted out to a concentration of 100,000 cells/ml. 100,000 cells in 1ml of DMEM were then placed in 24 well tissue culture plates[p] and returned to the incubator. The day before experimentation the PBMCs were isolated as previously described. On the day of experimentation all required DMEM and other samples were bought to room temperature. The PBMCs were centrifuged for 5 minutes at 3500 RPM. The supernatant was discarded and then cells were resuspended in DMEM to obtain the required concentration of 200,000 cells/ml (100,000 cells/500µl). LPS was prepared from the stock solutions to make concentrations of 2ng/ml, 20ng/ml, 200ng/ml and 400ng/ml. PBMCs were used as positive controls and 500µl of PBMCs (100,000 cells) were added to the 24 well tissue culture plates. All the DMEM was removed from the eMSCs and replaced with 500µl of fresh DMEM. For the controls the wells were topped up with a further 500µl of DMEM for a total volume of 1ml in each well. Further control wells were filled with 1ml of DMEM only. The remainder of the MSCs and PBMCs were then incubated with 1,10,100 or 200ng/ml of LPS. There was a total of 1ml of fluid in each well. The samples were returned to the incubator. All samples were preformed in triplicate. At 6, 12 and 24 hours media was collected from each well. The eMSCs were removed from the tissue culture wells by using 200µl/ well of TRIzol® Reagent[q]. The cells were lysed by pipetting the cells up and down several times. All samples were stored in the freezer at -80 degrees Celsius.

Objective 2: Cell proliferation

PBMCs and SCs prepared to concentration of 1x10^6 cells/ml in bone marrow media. Stimulator cells were treated with mitomycin C as previously described. 50µl responder cells and 50µl of stimulator cells added to each well. Stimulator cells were either mitomycin C treated SC or PBMCs. Multiple combinations of SC with each horse PBMCs and PBMCs with other horse

PBMCs were used. These wells were topped up with a further 200µl of DMEM. Positive controls were PBMCs exposed to 10ng of LPS. The LPS was prepared as previously described and added to each well. The final fluid volume in each well was 300µl.

Cell Proliferation ELISA, BrdU(colorimetric)[r]

The cells were placed in an incubator (37°C, 5% CO_2, 90% humidity) for 5 days. BrdU labeling reagent was prepared by diluting concentrate 1:100 in DMEM. 30µl of BrdU labeling reagent was added to each well and then the plate was reincubated for 24 hours. After 24 hours the plate was centrifuged at 300g for 10 minutes and the labeling medium removed by aspiration. 200µl of FixDenat was added to each well. The plate was then incubated for 30 minutes at room temperature. The FixDenat solution was removed by flicking off and blotting on an absorbant towel. To prepare anti-BrdU-POD stock solution it was dissolved in 1.1ml of distilled water for 10 minutes and mixed thoroughly. The working solution was then prepared by adding 100µl anti-BrdU-POD stock solution, in 10ml antibody dilution solution. 100µl of anti-BrdU-POD working solution was added to each well. The plate was incubated for 90 minutes at room temperature. The antibody conjugate was removed by flicking off. Washing solution was prepared by diluting 10ml of washing buffer concentrate with 90ml of distilled water. The wells were rinsed three times with the washing solution and then the remainder of the fluid blotted out on absorbent towel. 100µl of substrate solution was added to each well. The plate was incubated for 30 min for sufficient photometric detection. 25µl of 1M H_2SO_4 was added and the plate was mixed thoroughly by tapping the sides of the plate for 1 minute. Absorbance was then measured on a microplate reader at 450nm.

Objective 3: Influence of eMSCs on cytokine production

The stem cells were trypsonized as previously described and then diluted out to a concentration of 100,000 cells/ml. 100,000 cells in 1ml of DMEM were then placed in 24 well tissue culture plates and returned to the incubator. The day before experimentation the PBMCs were isolated as previously described. On the day of experimentation all required DMEM and other samples were bought to room temperature. The PBMCs were centrifuged for 5 minutes at 3500 RPM. The supernatant was discarded and then cells were resuspended in DMEM to obtain the required concentration of 2 million cells/ml (1 million cells/500µl). LPS was prepared from the stock solutions to make concentrations of 200ng/ml. All the DMEM was removed from the eMSCs.

The blank control wells were replaced with 1ml of DMEM. The co-incubated wells had Millicell 0.4µm cell culture inserts[s] placed within the wells. 1 million PBMCs in 500µl were then placed within each cell culture insert. The wells were then either filled with DMEM or 100ng of LPS for a total volume in each well of 1ml. The positive control PBMCs were placed in wells at 1million cells with either DMEM or 100ng of LPS. The samples were returned to the incubator. All samples were preformed in triplicate. At 6, 12 and 24 hours media was collected from each well. The eMSCs were removed from the tissue culture wells by using 200µl/ well of TRIzol® Reagent. Pipetting the cells up and down several times lysed the cells. The PBMCs were removed from the cell culture inserts the same way. All samples were then stored in the freezer at -80 degrees Celsius.

ELISAs

TNF alpha ELISA[t]

To prepare the plate, the coating antibody was diluted 1:100 in carbonate/bicarbonate buffer (110µl coating antibody in 10.89ml carbonate/bicarbonate buffer). 100µl of diluted coating antibody was added to each well. The plate was covered with a plate sealer and incubated overnight at room temperature. Following incubation the coating antibody solution was aspirated from each well and 300µl of blocking buffer was added to each well. Blocking buffer was prepared by adding 29ml of 35% bovine serum albumin to 221mls of Dulbecco's phosphate buffered saline. The plate was covered and incubated for 1 hour at room temperature. The blocking buffer was then aspirated from the plate and allowed to dry overnight at room temperature. The plates were then sealed with a plate sealer and stored in the refrigerator until use.

For the ELISA assay the standard was reconstituted in 1.12ml of reagent diluent to a concentration of 10,000pg/ml. The reconstituted standard is then further diluted 1:10 in reagent diluent to a concentration of 1,000pg/ml. Further serial dilutions of the high standard at a dilution of 1:2 were prepared. The final result was standards of 1,000pg/ml, 500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml, 31.25pg/ml, 15.625pg/ml and 7.8pg/ml. 100µl of sample or standard was added to each well. The plate was covered with a plate sealer and incubated for 1 hour at room temperature. After incubation the sample was aspirated and washed three times with a plate washer. Wash buffer was prepared by adding 0.5ml of Tween 20 to 1 liter of Dulbecco's

phosphate buffered saline. The detection antibody was then diluted in reagent diluent by adding 110µl of detection antibody to 10.89ml of reagent diluent. Then 100µl of detection antibody was added to each well. The plate was covered again with a plate sealer and incubated again for one hour. The samples were then aspirated and the plate washed with wash buffer three times as previously described. SA-HRP was prepared by adding 30µl SA-HRP to 12ml of reagent diluent (1:400). 100µl of diluted SA-HRP reagent was added to each well. The plate was covered with a plate sealer and incubated for 30 minutes at room temperature. The samples were then aspirated and the plate washed with wash buffer three times as previously described. 100µl of substrate solution was added to each well. The plate was covered again with a plate sealer and incubated in the dark for 20 minutes at room temperature. The reaction was stopped by adding 100µl of stop solution to each well. The absorbance was measured at A_{450} minus A_{550}. The detectable limits of the assay were 7.8 pg/ml to 1,000 pg/ml.

Interferon Gamma[u]

The equine IFN-gamma standard was reconstituted in 750µl of reagent diluent. Reagent diluent was prepared by adding 29ml of 35% bovine serum albumin to 221mls of Dulbecco's phosphate buffered saline. The standard now has a concentration of 37ng/ml. Serial dilutions of the standard were prepared by mixing 250µl of standard with 250 µl of reagent diluent. The dilutions produced samples at 37, 18.5, 9.25, 4.625, 2.312, 1.156 and 0.578ng/ml. 100 uL of Standard or sample was added to appropriate wells and covered with a plate sealer. The plate was incubated at room temperature for 1 hour. After incubation the sample was aspirated and washed four times with a plate washer. Wash buffer was prepared by adding 0.5ml of Tween 20 to 1 liter of Dulbecco's phosphate buffered saline. The plate was blotted on absorbent towel following washing. The equine IFN-gamma detection antibody working solution was prepared by reconstituting the detection antibody in 500µl reagent diluent. Dilute the 500µl of reconstituted detection antibody in 11.5ml reagent diluent. 100µl of detection antibody working solution was added to each well. The plate was covered and incubated at room temperature for 1 hour. After incubation the sample was aspirated and washed four times with wash buffer with a plate washer as previously described and blotted out following washing. Streptavidin-HRP working solution was prepared by diluting 500µl of Streptavidin-HRP in 11.5ml reagent diluent. 100µl of

Streptavidin-HPR working solution was added to each well. The plate was covered and incubated at room temperature for 30 minutes and then the plate was washed four times. 100 µl of TMB Substrate Solution was added to each well. The plate was developed in the dark for 30 minutes uncovered. The reaction was stopped by adding 100µl of Stop Solution to each well. Absorbance was measured on a plate reader at 450nm. The detectable limits of the assay were 0.578 pg/ml to 37 pg/ml.

IL-6[v]

The kit components and samples were warmed to room temperature before use. The standard was reconstituted with 1.0 mL of standard diluent and shaken gently. The concentration of the standard in the stock solution is 8,000pg/mL. The standard stock was then further diluted out to 2,000pg/mL by adding 250µl of stock solution to 750µl of standard diluent. This sample was serially diluted out to have final standard concentrations of 2,000, 1,000, 500, 250, 125, 62.5 and 31.2pg/ml. 100µL each of dilutions of standard, blank and samples were placed into the wells. The plate was covered with a plate sealer and incubated at 37 degrees Celsius for 2 hours. The samples were then aspirated from the wells. To prepare detection reagent A, assay diluent A concentrate was diluted with 6ml of distilled water. The detection reagent A was briefly centrifuged and then 120µl of detection reagent A was added to 11.88ml of assay diluent A. 100µL of detection reagent A working solution was added to each well. The plate was covered with a plate sealer and incubated for 1 hour at 37°C. Wash solution was prepared by diluting 20mL of wash solution concentrate (30x) with 580mL of distilled water to prepare the working solution. The samples were aspirated and the plate was washed three times using a plate washer. After the last wash the remaining liquid was removed by blotting the plate on absorbent paper. To prepare detection reagent B, assay diluent B concentrate was diluted with 6ml of distilled water. The detection reagent A was briefly centrifuged and then120µl of detection reagent A was added to 11.88ml of assay diluent A. 100µL of detection reagent A working solution was added to each well. The plate was covered with a plate sealer and incubated for 30 minutes at 37°C. The plate was then washed five times as previously described. 90µL of substrate solution was added to each well. The plate was incubated for 25 minutes at 37°C in a dark room. 50µL of stop solution was then added to each well. The wells were gently mixed by tapping the side of the

plate. The plate was then placed in a microplate reader and absorbance measured at 450nm immediately.

IL-10[w]

All reagents and samples were bought to room temperature (18–25 °C) before use. The standards are prepared diluting out assay diluent B (1ml diluent in 4ml of distilled water) and then briefly spinning down the vial containing the standard. 800µl of assay diluent B is added to the standard vial to prepare a 25ng/ml standard. A dilution series was then prepared by placing 300 µl of assay diluent B into eppendorfs and then adding 200 µl of previous concentration to each eppendorf. This creates final standard concentrations of 25, 10, 4, 1.6, 0.640, 0.256 and 0.102ng/ml. 100µl of each standard and sample were placed into appropriate wells. The plate was covered and incubated for 2.5 hours at room temperature. The samples were then aspirated from each well and the plate washed four times with wash solution using a plate washer. Wash solution was prepared by diluting 20ml of wash buffer concentrate in 400ml of distilled water. The plate was blotted following the last wash. The detection antibody was prepared by briefly spinning the vial and then 100 µl of diluted assay diluent B into the vial to prepare a detection antibody concentrate. The concentrate is mixed gently by pipetting and then 100µl of concentrate was added to 8ml of assay diluent B. 100 µl of the prepared detection antibody was added to each well. The plate was incubated for 1 hour at room temperature with gentle shaking. The solution was aspirated and then the plate was washed four times and blotted after. The HRP-Streptavidin concentrate vial was gently mixed and then 20 µl of HRP- Streptavidin concentrate was added to into a tube with 10 ml of diluted assay diluent B. 100 µl of prepared HRP-Streptavidin solution was added to each well. The plate was incubated for 45 minutes at room temperature with gentle shaking. The solution was then aspirated and plate washed four times as previously described. 100 µl of TMB one-step substrate reagent was added to each well. The plate was incubated for 30 minutes at room temperature in the dark with gentle shaking. 50 µl of Stop Solution was added to each well. The plate was read immediately on a microplate reader at 450 nm. The detectable limits of the assay were 0.102 ng/ml to 25 ng/ml.

Statistical Analysis

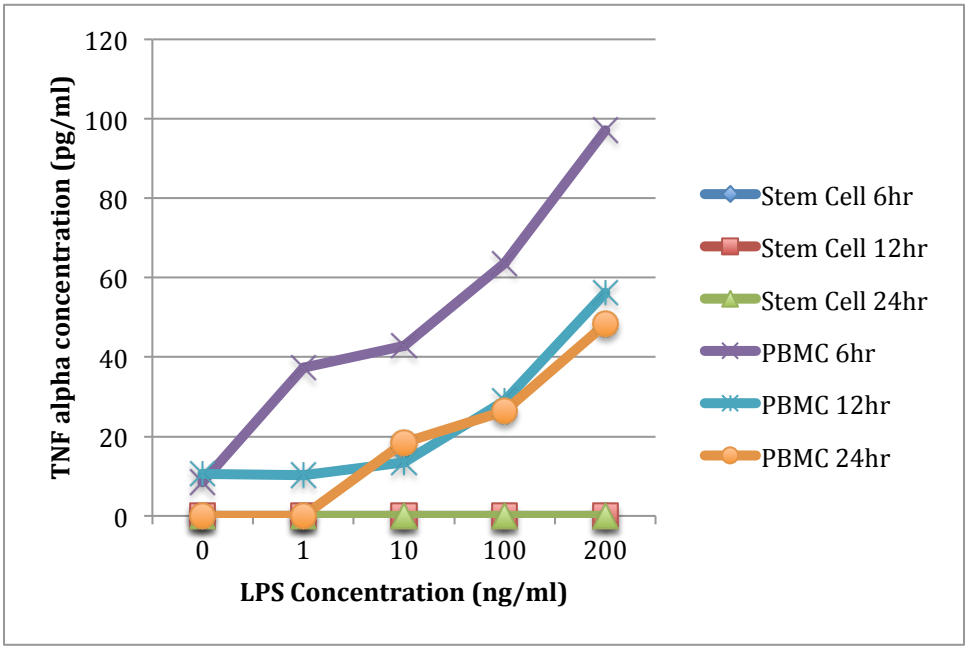
Data was entered into a commercially available desktop statistical analysis program[x]. End points were measured by repeated ANOVA. Posterior pair wise comparison was adjusted by the method of Tukey**.** Assumptions of ANOVA made by examining plot of residual vs. predictive values**.** Non-normally distributed data transformed as necessary**.** *A priori* significance was set a P <0.05

Results

The results confirm previous findings that stem cells do not produce TNF-α in response to LPS. They do produce some IL-6 and production of anti-inflammatory cytokine IL-10 is variable between stem cell lines. MSCs do alter the cytokine production and proliferation of PBMCs *in vitro*.

An initial dose titration curve was performed using 100,000 cells/ml to assess the best dose for stimulation. Both PBMCs and stem cells were utilized. SC line 103258 did not produce any TNF-α while the PBMCs produced a dose dependent response (Figure 4). TNF-α production peaked at six hours. Results for SC line 103136 were more variable with the SCs making a small amount of TNF-α at some time points and the PBMCs had a very variable response. Based on these findings, a stimulating dose of 100ng/ml LPS was selected for further use. Based on the poor response of PBMCs to stimulation, the cell count of future experiments was increased to one million cells/ml.

Figure 4: SC line 103258 TNF-α (100,000 cells/ml) dose titration curve



Objective I: Response of MSC to LPS

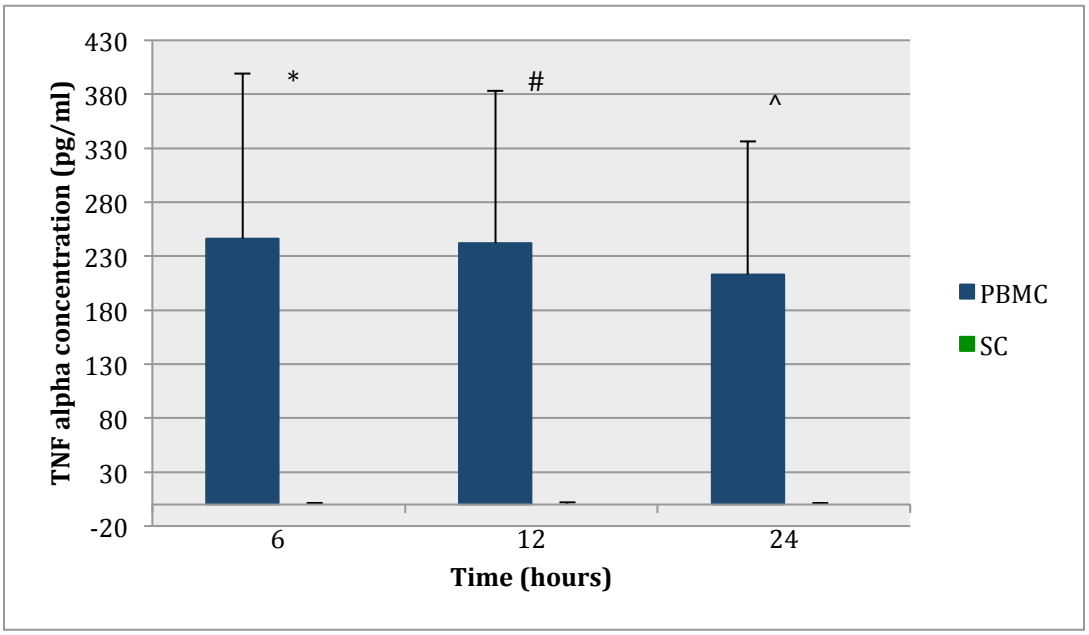
TNF-α

Consistent with preliminary dose response studies, the response of stem cells to LPS stimulation was poor, while the PBMC's had a robust response peaking at 6 hours. There was no statistically significant difference between stem cell lines for analysis of TNF-α so results for both stem cell lines were combined. The concentration of TNF-α was below the limits of detection in the media and negative controls at all time points. At six hours post stimulation there was a statistically significant difference (p=0.045) in concentration of TNF-α between SC and PBMCs. The same was found at twelve hours (p=0.016) and twenty-four hours (p=0.024) with the PBMCs producing TNF-α while the SC did not. (See Table 1 and Figure 5)

Table 1: Mean TNF-α concentration (pg/ml) of cell populations (100,000 cells/ml) stimulated with 100ng/ml LPS

Time (hour)	PBMC	SC	SE PBMC	SE SC	P value
0	0	0	0	0	
6	246.077	0	152.998	1.611	0.045
12	242.237	0	141.091	1.8942	0.016
24	212.81	0	123.872	1.646	0.024

Figure 5: TNF-α concentration of cell populations (100,000 cells/ml) stimulated with 100ng/ml LPS (* p = 0.045, # p = 0.0016, ^p = 0.0024)



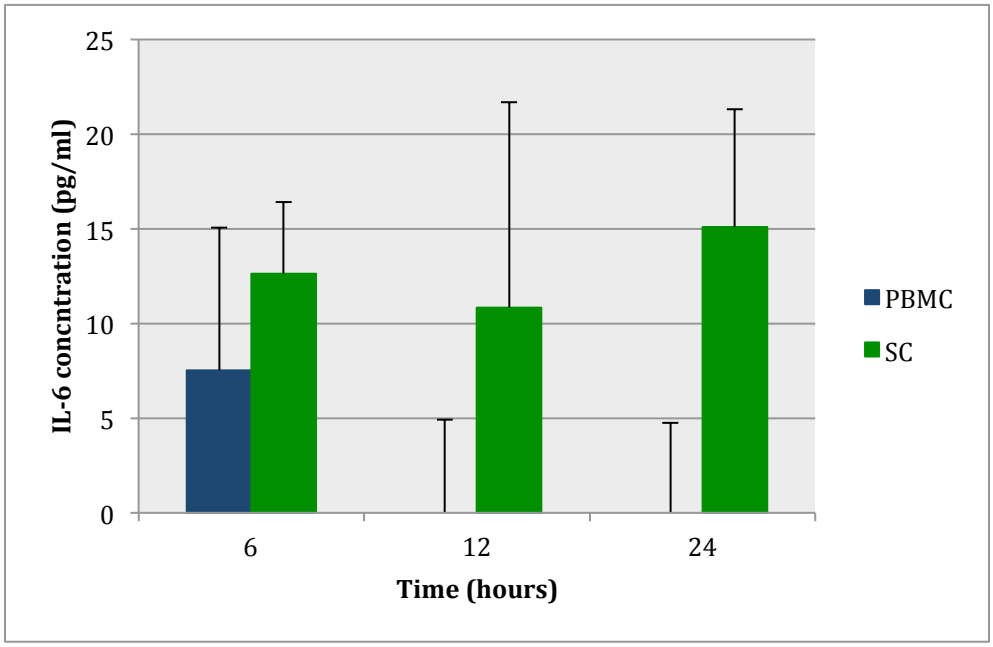
IL-6

There was no statistically significant difference between stem cell lines for analysis of IL-6 so results for both the stem cell line were combined. The concentration of IL-6 was below the limits of detection in the media and the negative controls at all time points. There was no significant difference between the SCs and PBMCs in the production of IL-6 (See Table 2 and Figure 6).

Table 2: Mean IL-6 concentration (pg/ml) of cell populations (100,000 cells/ml) stimulated with 100ng/ml LPS

Time (hour)	PBMC	SC	SE PBMC	SE SC	P value
0	0	0	0	0	0.999
6	7.527	12.648	7.257	3.755	1.000
12	0	10.846	4.932	10.846	0.9868
24	0	15.104	4.75	6.211	1.000

Figure 6: IL-6 concentration of cell populations (100,000 cells/ml) after stimulation with 100ng/ml LPS.



IL-10

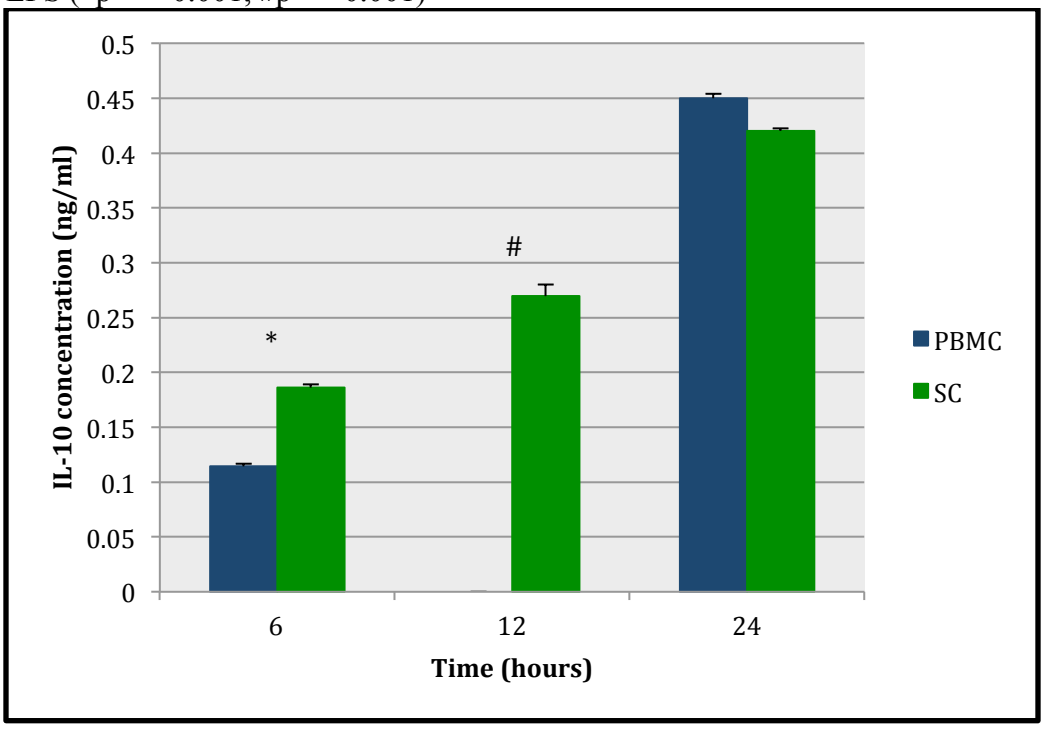
SC line 103136 and the associated PBMCs did not produce detectable concentrations of IL-10. At the lower cell counts (100,000 cells/ml and 500,000 cells/ml), there was no detectable IL-10 produced for SC line 103258 and the PBMCs. The level of IL-10 was below the limits of detection in the media and the negative controls at each time point. At 1,000,000 cells/ml there was low-level production of IL-10. At six and twelve hours, the stem cells produced a significant

(p = < 0.0001) amount more IL-10 than PBMCs. There was similar production of IL-10 between the SC and PBMCs by twenty-four hours (See Table 3 and Figure 7).

Table 3: Mean IL-10 concentration of SC 103258 (1,000,000 cells/ml) stimulated with 100ng/ml LPS

Time (hour)	PBMC	SC	SE PBMC	SE SC	P value
0	0	0	0	0	1.000
6	0.1143	0.186	0.0027	0.003	<0.0001
12	0	0.269	0	0.0107	<0.0001
24	0.453	0.420	0.004	0.0025	0.0002

Figure 7: IL-10 concentration of SC line 103258 (1,000,000 cells/ml) stimulated with 100ng/ml LPS (*p = < 0.001, #p = <0.001)



IFN-γ

Both SC lines and PBMCs did not produce IFN-γ above the detectable limits of the assay.

Objective II: Cell Proliferation

Figure 8 and 9 demonstrate the stem cell proliferation of SC line 103258 and 103136 respectively. In both lines, the stem cells continue to proliferate without stimulation and

proliferation was inhibited when they were treated with mitomycin. In SC line 103136, LPS stimulation increased stem cell proliferation. The opposite occurred for SC line 103258 with LPS stimulation leading to a decrease in proliferation. Unstimulated PBMCs did not proliferate. However, there was minimal proliferation when they were treated with LPS. When mitomycin stem cells were incubated with PBMCs there was no proliferation of the PBMCs.

Figure 8: SC line 103258 Cell proliferation

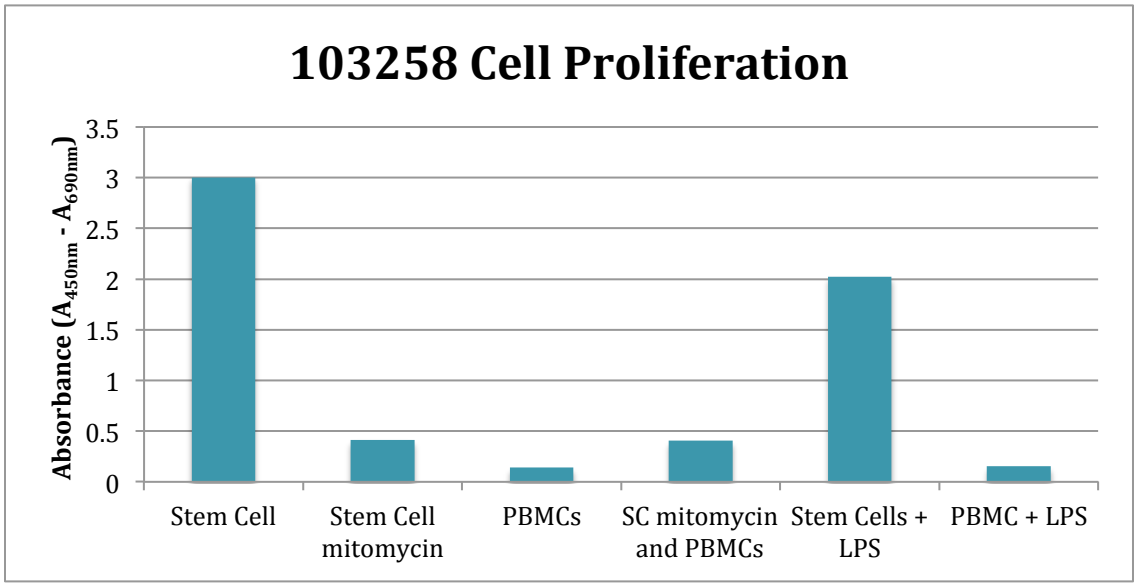
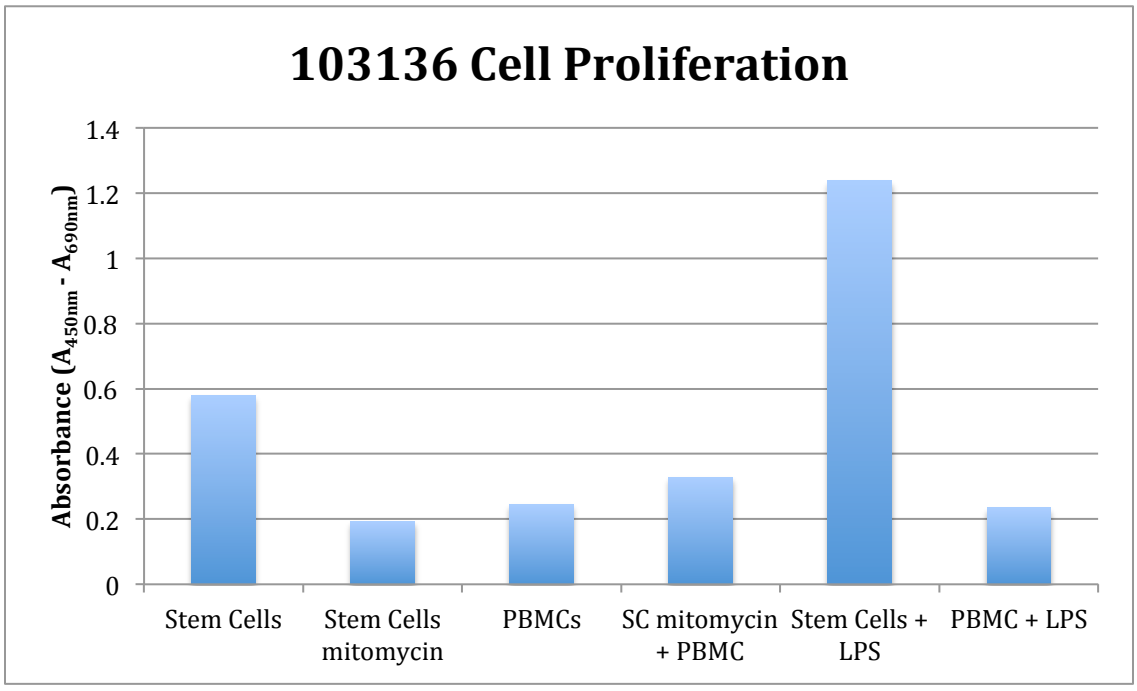


Figure 9: SC line 103136 Cell proliferation



Objective III: Influence of eMSC on cytokine production

TNF-α

Analysis of results found that there was a significant (p = 0.0146) effect of stem cell line on the production of TNF-α, hence results were analyzed separately. The negative controls as well as time zero PBMC's and SC's were negative.

Table 4 shows that unstimulated PBMCs produced low concentrations of TNF-α when compared to stimulated PBMCs. Production of TNF-α was decreased when PBMCs were co-incubated with SC (line 103136) and stimulated with LPS, but this difference did not achieve statistical significance (P> 0.05)(Figure 10). The magnitude of the response was decreased with SC line 103136 (See Table 5 and Figure 11) when compared to SC line 103258, but the trend was consistent between both stem cell lines.

Table 4: Mean TNF-α concentration (pg/ml) of PBMCs coincubated with MSCs (line 103258) and 100ng/ml LPS

Time (Hours)	PBMC	PBMC LPS	SC:PBMC LPS	SE PBMC	SE PBMC LPS	SE SC:PBMC
0	0	0	0	0	0	0
6	99.607	672.59	425.67	46.727	218.744	143.44
12	110.268	576.312	275.248	46.175	189.214	86.38

Figure 10: TNF-α concentration (pg/ml) of PBMCs coincubated with MSCs (line 103258) and 100ng/ml LPS

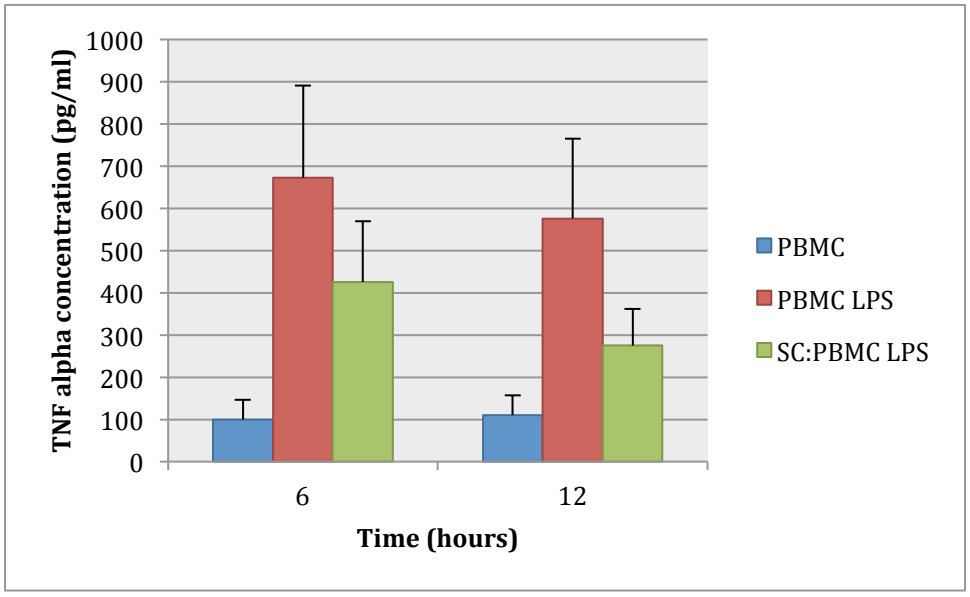
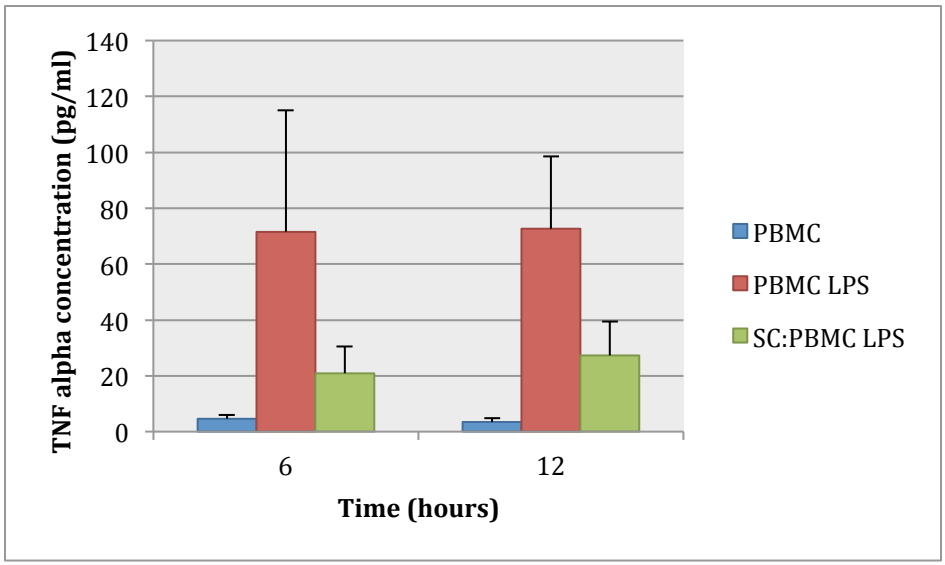


Table 5: Mean TNF-α concentration (pg/ml) of PBMCs coincubated with MSCs (line 103136) and 100ng/ml LPS

Time (Hours)	PBMC	PBMC LPS	SC:PBMC LPS	SE PBMC	SE PBMC LPS	SE SC:PBMC
0	0	0	0	0	0	0
6	0	71.599	20.933	1.268	43.405	9.509
12	0	72.583	27.314	1.302	25.927	12.096

Figure 11: TNF-α concentration (pg/ml) of PBMCs coincubated with MSCs (line 103136) and 100ng/ml LPS



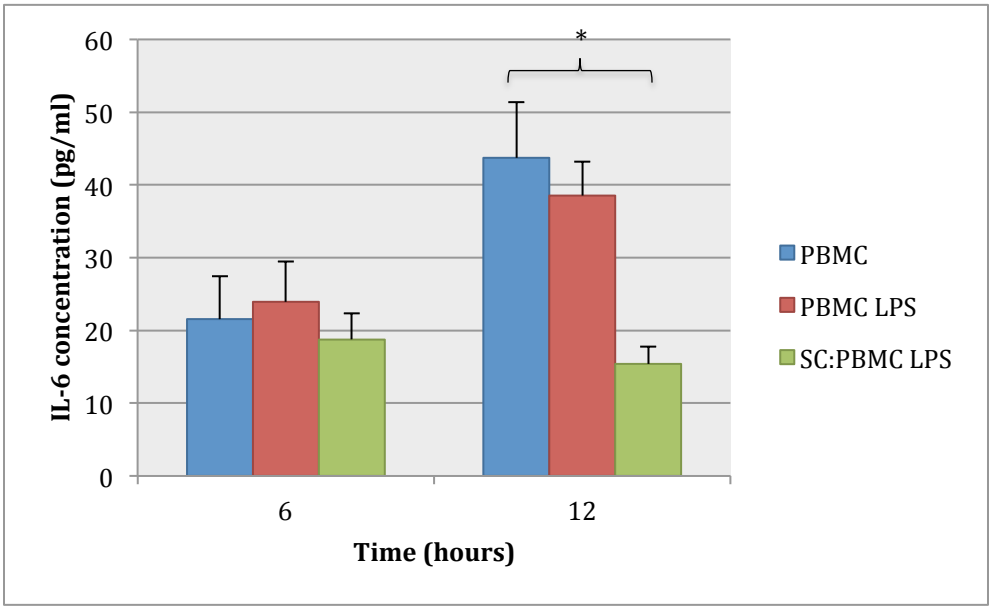
IL-6

There was no significant difference between the stem cell lines and PBMCs in the production of IL-6 so they were analyzed together. The media and negative controls at each time point were below the detectable limit of the assay. PBMCs and SCs at time zero also had IL-6 levels below the detectable limit of the assay. There was no significant difference between production of IL-6 with any cell types at all at six hours. There was a statistically significant difference ($p = 0.0014$) between unstimulated PBMCs and co-incubated SC and PBMCs stimulated with LPS at twelve hours. (See Table 6 and Figure 12)

Table 6: Mean IL-6 concentration (pg/ml) of PBMCs coincubated with MSCs and 100ng/ml LPS

Time (Hours)	PBMC	PBMC LPS	SC:PBMC LPS	SE PBMC	SE PBMC LPS	SE SC:PBMC
0	0	0	0	0	0	0
6	21.57	23.907	18.7925	5.93	5.619	3.552
12	43.73	38.56	15.383	7.64	4.66	2.441

Figure 12: IL-6 concentration (pg/ml) of PBMCs coincubated with MSCs and 100ng/ml LPS (*p = 0.0014)



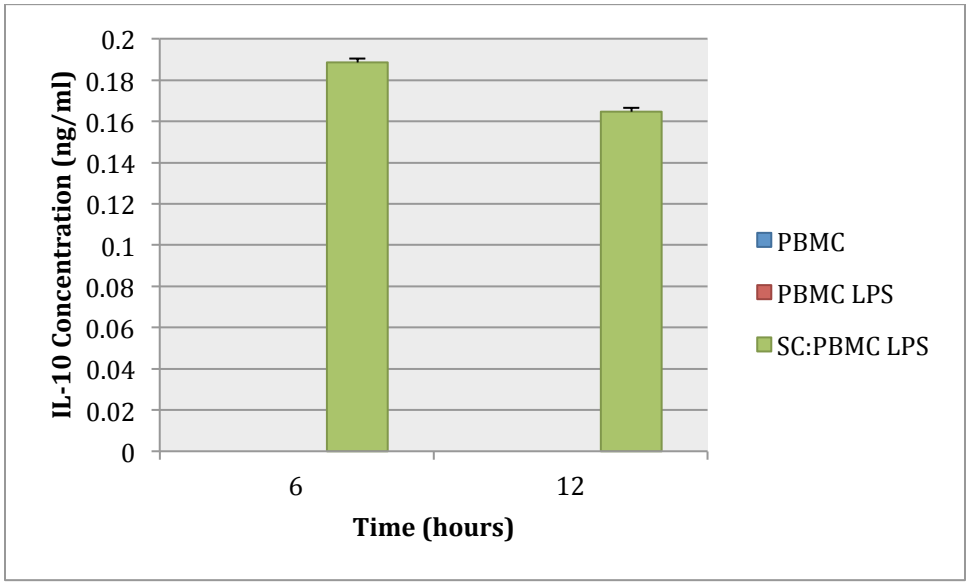
IL-10

SC line 103136 and the associated PBMCs did not produce detectable levels of IL-10. For SC line 103258, detectable IL-10 concentrations were only produced with coincubated SC and PBMCs that were stimulated. There was no statistical significant difference between coincubated SC and PBMCs when compared to unstimulated and stimulated PBMCs. (see Table 7 and Figure 13)

Table 7: Mean IL-10 concentration (pg/ml) of PBMCs coincubated with MSCs (line 103258) and 100ng/ml LPS

Time (Hours)	PBMC	PBMC LPS	SC:PBMC LPS	SE PBMC	SE PBMC LPS	SE SC:PBMC
0	0	0	0	0	0	0
6	0	0	0.1885	0	0	0.00193
12	0	0	0.1645	0	0	0.00205

Figure 13: IL-10 concentration of SC line 103258 co-incubated with PBMCs and 100ng/ml LPS



IFN-γ

Both SC lines and PBMCs did not produce IFN-γ above the detectable limits of the assay.

Discussion

In the current study, the ability of autologous mesenchymal stem cells to influence the response of cells to LPS was investigated; two separate stem cell lines were studied. Results indicated that both stem cells lines demonstrated a similar response in TNF-α and IL-6 production. LPS did not initiate production of the pro-inflammatory cytokine TNF-α. While there was no statistically significant difference in IL-6 production, the MSCs, when stimulated with LPS, tended to produce higher levels of IL-6 when compared to PBMCs. IL-10 production appeared to be variable between stem cells lines.

Previous studies have demonstrated that stimulation of MSCs derived from multiple sources (adipose tissue, bone marrow, umbilical cord tissue and blood) did not produce TNF-α when stimulated (Carrade et al. 2012) and the current findings provide further support. In this study, co-incubation of MSCs and PBMCs with stimulation by LPS causes a decrease in the production of TNF-α. This was observed consistently between both stem cell lines. Despite the magnitude of difference in the production TNF-α between stem cell lines these is clear evidence that MSCs may play a beneficial role in suppressing TNF-α production.

IL-10 production was only detected in one cell line and then only when using a large number of cells – clearly the two stem cells used in this study produce very little IL-10 following stimulation with LPS. However, some stem cell lines may produce IL-10 when there is a higher concentration of MSCs. This one SC line produced a statistically significant amount more IL-10 then PBMCs at six and twelve hours, but by twenty-four hours there was minimal difference between the PBMCs and MSCs. When co-incubated there was an increased production of IL-10 when compared to MSCs and PBMCs alone at both six and twelve hours. It is undetermined at this time which cell was responsible for the observed increased in IL-10 concentration. Other studies have shown that it appears stem cells do not make large amounts of IL-10, but induce the production of IL-10 by other cells (Aggarwal et al. 2005). In mice MSCs it appear that IL-10 may be responsible for inhibiting T cell proliferation (Yang et al., 2009). The role of IL-10 is different between mice and humans in that IL-10 is produced in humans as part of both the Th1 and Th2 response, but only in the Th2 responses by rodents (Mestas and Hughes, 2004). The variation between stem cell lines in the IL-10 production may indicate IL-10 may not play a role in modulating the equine response.

IL-6 production was not statistically significantly different between stimulated SC and PBMCs although there was a trend towards the SCs producing more IL-6 when stimulated. These findings are consistent with other studies that showed that stimulated MSCs secrete significantly more IL-6 when compared to stimulated T cells (Carrade et al. 2012). This is in contrast to other studies which have shown that equine MSCs constitutively express IL-6 (Paterson et al. 2014). The current findings support that fact that MSCs do produce IL-6 when stimulated. When coincubated with PBMCs, by twelve hours, there was a decrease in the secretion of IL-6 suggesting that there may be some influence of the cell types on each other.

Analysis of IFN-γ in both objectives of this study yielded results that were below the detectable limit of the assay. It has been reported that equine MSCs produce low levels of IFN-γ when stimulated by a mitogen and coincubation does not suppress production of IFN-γ (Carrade et al. 2012). In canine MSCs, conicubation with stimulated leukocytes actually increased the production of IFN-γ (Kang et al. 2008).PBMCs stimulated with LPS would be expected to produce IFN-γ. It has been shown that equine PBMCs do produce IFN-γ *in vitro* (Ryan et al. 2010). The lack of response by PBMCs to stimulation with LPS and production of IFN-γ may be related to low cell numbers that did not produce a detectable response. It has also been demonstrated that human PBMCs alter in their response based on the type of mitogen used for stimulation (Brown et al. 2003). This was also shown in equine PBMCs with age and the type of mitogen affecting the amount of IFN-γ produced (Ryan et al. 2010). A more sensitive technique such as PCR may have been more beneficial to detect lower levels of gene expression.

Due to limitations in cell numbers co-incubation of MSCs and PBMCs was not carried out beyond twelve hours. There may have been an alteration in the cytokine response by twenty-four hours. The stem cells may have continued to exert their effect or their efficacy may have decreased suggesting that frequent treatment is required for the most beneficial response.

In this study there was a large variation of the magnitude of production of TNF-α between the two stem cell lines and PBMCs. Experimental variability may have been responsible. Multiple steps are required are for processing the samples and the potential for skipping a washing step or incorrect dilution of a reagent are possible contributing factors to why variability exists. There is also the potential for sample denaturation during storage. Both cell lines were stored in the same conditions so this is less likely.

When MSCs and PBMCs were co-incubated they were not in direct contact with each other. They remained separated by the use of the filter and could only communicate through the media. A positive response was observed by the reduction of TNF-α production by PBMCs. This provides further evidence that the MSCs do not require direct contact to exert their influence. It is unknown if the magnitude of the response may be different if the cells were in direct contact with each other. Injecting the MSCs directly at the source of concern (lungs, kidney, etc.) is not always possible. The soluble effects show that we don't have to administer the MSCs directly to the affected area, but a safer less invasive route of administration may still be beneficial.

Human and mice stem cells have been recognized to be poorly immune responsive and poorly immunogenic. When using a mixed lymphocyte reaction cell proliferation of the responder cell (PBMC) should be minimal to none if the MSCs are poorly immunogenic. Low level of immunogenicity for equine MSCs was also confirmed in the present study. Stem cells from both cell lines showed large amounts of proliferation, which was blocked by mitomycin. The PBMCs, when incubated with the mitomycin treated stem cells, did not elicit any proliferation. However, the positive control cells (PBMC stimulated with LPS) did not demonstrate a strong response, suggesting: possible lack of sensitivity of assay, too low cell numbers or use of all available substrate. This commercially available kit required incubation for five days. If the cells were proliferating rapidly, they could have used up the available media and by the end of the incubation period the viability of the PBMCs was decreased. Overall, the findings suggest that these equine MSCs are poorly immunogenic and there is potential for allogeneic use.

There are a few limitations to this study. Only two stem cell lines were analyzed and characterizing the response with more stem cell lines would have been more beneficial. Using multiple stem cell lines could provide further evidence if the variable responses seen in this study were due to differences in the horse or experimental error. PBMCs were collected from four donor horses. They were considered to be healthy based on physical examination and CBC prior to collection of blood. It is not possible to rule out natural exposure to endotoxin that could influence future cytokine responses. PBMCs were only collected from four different horses. While the PBMC response was similar enough between horses that they were analyzed together a larger population of horses for analysis would be beneficial as there could be some horses that have a different response when exposed to MSCs. The PBMCs in this study were collected the

day prior to the start of the experiment. They were incubated for at least twelve hours prior to treatment with LPS. The cell counts were performed at the time of isolation and there is a chance that the prolonged incubation prior to experimentation may have affected the viability of the PBMCs. Only a small number of mediators involved in the immune response have been evaluated in this study. It has been demonstrated that inhibition of PGE_2 can reverse the MSC inhibition of T cell proliferation and restore the secretion of inflammatory cytokines (Carrade Holt et al. 2014) suggesting that there may be other mediators that play a more essential role in mediating the interaction of MSCs with the immune response in the horse.

Conclusions and Future Directions

MSCs secrete soluble factors that can suppress PBMC cytokine production. In an inflammatory reaction MSCs may be beneficial in down regulating the lymphocyte response, in particular production of TNF-α. MSCs have potential for immune regulation for the treatment of immune mediated and inflammatory disease in the horse. There is potential that there may be variation between some stem cell lines. For allogeneic use there may be some MSC donors that are better than others. Further studies are required to expand our understanding of equine MSCs interactions with other cells of the immune system and evaluate further mediators that may play a role in the equine response. It is essential to evaluate if *in vitro* function correlates with *in vivo* functions and efficacy as well as understanding if the concentrations of mediators produced by the equine MSCs are adequate to have a physiologic effect. The ideal dose of MSCs to treat SIRS and other inflammatory conditions along with the frequency of treatments needs further study.

Footnotes:

a. DMEM (1x)+ GlutaMax™-l, Gibco® by Life technologies, Grand Island, NY

b. Fetal Bovine Serum, Gibco® by Life technologies, Grand Island, NY

c. Penicillin-Streptomycin, Gibco® by Life technologies, Grand Island, NY

d. Nalgene™ Rapid flow™ Sterile Disposable Filter with SCFA membrane, Thermo Fisher Scientific, Waltham, MA

e. Nunc™ Cell Culture Treated EasYFlasks™, Thermo Fisher Scientific, Waltham, MA

f. 0.05% Trypsin-EDTA, Gibco® by Life technologies, Grand Island, NY

g. AC-T diff™ Analyzer, Beckman Coulter®, Brea, CA.

h. BD Vacutainer™ Lithium Heparin, BD Medical, Franklin Lakes, NJ

i. RPMI-1640 Medium, Sigma-Aldrich, St. Louis, MO.

j. Penicillin-streptomycin solution, Sigma-Aldrich, St. Louis, MO

k. Gentamicin solution, Sigma-Aldrich, St. Louis, MO

l. Lymphoprep™, Accurate Chemical & Scientific Corp., Westbury, NY

m. Harleco™ Hemacolor™ Stain Set, EMD Millipore, Gibbstown, NJ.

n. Lipopolysaccharide from *Escherichia coli* O55:B5, Sigma-Aldrich, St. Louis, MO

o. Mitomycin C, Fisher scientific, Fair Lawn, NJ

p. Falcon Tissue culture plate, 24 well, BD labware, Frankline Lakes, NJ

q. Trizol® Reagent, Ambion by Life technologies, Carlsbad, CA

r. Cell Proliferation ELISA, BrdU (colorimetric), Roche Diagnostics, Indianapolis, IN

s. Millicell® Cell culture inserts, Millipore, Cork, IRL

t. Equine TNF-α Reagent Kit, Thermo Scientific, Rockford, IL

u. Equine IFN gamma Elisa Kit, GenWay, San Diego, CA

v. Equine Interleukin-6 Elisa, Uscn Life Science Inc, Hubei, PRC

w. Equine Interleukin-10 Elisa Kit, Sigma-Aldrich, St. Louis, MO

x. SAS, SAS Institute Inc, Cary, NC

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