The Role of the CD14 molecule in equine endotoxemia

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ABSTRACT

Objectives – To evaluate the effects of equine sCD14 and monoclonal antibodies (mAbs) to equine CD14 on LPS-induced TNFα expression of equine peripheral blood mononuclear cells (PBMCs). To determine serum concentrations of soluble (sCD14) in a population of horses with gastrointestinal diseases or other illnesses likely to result in endotoxemia; and identify relationships with clinical data.

Animals – Part 1; 10 healthy horses. Part 2; 55 clinical cases and 23 healthy control horses.

Procedure – Part 1; PBMCs were incubated with *Escherichia coli* LPS, CD14 mAb, sCD14, CD14 mAb plus *E coli* LPS or sCD14 plus *E coli* LPS. Supernatants were collected at 6 hours and assayed for tumor necrosis factor α (TNFα) activity. Part 2; Serum sCD14 was measured at admission and then at 24 and 48 hours after admission using a bead-based multiplex assay.

Results – Part 1; Pre-incubation with CD14 mAb did not inhibit LPS-induced TNFα protein production in isolated equine monocytes. Use of sCD14 inhibited LPS-induced TNFα protein production in isolated monocytes in a concentration-dependent manner. Part 2; Serum concentration of sCD14 was positively related to duration of clinical signs ($P = 0.007$), respiratory rate ($P=0.04$) and band neutrophil count ($P = 0.0002$). There was no correlation between serum concentration of sCD14 and heart rate, temperature, hematocrit, lactate, white blood cell count, fibrinogen, creatinine, urea nitrogen, glucose and anion gap values. Serum sCD14 did not correlate with outcome at any time point for clinical cases.

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ATRIBUTION

Several colleagues and coworkers aided in the writing and research of this dissertation. A brief description of their background and their contributions are included here.

Dr Martin Furr PhD DVM DACIM is the primary Advisor and Committee Chair. Dr Furr was key in the planning and execution of the study. Furthermore, Dr Furr also provided invaluable assistance in the statistical analysis.

Dr Harold McKenzie III DVM MS DACVIM was integral in the review process of the thesis.

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Chapter 1: Literature Review

Endotoxemia

In its literal sense, the term endotoxemia describes the presence of endotoxin in the bloodstream; however it is more usual to associate endotoxemia with the acute systemic pathophysiologic responses to endotoxin. In equine veterinary practice, the concept of endotoxemia is often recognized as "inflammation gone awry".[2] Consequently, it is habitually used to describe the inflammatory response associated with severe illness, regardless of the underlying etiology. The response to endotoxin is an important component of the mammalian innate defense against Gram-negative bacteria, and successful inflammatory response removes offending pathogens in a locally contained and self-limiting reaction. Clinically relevant endotoxemia on the other hand develops as the result of an unchecked systemic activation of inflammatory cells, which may be attributable to overwhelming amounts of endotoxin or bacteria, or an inability of local inflammatory events to eliminate the offending pathogens.[3]

Sensitivity to endotoxin is widespread in mammals but varies distinctly between species. By comparative analysis humans, chimpanzees and horses are particularly susceptible to LPS, whereas mice and rats are relatively resistant to LPS-mediated toxicity.[4] The lethal LPS dose in ponies is 200 to 400 μg/kg, whereas the lethal dose for rabbits and guinea pigs is 3 and 10 mg/kg respectively.[5] In addition to the species-specific sensitivity, individual response to endotoxin is quite variable. Wurfel et al[6] showed considerable variability in responses of whole blood leukocytes to LPS in a population of 102 healthy human subjects, with individuals showing consistently high or low cytokine responses to the same dose of LPS measured by ELISA. In that study, values for TNFα varied more than three orders of magnitude, and values for IL-1β spanned a 300-fold range. The authors of that study suggested that this variation was much greater than would be expected due to technical disparity and could not be explained only by variation in monocyte counts.

Sources of endotoxin include exogenous Gram-negative pathogens causing infection (e.g. colitis, pleuropneumonia, and metritis) or endogenous Gram-negative bacteria and/or LPS from the gastrointestinal tract. Even in health, the host is constantly exposed to small amounts of endotoxin that translocate across the intestinal wall.[7] In healthy humans, plasma endotoxin

concentrations of 3-10 pg/ml have been measured, while concentrations exceeded 300 pg/ml in human patients diagnosed with septic shock.[8] Translocated endotoxin is normally taken up and cleared by the mononuclear phagocytic system in the liver, such that it does not activate a systemic inflammatory response. Increased translocation of endotoxin, which overwhelms hepatic removal mechanisms and causes clinical disease, is thought to be responsible for the development of endotoxemia in primarily non-infectious conditions such as severe thermal injury[9], strenuous exercise[10,11] or any other disease that compromises mucosal barrier function. Translocation mechanisms are particularly important in horses, where large number of Gram-negative bacteria and therefore large amounts of LPS reside in the intestinal tract[12-14] and acute gastrointestinal disease is a common occurrence. There are several mechanisms present to prevent LPS from reaching the systemic circulation during normalcy. Tight mucosal junctions between epithelial cells, secretion from epithelial cells and resident bacteria comprise the intestinal mucosal barrier that prevent absorption of significant amounts of LPS into the circulation.[2] However, under disease conditions, endotoxin can overwhelm these mechanisms and play a major role in the systemic inflammatory response in the horse.[2]

Endotoxemia in the horse most often occurs secondary to compromise of the intestinal mucosal barrier. The quantity of endotoxin released into the circulation can become so great that it cannot be cleared and thus overwhelms the normal protective mechanisms. Endotoxin can also cross hypoperfused or inflamed intestine, enter the peritoneal cavity, and gain access to the systemic circulation via the thoracic duct.[2] Alternatively, LPS that originates from sites in the body other than the gastrointestinal tract, such as the peritoneal cavity, pleural cavity or uterus, may bypass the portal circulation and directly enter the systemic circulation.[15] Environmental endotoxin may gain access to the host via inhalation. When housed in traditional indoor management systems, horses may be exposed to sufficiently high enough concentrations of LPS via inhalation to contribute significantly to pulmonary inflammation and bronchial hyper-responsiveness.[16,17] There are many disease processes of the horse that may lead to the development of endotoxemia. Acute abdominal disease, colitis, post-operative ileus, enteritis, peritonitis, pleuropneumonia, metritis, and grain overload are all capable of inducing secondary endotoxemia in the horse. In the neonatal foal, endotoxemia is most commonly resultant of Gram-negative sepsis and can be acquired during gestation, parturition or in the early neonatal period.[2] Most commonly, equine endotoxemia results from an acute gastrointestinal tract

disturbance. Endotoxin has been detected in the plasma of approximately 25%[13,18,19] of adult horses admitted to teaching hospitals for acute gastrointestinal tract disease and in 40% to 50% of neonatal foals presented with suspected septicemia.[20] Experimentally, intravenous infusion of LPS decreases cecal blood flow and increases intestinal permeability, thus allowing endotoxin access to the systemic circulation.[21] In an experimental model of small intestinal ischemia/reperfusion injury, circulating endotoxin was detected at 60 and 120 minutes after reperfusion.[22] Clinical studies have shown that horses are endotoxemic during colic surgery, and that circulating LPS can be detected in a large proportion of horses up to 5 days after colic surgery.[23]

Intravenous infusion of LPS has been used extensively in the past as a method for inducing experimental endotoxemia. Although experimentally-induced endotoxemia does not exactly replicate or mimic clinical disease, it allows for investigation of various aspects that cannot be studied or controlled in the clinical arena. Typically following administration of low dose LPS (10-35 ng/kg), horses become mildly colicky and develop fever, tachypnea, tachycardia, an initial neutropenia with a rebound neutrophilia, thrombocytopenia, increased capillary refill time, hemoconcentration, and lactic acidosis.[24,25] Leukopenia is one of the cardinal signs of endotoxemia and can occur between 30 minutes and 4 hours post-infusion.[26,27] Higher doses of LPS (over 10 μg/kg) have resulted in mucous membrane cyanosis, hyper- and hypoglycemia, hyper- and hypothermia, and extreme coldness of the lower limbs.[28] Endotoxemia has been shown to cause decreased gastrointestinal tract motility in horses, specifically decreased intestinal muscular activity due to edematous degeneration and coagulative necrosis of smooth muscle cells of the intestinal muscularis layer and possibly degeneration of the central, autonomic, and myenteric nervous systems.[29,30] Prostaglandin E2 decreases gastric contraction amplitude and rate in endotoxemic ponies.[31] This decrease in gastric activity can be prevented by pre-treatment with phenylbutazone, a known COX-1 inhibitor.[32] Insulin resistance is caused by endotoxemia in humans and rats. In horses, administration of endotoxin resulted in decreased insulin sensitivity for 24 hours along with a compensatory pancreatic response.[33]

One major complication of diseases accompanied by endotoxemia in horses is the development of laminitis. Endotoxin has been detected in both cecal contents and plasma of horses with experimentally-induced carbohydrate overload laminitis, and has been associated with Obel Grade 3 lameness in this model.[12,34] Severe compromise of the cecal mucosa allows

numerous toxins, including endotoxin, to enter the circulation and stimulate the release of pro-inflammatory cytokines, as demonstrated by the increased plasma endotoxin concentrations seen in CHO-induced laminitis.[34] Experimentally-induced endotoxemia leads to decreased digital arterial and venous blood flow, decreased digital laminar blood flow, and decreased coronary band and hoof wall temperature.[23] Endotoxemia has also been implicated as an important risk factor for the development of acute laminitis in horses hospitalized for medical or surgical conditions.[35]

Major pathophysiologic events during the development of endotoxemia include the acute phase response; neutrophil activation, margination and extravasation; activation of the coagulation, fibrinolytic and complement cascades; as well as endothelial injury and microvascular failure.[36] If allowed to proceed in an uncontrolled fashion, these responses culminate in cardiovascular insufficiency (shock), organ failure and ultimately death. Pathophysiologic events are in part mediated directly by endotoxin, but predominantly by a multitude of cytokines and other inflammatory mediators. These include tumor necrosis factor α (TNFα), the interleukins IL-1, IL-6, and IL-8, platelet activating factor, the arachidonic acid metabolites (prostanoids), kinins, complement components, reactive oxygen species, histamine, and colony stimulating factors which induce leukocyte production.[37] Mediators that primarily have an anti-inflammatory effect include IL-10, IL-4 and IL-1 receptor antagonist.[38] It is important to recognize that endotoxin stimulates the release of pro-inflammatory as well as anti-inflammatory mediators simultaneously, and that it is the balance of these mediators– or the lack thereof – which determines the outcome of an inflammatory reaction. While endotoxemia is typically interpreted as an excessive stimulation of pro-inflammatory responses, a "compensatory anti-inflammatory response syndrome" has been described and constitutes an overwhelming production of anti-inflammatory mediators leading to a state of immunological anergy and therefore increased susceptibility to infection.[39,40]

Endotoxin

During studies of the pathogen *Vibrio cholera* in the late 1890s, the German physician and microbiologist, Richard Pfeiffer, found that even killed vibrios could evoke illness in laboratory animals. He hypothesized that the cause was a toxin in the bacterial cell wall. Because it was endogenous to the microorganism and toxic to the host, he termed this factor 'endotoxin' (from the Greek "endo", meaning within).[41] Interestingly, the Italian pathologist Eugenio Centanni was making a similar discovery. Centanni extracted a heat stable toxin from *Salmonella typhi* and named it 'pyrotoxina' (from the Greek "pyros", meaning fire) for its ability to induce fever.[41] A great deal of work was done through the next decade analyzing these bacterial extracts. However, it was not until the 1920's and 30's that chemists determined the bacterial extracts were complex structures composed of polysaccharide, phospholipid and protein.[42]

Endotoxin or bacterial lypopolysaccharide (LPS) is an intrinsic component of the outer membrane of Gram-negative bacteria and is essential for the viability of enteric bacteria. LPS makes up about 75% of the entire outer membrane of enteric bacteria, and up to 4 million LPS molecules are found in each bacterial cell wall.[3] LPS molecules are released from the cell wall during bacterial growth and in large numbers upon bacterial death; which can be exacerbated by antibiotic-induced lysis of bacterial cells.[43-45]

LPS is an amphiphilic molecule, which forms large aggregates in solution. Structurally, LPS is composed of three interconnected structures that give the molecule its polar and amphipathic characteristics. The basic structure consists of a hydrophobic lipid A domain, an oligosaccharide core, and a distal polysaccharide (O-antigen) (Figure 1). The lipid A is linked through the core region to the O-antigen. This O-antigen is hydrophilic and the most surface exposed part of LPS. It is usually heterogeneous in length and quite variable in structure from one bacterial strain to the next. Among enteric Gram-negative bacteria, the O-chain is highly variable and is responsible for the serologic characteristics of bacterial strains. Colonies of bacteria producing LPS that contain the O-chain have a characteristic "smooth" appearance, which led to use of the term "smooth LPS" for these molecules. In comparison, mutant bacteria with a defect or lack of the gene cluster responsible for O-chain synthesis exhibit different colony morphology and their LPS is denoted as "rough". [42] Although rough LPS mutants are

viable *in vitro*, the O-chain is required for survival of bacteria in host organisms, by enabling bacteria to evade phagocytosis and complement-mediated destruction.[46] The lipid A domain is characterized by four to seven acyl side chains with slightly different lengths and phosporylation of one or two glycosamines.[47] The structure of lipid A is highly conserved but not identical among Gram-negative bacteria genera, and it is responsible for the major part of the biological activities of the LPS molecule. The variety of lipid A structures between bacteria explains the difference in the biological activity of LPS. Changing the number of acyl groups from six to five or seven decreased the biological activity of LPS 100-fold.[48]

The innate immune system is able to detect picomolar concentrations of LPS to trigger the cellular response.[49] LPS spontaneously forms aggregates (micelles) in aqueous solutions with its hydrophobic lipid section in the center of the micelle and the hydrophilic polysaccharide components displayed on the outside surface. In biologic fluids, such as plasma, LPS rapidly interacts with a variety of serum or membrane-bound lipophilic proteins. Very little LPS circulates freely in the plasma as virtually all LPS molecules are rapidly complexed with circulating proteins and lipoproteins. LPS receptors are targeted against the conserved lipid A moiety. Three receptors for LPS have been recognized, which are the soluble or membrane-bound CD14-MD2-TLR4 molecules, the CD11/CD18-complex and the scavenger receptors for lipid molecules.[8]

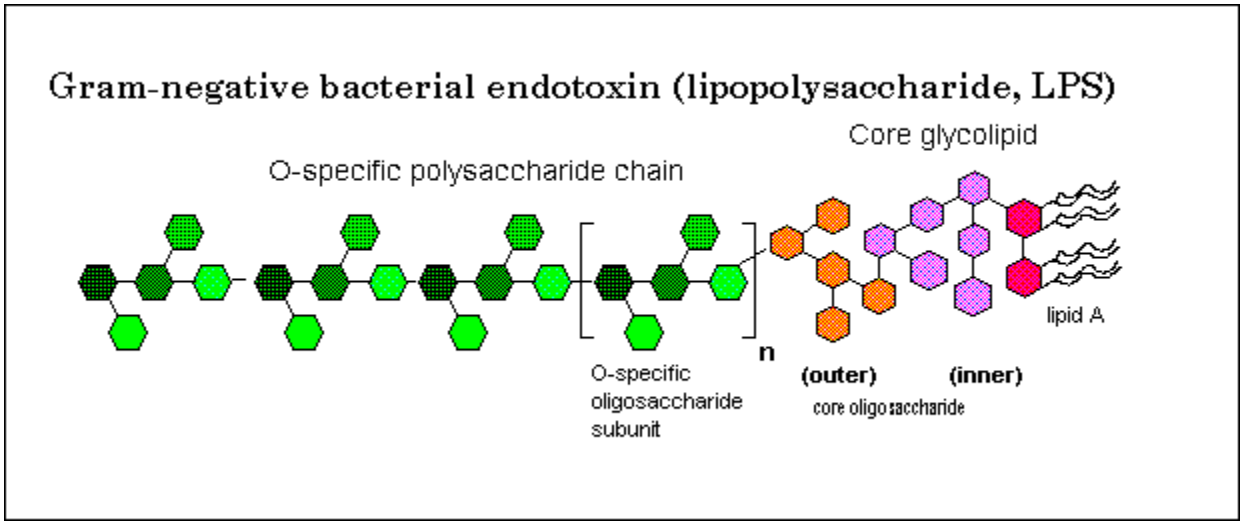

Figure 1 - Structure of Lipopolysaccharide

Cellular activation by LPS

The immune response

The goal of the immune response is to eliminate danger signals to the organism; this requires as a first step recognition of those danger signals. To that end, the mammalian immune system has evolved a number of mechanisms to recognize threats. Anything that causes tissue stress or damage is recognized as danger by the immune system.[50] Pathogen-associated molecular patterns (PAMPs) being exogenous molecules derived from micro-organisms, such as endotoxin, can activate immune cells. Besides PAMPs, endogenous molecules (alarmins) produced in stressed or damaged tissue resultant from trauma, burns, ischemia, hemorrhage or other condition of altered homeostasis can also activate immune competent cells. PAMPs and alarmins comprise the danger-associated molecular patterns (DAMPs), to which inflammation is the primary response. DAMPs are recognize by pattern-recognition receptors (PRRs), that when activated will trigger the production of inflammatory mediators altering function of tissues and organs.[51]

The immune system uses both innate and adaptive immunity to mount a response to danger signals. The innate immune system is the first line of defense. The innate response recognizes DAMPs and activates preformed proteins in a cascade fashion in the plasma and immune competent cells such as monocytes, macrophages, dendritic cells, lymphocytes and neutrophils. This activation results in prompt production of pro-inflammatory and anti-inflammatory mediators.[51] The components of the innate immune system in the critically ill are interwoven, pleiotropic and redundant, depicting a nonlinear dynamic system with great connectivity, cross-regulation and variability overtime.[52] The series of events that leads to the activation of the innate immune response following exposure to LPS is not completely elucidated, however several key molecules are known to be involved in this process, including: the serum proteins LPS binding protein (LBP) and soluble CD14 (sCD14), membrane bound CD14 (mCD14), the CD11/CD18-complex and the Toll like receptors (TLRs) on the surface of effector cells (e.g. monocytes, macrophages etc). A review of the current proposed pathway is provided below (Figure 2).

Once endotoxin enters the circulation it can interact with plasma proteins or blood cells, or it can be removed by macrophages in the liver, spleen or pulmonary vasculature.[2] Interaction

with LBP is the most significant for cellular activation. LBP binds to the LPS aggregates, extracting an LPS monomer that is then transferred to the cellular receptor cluster of differentiation antigen 14. CD14 is required to efficiently transfer the LPS monomer to the Toll-like receptor 4 (TLR4), which in collaboration with the myeloid differentiation factor 2 (MD2) transmits the extracellular signal into an intracellular signal. Transduction of extracellular signals into intracellular responses involves an extensive group of intracellular signaling pathways. Rearrangement of the MD-2/TLR4 complex allows the recruitment of adapter proteins to the intracellular domains of TLR4. This triggers the signaling cascade, leading to activation of genes through the translocation of transcription factors to the nucleus ultimately resulting in the production of a plethora of inflammatory mediators and enzymes.[47]

Lipopolysaccharide binding proteins

Cells of the innate immune system encounter LPS either as an integral component of intact bacteria, or in the form of free molecules. Cell-associated LPS is a target for opsonization, whereas the toxic entity of LPS (lipid A moiety) is only able to interact with and activate inflammatory cells after LPS has been released from the bacterial cell. Because LPS is amphipathic, LPS released from Gram-negative bacteria is present in aggregates in solution. Within the cascade of LPS recognition molecules, LBP is the only one with the ability to recognize LPS aggregates formed immediately after LPS release in to the bloodstream. Although several plasma proteins (e.g. albumin, lactoferrin) interact with LPS, the binding of LPS with LBP and the subsequent interaction with the CD14 receptor is predominantly responsible for cellular activation.[53-55]

LBP is a 60 kDa glycoprotein that belongs to the acute-phase proteins and is primarily synthesized by hepatocytes but also by epithelial and muscle cells.[56] Its normal concentration in human serum is 5-15 μg/l and it increases in concentration by 10 to 100 fold in response to infection or inflammation.[57,58] This rise in LBP is caused by transcriptional activation of LBP mediated by IL-1 alone or synergistically by IL-1 and IL-6.[59] Other stimuli that induce LBP synthesis *in vivo* include Gram-negative bacteria, LPS and non-infectious agents such as turpentine.[59] Maximal LBP concentrations are detected within 24 to 48 hours after stimulation with cytokines in hepatoma cell lines and the maximal increase of serum LBP concentration in trauma or sepsis human patients is reached at day 2 or 3 after the onset of the acute phase.[59,60]

The hepatic transcriptional induction of LBP is inhibited by the anti-inflammatory cytokine transforming growth factor β1(TGF-β1).[61]

LBP plays a crucial role in mediating LPS responses. LBP can bind to LPS in essentially any LPS physical state. LBP is able to bind LPS on the surface of Gram-negative bacteria functioning as an opsonin that can then enhance bacterial phagocytosis by permitting whole bacteria to attach to mCD14.[62] LBP can also combine with large LPS aggregates and promote their internalization by mCD14.[63] The potency of LPS is dramatically enhanced by LBP, by accelerating the binding of LPS to CD14. *In vitro* studies have shown that the sensitivity to LPS of CD14-positive cells in culture increases as much as 1000 fold in the presence of LBP.[53,54] The mechanism by which LBP enhances LPS-mediated cellular activation involves removal of monomeric LPS from LPS aggregates and delivery of these monomers to the CD14 receptor. Additionally, LBP accelerates binding of LPS to sCD14.[64]

Absence of LBP lowers the sensitivity of monocytes to LPS by approximately 250 fold compared with concentrations normally found in plasma.[54] Early studies using antibodies against LBP in murine models of experimental endotoxemia supported the hypothesis that blocking LBP-LPS interactions would be protective by decreasing overwhelming systemic inflammation.[65,66] LBP-deficient mice were protected against septic shock in response to intraperitoneal injection of *Salmonella* LPS.[67] However, further studies using LBP knockout mice showed that these animals had impaired resistance to intraperitoneal infection with *Salmonella typhmurium*[68,69], *Escherichia coli*[70] and to pulmonary infection with *Klebsiella pneumoniae*.[71] While the blockade of LBP-LPS interactions can rescue animals from the toxic effects of LPS, it can impair host defense to Gram-negative infection. Taken together, LBP is extremely important in bacterial recognition and the control of bacterial growth.[72]

At high concentrations, such as those identified during the acute phase response, LBP inhibits host cellular activation by LPS and aids in the sequestration and removal of LPS in order to prevent the detrimental effects of unrestrained inflammation. Experiments with mouse macrophages showed that while low concentrations of LBP enhanced TNFα expression, increasing the LBP concentration to acute-phase concentrations blocked the TNFα release.[73] Moreover, *in vivo*, mice were protected by high-dose LBP after intraperitoneal injection of an otherwise lethal dose of LPS or infection with *Escherichia coli*.[73] Additionally, human subjects with severe sepsis or septic shock that had higher concentrations of LBP exhibited a significant

reduction of LPS-mediated activation of their monocytes.[57] Mechanisms suggested to be responsible for the protective effect of LBP at high concentrations include increased LPS transfer to high-density lipoproteins (HDL),[74] as well as opsonization and enhanced CD14-dependent phagocytosis of LPS aggregate structures, LPS-bearing particles and intact Gram-negative bacteria.[63,75] Similarly to LBP-mediated movement of LPS to CD14, LBP acts catalytically in the transfer of LPS into high-density lipoprotein (HDL) and low-density lipoprotein (LDL), resulting in acceleration of LPS clearance from the bloodstream. Moreover, neutralization of LPS by HDL is accelerated more than 30-fold by addition of sCD14.[76] Binding of LPS to lipoproteins results in increased LPS clearance and decreased LPS binding to cells. Consequently, there is decreased production and release of proinflammatory cytokines, which may represent an endogenous mechanism for clearance of LPS.[77]

Serum LBP concentrations, measured by means of an immunoblot assay, were evaluated in healthy horses and those with colic.[78] Median serum concentrations of LBP did not differ significantly between the 765 horses with acute gastrointestinal problems and the 79 healthy controls (7.1 μg/mL versus 3.7 μg/mL; respectively). Furthermore, serum concentrations of LBP did not correlate with outcome, disease process or portion of the gastrointestinal tract affected in the colic horses.

LPS binds to many other proteins, which, like LBP, may play a role in enhancing the cellular activation by LPS, and/or in removing LPS by sequestration and phagocytosis. Bactericidal-permeability increasing-protein (BPI), a protein of similar structure to LBP, has shown promise as a potential treatment for endotoxemia and sepsis. BPI, which is present in neutrophil azurophilic granules as well as in monocytes and macrophages, is bactericidal for Gram-negative bacteria by increasing outer cell wall permeability and activating enzymes that degrade the bacterial cell wall.[79] These effects of BPI lead to growth arrest, impaired energy metabolism and bacterial cell death.[79,80] Binding of LPS by BPI blocks the delivery of LPS to its cellular receptors, inhibits cytokine release by mononuclear cells and inhibits neutrophil activation. BPI competes with LBP for LPS-binding and can prevent excessive activation of the inflammatory response and death from endotoxic shock in animals.[81] Although plasma concentrations of LBP greatly exceed those of BPI under normal conditions, BPI may play an important role during local infection. Clinical studies using the N-terminal domain of recombinant human BPI in septic patients have been promising.[82]

Other proteins that bind LPS and/or bacteria include lactoferrin, lysozyme, surfactants A and D, serum amyloid P, albumin, transferrin, and hemoglobin. Some of these proteins, such as serum amyloid P, may promote chemotaxis and opsonization, thereby enhancing cellular interaction with LPS. Other proteins may exhibit primarily anti-inflammatory effects by binding and sequestering LPS.[37]

Cluster-of-differentiation antigen 14

As mentioned before, the innate immune system uses a variety of pattern recognition receptors (PRRs) to recognize molecular structures of bacteria, viruses, fungi and protozoa (pathogen-associated molecular patterns) as well as endogenous structures and proteins released during injurious processes, such as trauma, ischemia or necrosis (alarmins). Pattern recognition receptors can be expressed on the cell surface, in intracellular compartments or secreted into the bloodstream and tissue fluids. The main functions of PRRs include activation of inflammatory signaling pathways, opsonization, and phagocytosis, activation of complement and coagulation and induction of apoptosis.[51,83] Some of the PRRs currently identified are the complement receptors, scavenger receptors, the mannose receptor, CD14 and the TLRs.[83,84]

CD14 was characterized as a receptor for LPS in 1990 and can be regarded as the first described pattern-recognition receptor.[85,86] The protein was first identified as a differentiation marker on the surface of monocytes and macrophages and was designated CD14 in the First Leukocyte Typing Conference in Paris in 1982.[87] The genomic DNA of human CD14 was cloned in 1988[88] followed by identification of the *CD* gene of several mammals including mice, rabbits, cows[89], pigs [90] and horses.[91] The amino acid sequence of equine CD14 is highly homologous (approximately 70%) to its human, mouse, rabbit, cow and pig counterparts.[90,91] The general structure of the CD14 protein is quite similar in all these species.[90] The CD14 protein is composed of eleven leucine-rich repeats which are important in PAMP binding. In addition, the crystal structure of CD14 revealed that the protein has a horseshoe shape containing a large hydrophobic pocket which is the main site for LPS binding and signaling.[92,93]

CD14 is expressed predominantly on the surface of myeloid cells, such as monocytes, macrophages and neutrophils, but at lower concentrations also on non-myeloid cells such as epithelial cells, endothelial cells and fibroblasts.[84,94-97] The level of expression of CD14 on human monocytes was reported to be approximately 110,000 molecules per monocyte, whereas

expression in neutrophils is much lower (approximately 3,000 receptors per cell).[98] The CD14 protein is processed in the endoplasmatic reticulum and expressed as a 55 kDa glycoprotein on the cell surface via a glycosylphosphatidylinositol (GPI) anchor.[85,99] Thus, CD14 is not a transmembrane protein but is anchored to the cellular membrane through GPI linkage. GPI anchored proteins, such as CD14, are clustered in membrane micro-domains called ‘‘lipid rafts’’[100] which are implicated in a broad range of cellular processes including transmembrane signaling.[101] Lipid rafts enable efficient and specific signaling in response to stimuli by localizing all of the components of specific signaling pathways within a membrane compartment.[100]

In addition to being expressed as a GPI-anchored membrane protein (mCD14), CD14 also exists as a soluble molecule. Soluble CD14 (sCD14) can be found with two different molecular weights in serum[102], a high molecular mass form of 53-56 kDa and a low molecular mass form of 48-50 kDa.[103,104] Soluble CD14 may result from secretion of the protein before coupling to the GPI anchor or from shedding or cleavage from the surface of monocytes.[105] In the year 2000, another 48 kDa isoform was discovered in the colostrum of cows and women. This colostral CD14 was present at concentrations roughly 25 times that found in plasma and it is produced by mammary epithelial cells.[106]

Biological function of CD14

CD14 is a molecule with a wide range of functions. It is recognized that CD14 serves as a pattern recognition receptor for a variety of ligands, ranging from parts of microbial cell wall, bacterial products, to apoptotic cells and fungi.[107-110] In particular, CD14 is a key receptor for the LPS of gram-negative bacteria. The crucial role of CD14 in LPS responsiveness has been confirmed in studies with knock-out mice. The CD14 deficient mice are highly resistant to septic shock initiated by injection of either LPS or live bacteria. Furthermore, monocytes and macrophages derived from these mice are greatly hyporesponsive to LPS, producing only modest amounts of TNFα and insignificant amounts of IL-1β and IL-6 in response to high (1 and 10 μg/ml) concentrations of LPS.[111] CD14 is also capable of binding and signaling internalization of whole gram-negative bacteria by a LBP dependent pathway, suggesting that free endotoxin is not required for CD14 recognition.[112]

The recognition of LPS by CD14 is catalyzed by lipopolysaccharide-binding protein (LBP), a 60 kDa acute-phase response glycoprotein synthesized mainly in the liver and lung. Although CD14 receptor by itself binds LPS, the presence of LBP enhances this interaction 100 to 1000 times.[53,64] LBP binds LPS aggregates and catalytically transfers several hundred LPS monomers per LBP molecule, forming a large ternary complex with the CD14 receptor.[113]

In vitro studies have shown that certain anti-CD14 or anti-LBP antibodies that inhibit LPS binding also inhibit cell activation, and high concentrations of LPS are generally required to overcome the suppressive effects of the antibodies.[54,85] *In vivo* studies also show that antibodies to either LBP[65] or CD14[114,115] can suppress LPS responses and rescue animals from the lethal effects of LPS. Schimke et al[115], using a rabbit model of endotoxic shock, established that treatment with anti-rabbit CD14 monoclonal antibodies (mAbs) protects against organ injury and death even when the antibody is administered after initial exposure with LPS. Blockade of CD14 with anti-CD14 antibodies in rabbits infected with *E. coli* pneumonia was effective at preventing hypotension and reducing intravenous fluids requirements. Although the deleterious systemic responses were prevented, the dissemination of bacteria was increased by the blockade of CD14.[116] This observation may indicate impaired clearance of bacterial pathogens if the CD14-dependent pathways are blocked. Pretreatment with anti-CD14 mAbs prevented LPS-induced hypotension and reduced plasma cytokine concentrations in cynomolgus monkeys[114] and inhibited endotoxin-mediated symptoms, proinflammatory cytokine release and leukocyte activation in response to 4 ng/kg of endotoxin in healthy humans. IC14, a recombinant chimeric monoclonal antibody that recognizes the human membrane bound and the soluble forms of CD14, was administered as single and multiple dose regimen to 40 patients with severe sepsis.[117] IC14 doses of 2-4 mg/kg given for 4 days were sufficient to saturate the mCD14 receptors of human peripheral blood monocytes by more than 90% for 7 days. Results of this therapeutic trial showed that IC14 administration was well tolerated and did not increase the incidence of secondary bacterial infection. Moreover, although there was a high degree of patient variability, the concentrations of several proinflammatory (including TNFα, IL-1 and IL-6) mediators were decreased from baseline after administration of IC14.[117] The study was not powered to show effects on survival but showed a trend toward a reduction in multiple organ dysfunction syndrome scores under IC14 treatment.

Soluble CD14 is present in sera of healthy adult humans at concentrations of 1.5 to 5μg/mL[118,119], whereas sCD14 concentrations in umbilical cord blood or plasma of neonates at the first day of life is decreased compared to adult concentrations.[120] Soluble CD14 readily binds LPS monomers from LPS-LBP complexes and sCD14 can participate in cell activation by transferring LPS to mCD14 or by transferring LPS directly to the MD-2/TLR-4 complex on cells with low mCD14 expression, such as endothelial and epithelial cells.[121-123] However, at high concentrations, LBP and sCD14 are also able to down regulate LPS-induced responses by catalytically transferring LPS to high density lipoproteins for subsequent removal.[124] Administration of sCD14 has been demonstrated to inhibit LPS induced TNFα production in human mononuclear cells and decrease fatality in LPS challenged mice.[125-127] Administration of recombinant bovine sCD14 (rbosCD14) decreased mortality of mice challenged with intraperitoneal injection of LPS, and reduced severity of mastitis in mice after intramammary challenge with *E. coli*.[128] Furthermore, rbosCD14 was able to reduce the severity of infection in dairy cows after intramammary challenge with *E. coli*.[129] Additionally, enriched sCD14 in milk has been reported to act as a B-cell mitogen and play a role in breast feeding-associated benefits, such as reduced gastrointestinal infections in infants.[106,130]

Kitchens et al[124] found that sCD14 concentrations increase during acute inflammation and sepsis in humans, and this increases the LPS efflux from mCD14 on monocytes. Increased LPS efflux causes decreased or much more transient LPS association with the cells. Moreover, it was demonstrated that elevated concentrations of sCD14 (within the range of those found in plasma of septic patients) markedly inhibit monocyte responses to cell-bound LPS in whole blood or undiluted serum.[124] Therefore, depending on its concentration, sCD14 can either enhance or inhibit responses of cells that express mCD14.[124]

Increased concentrations of sCD14 have been reported in a number of clinical situations in humans. These situations include chronic inflammatory diseases such as psoriasis[131], sarcoidosis[132] and lupus erythematosus[133]. Subjects with malaria[134] and tuberculosis[135] were also reported to have elevated concentrations of sCD14. Increased sCD14 was also detected in the bronchoalveolar lavage fluid from patients with acute respiratory distress syndrome[136] and in cerebrospinal fluid of patients with bacterial meningitis[137]. Kruger et al[118] detected elevated sCD14 concentrations in the serum of patients with polytrauma and burns and Endo et al[138] found higher sCD14 concentrations in septic patients with multiorgan failure. Increased

concentrations of sCD14 and reduced expression of mCD14 were correlated with increased mortality in gram-negative septic shock[139] in 54 human patients. Reduced expression of mCD14 during sepsis is accompanied by an enhanced secretion of sCD14 that represents a compensatory mechanism.[140] Soluble CD14 has been described as an independent predictor of mortality in human patients with HIV infection[141] and hemodialysis patients.[142] In a study of patients infected with HIV, including 74 subjects who died, 120 of whom developed cardiovascular disease, and 81 that developed AIDS, these individuals were found to have increased sCD14 concentrations compared to matched controls; with patients who died having significantly higher concentrations than the controls subjects (2.47 versus 2.23 μg/mL). In patients undergoing hemodialysis, sCD14 correlated positively with C-reactive protein, IL-6 and endotoxin; and although sCD14 was associated with mortality, only patients with the highest sCD14 tertile (>3.63 μg/mL) had increased mortality rate.[142] Children with pneumonia had higher serum sCD14 concentrations compared to cystic fibrosis, asthma and control subjects and the authors of this study suggested sCD14 as a potential marker for pneumonia in children.[143] Similarly, Berner et al[120] suggested LBP and sCD14 as a possible auxiliary diagnostic markers for bacterial sepsis in the newborn, and Lakatos et al[144] proposed LBP and sCD14 as markers of disease activity in patients with Crohn's disease.

In contrast to humans and a few other species, little is known regarding CD14 in horses. Barton et al[145] collected pre-suckle and post-suckle serum from 10 healthy foals and used Western blot analysis to quantify sCD14 in the samples. They found that pre-suckle samples did not contain CD14, however the post-suckle samples had concentration equivalent or greater than that found in the mare's serum. These results suggested that colostral CD14 is passively transferred to newborn foals and may play a critical role in conferring innate immunity. Kiku et al[26] found that the percentage of CD14 positive cells increased significantly between 50 minutes and 3 hours after LPS (30 ng/kg/BW) infusion in horses and this increase corresponded well to changes in clinical findings (tachycardia, tachypnea, fever and leukopenia). Recently, the production of the first monoclonal antibodies to equine CD14 and the expression of equine recombinant CD14 were described, providing new tools for the analysis of CD14 in horses.[146]

Toll-Like Receptors (TLRs)

Despite the indisputable significance of CD14 for LPS binding, CD14 is not the signaling component of the LPS receptor complex. CD14 is a GPI-anchored protein that lacks a transmembrane domain, and therefore cannot transmit a signal to the cytosol. Instead, CD14 facilitate the transfer of LPS to Toll-like receptors (TLRs) which are then responsible for intracellular signaling.[147]

The TLRs are receptors that activate signaling pathways, inducing antimicrobial effector responses, and initiate inflammation upon recognition of the microbial patterns.[51] The TLR family is unique primarily because of its capacity for initiating an adaptive immune response through activation of professional antigen-presenting cells.[148] In mammalian species, there are at least 13 cloned TLRs, ten of them identified in humans.[149] Equine TLR2, 3, 4, 5, 7 and 8 have been fully sequenced and compared with human TLR genes, the equine counterparts have a nucleotide homology of 65–77%.[150] All TLRs express a series of leucine-rich repeats in their ectodomain, a transmembrane domain, and an intracellular domain that bears remarkable homology to the intracellular domain of the interleukin-1 type 1 receptor. This region of homology is known as the toll like receptor interleukin-1 receptor (TIR) domain.[149] The leucine-rich repeats are involved in ligand recognition and signal transduction, and are present in both cytoplasmic and transmembrane proteins.[151] The TIR intracellular domain is essential for cellular signaling, by recruiting a group of adaptor proteins based on specific protein interactions. This group of adaptor proteins includes myeloid differentiation factor 88 (MyD88), MyD88 adaptor-like (Mal), TIR-domain-containing adaptor inducing interferon-β (TRIF) and TRIF-related adaptor molecule (TRAM).[152]

TLRs share extracellular and intracellular structural similarities but differ from each other by ligand specificity, expression patterns and in some cases signaling pathways. For example, TLR2 recognizes lipotechoic acid, lipoproteins, lipoarabinomannan, peptidoglycan, porins and zymosan while TLR4 recognizes most forms of Gram-negative bacteria LPS, mannan from fungi, glycoinositolphospholipids from protozoan parasites and several viral proteins.[149]

TLR4 was the first human homolog of the *Drosophila* Toll gene to be identified.[153] Evidence for the participation of TLR4 in LPS signaling is based on the observation that LPS-resistant C3H/HeJ mice have a point mutation in the Toll domain of the *Tlr4* gene and that another LPS-resistant mouse strain, C57BL/10ScCr, exhibits a total deletion of the *Tlr4* gene.[154]

Shortly thereafter, other strains of mice with targeted deletions of the *Tlr4* gene were reported to be unresponsive to LPS.[155,156] Similarly, hyporesponsiveness of humans to LPS has been associated with point mutations in the TLR4 domain.[157] Collectively, the results of these studies demonstrated the essential role for TLR4 in recognition of LPS from Gram-negative bacteria. Werners et al[158] investigated the genetic sequences of TLR4, MD2 and CD14 in a group of healthy horses. Though TLR4 polymorphisms were found, these mutations did not influence TLR4 function and were not related to an altered LPS response in individual horses.

TLR4 is expressed constitutively on neutrophils, monocytes, macrophages and dendritic cells, but also on epithelial and endothelial cells.[149] LPS signaling through TLR4 not only requires the presence of CD14, but is also dependent on the presence of MD2. Myeloid differentiation factor 2 is a secreted 160 amino acid glycoprotein, which associates with TLR4 on the cellular surface.[159] TLR4 and MD2 become associated in the Golgi with the assistance of the endoplasmic reticulum chaperone gp96. MD2 has also been implicated in the glycosylation of TLR4, which is an essential step in the migration of TLR4 to the cell surface.[160] Although it is still not clear if LPS directly binds to TLR4, there is strong evidence to support an interaction between TLR4 and MD2. The results of in vitro studies have demonstrated that cells expressing TLR4 alone or with a mutant form of MD2 are hyporesponsive to LPS. However, the response to LPS is rescued when cells lacking MD2 are either co-transfected with MD2 cDNA or when soluble MD2 protein is provided in the medium.[159]

Additional receptors

Several other receptors have been implicated in the binding of LPS and/or cellular responses to an LPS challenge. One family represents the β2-integrins, or CD18 antigens, which comprise glycoproteins CD11a/CD18 (α1β2-integrin; leukocyte function-associated antigen 1, LFA-1), CD11b/CD18 (α2β2-integrin; complement receptor type 3, CR3) and CD11c/CD18 (α3β2-integrin, CR4). Several lines of evidence suggest binding of LPS and lipid A as well as intact bacteria by CD18.[37] Cellular activation by LPS may in part depend on ligand interaction with β2-integrins, however, antibodies against these antigens do not entirely inhibit LPS-mediated activation,[161] and the *in vivo* relevance of the activation pathways may be limited.[37] In radioligand binding assays, monoclonal antibodies against the CD11/CD18 complex did not inhibit LPS binding to cells and, vice versa, LPS did not inhibit antibody binding.[162] P-Selectin

and L-Selectin are also able to bind LPS.[163] Class A scavenger receptors (SR-A) are thought to be involved in uptake and detoxification of LPS and lipid A, and in cellular activation of hepatic Kupffer cells. LPS induces up-regulation of SR-A via TLR4 in macrophages and SR-A deficient mice survived an LPS endotoxin challenge better than their wide-type mice counterparts. [164] Aside from SR-A, other scavenger receptors have been identified on Kupffer cells and hepatic sinusoidal cells, but the relative importance of different scavenger receptors for hepatic LPS uptake has not been resolved.[165]

In addition to membrane proteins serving as receptors for LPS, cytoplasmatic proteins have been identified that can bind LPS and lead to cellular activation. NOD1 and NOD2 are members of the NOD (nucleotide-binding oligomerization domain) family, whose members are implicated in innate immune responses to bacteria.[166] Similar to CD14 and TLR4, NOD1 and NOD2 contain leucine-rich repeats in their ligand-recognition domain. Activation of the transcription factor NF-κB as well as pro-apoptotic signaling pathways in response to LPS have been demonstrated to involve NOD1 and NOD2. These proteins may therefore confer upon cells the ability to respond to intracellular infection, and may link innate and adaptive immune responses similar to TLRs.[166]

Intracellular signaling pathways

Upon binding of LPS to the TLR4/MD-2 complex, conformational changes in the receptor structure allow recruitment of adapter proteins and a number of kinases that form a signaling complex.[149] Ultimately, transcription factors such as NF-κB and AP-1 (activator protein 1) as well as MAPK (Mitogen-activated protein kinases) are activated, resulting in increased transcription of pro- and anti-inflammatory genes (Figure 2).[149] MyD88, Mal and TRIF have been identified as initial adapter proteins involved in signaling via TLR4.[149]

MyD88-dependent pathways are responsible for signaling events that result in increased cytokine production, while a MyD88-independent pathway is involved in activation of IRF-3 (IFN-regulatory factor-3) and induction of IFN-β and IFN-inducible genes.[149]

MAP kinase activation pathways utilize MyD88 and the adapter protein TRAF-6 (TNFα receptor associated factor-6). MAPK pathways are activated through sequential phosphorylations, beginning with the activation of MAPK kinase kinase (MAPKKK) which

phosphorylates and activates MAPK kinase (MAPKK) which in turn activates MAPK by phosphorylation. MAPK pathways include ERK (extracellular regulated signaling kinase), p38 and JNK (c-Jun N-terminal kinase) which can be activated by many endogenous and exogenous substances. These different MAPK pathways have differential effects. ERK cascade is essential in regulation of cell division; JNK activates transcription factor AP-1 which is important in TNF synthesis and expression of iNOS by monocytes and macrophages as well as T-cell proliferation and differentiation. The p38 proteins which can be activated by ischemia-reperfusion and LPS are important for production of TNF, IL-1, IL-6 and IL-8.[167]

After MyD88/Mal binds to TLR4, several kinases and additional adapter proteins are recruited to form a signaling cascade. IRAK-1 (IL-1 receptor associated kinase), IRAK-4 and IRAK-2 are proximal kinases that are recruited to MyD88 and are subsequently phosphorylated. After IRAK phosphorylation, the adapter protein TRAF-6 is recruited and activated, and via multiple kinase steps leads to activation of the IKK (I-κB kinase) complex. Activated IKK phosphorylates I-κB (inhibitory factor κB), which results in the degradation of I-κB. Because I-κB sequesters NF-κB in unstimulated cells, its degradation frees NF-κB, which then translocate into the nucleus leading to increased gene transcription and expression.[168] NFκB is a dimeric protein of either identical or structurally homologous protein subunits of about 50 to 75 kDa. NFκB binds to the promoter region of genes encoding for inflammatory mediators, such as, tumor necrosis factor-α, inducible nitric oxide synthase, cyclooxygenase-2 and many other cytokines and chemokines that play a vital role in the host response to inflammation.[168]

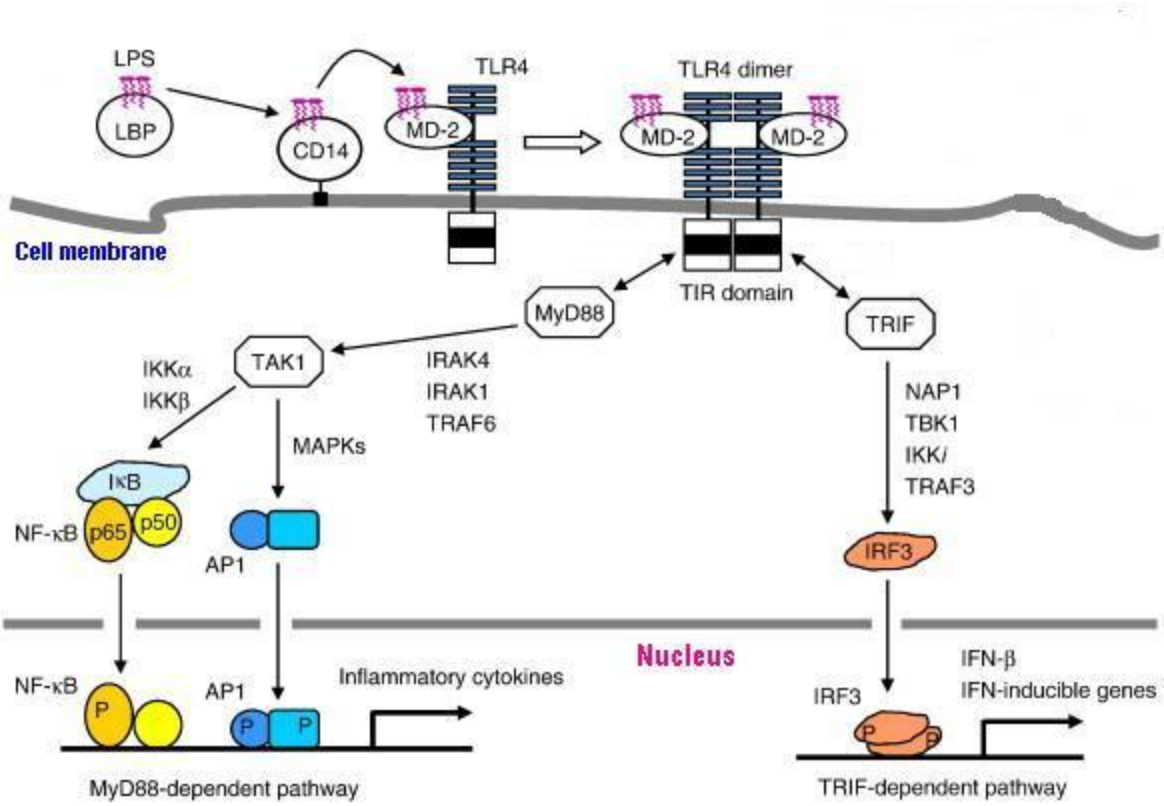

Figure 2 Schematic diagram of LPS binding and TLR4-mediated inflammatory signaling in the mammalian host. Modified from Park et al, 2012.

LBP transfers LPS to CD14. CD14 concentrates LPS molecules and presents LPS monomers to the receptor complex MD-2/TLR4. The MD-2/TLR4 complex undergoes conformational changes allowing recruitment of adapter proteins and a number of kinases that form a signaling complex. Intracellular adaptor molecules are MyD88 and TRIF. MyD88-dependent pathway activates the transcription factors of NF-κB and AP1 for the induction of inflammatory cytokines through multiple signaling molecules such as IRAK4 (IL-1 receptor-associated kinase 4), IRAK1, TRAF6 (TNF receptor-activated factor 6), TAK1 (transforming growth factor-β-activated kinase 1), IKKα (inhibitory κB kinase α), IKKβ, and MAPKs (mitogen-activated protein kinases). TRIF-dependent pathway activates the transcription factor of IRF3 for the up-regulation of IFN-β and IFN-inducible genes.

Cytokines and secreted inflammatory mediators

The cytokines that are central to the inflammatory response are polypeptides produced prominently by mononuclear phagocytes. These cytokines are synthesized in response to invading microorganims, and are involved in mediating and regulating immune and inflammatory responses. Their secretion is usually rapid and self-limited, and their synthesis is initiated by gene transcription as a consequence of cellular activation. They often act on different cell types and can influence the synthesis and actions of other cytokines.[169] Several cytokines play key roles in mediating acute inflammation; three of the most studied cytokines are IL-1, IL-6 and TNFα. They have a similar broad range of physiological effects, which are exerted locally at the site of their production or systemically when produced in large amounts as in the case of endotoxemia and sepsis.

TNFα has been recognized as one of the central mediators of endotoxemia and is responsible for many of the systemic complications observed during endotoxemia and sepsis. TNFα is produced primarily by monocytes and macrophages, but also by many other cell types including T- and B-lymphocytes, natural killer cells, mast cells and intestinal epithelial cells.[170] TNFα is a proximal mediator produced early in the course of endotoxemia, and in addition to having direct effects it also stimulates the release of other mediators, thereby potentiating the inflammatory cascade. TNFα mediates a plethora of biological responses in the host. Its main physiological functions are to stimulate the recruitment of neutrophils and monocytes to sites of infections and to activate these cells to eliminate invading microorganisms. This is due to its stimulating production of chemotactic factors (e.g. IL8 and platelet activating factor) and induction of adhesion molecules for leucocytes on vascular endothelium. TNFα also stimulates the production of acute phase proteins, such as serum amyloid A and fibrinogen, by hepatocytes.[171] Therefore, TNFα is often measured experimentally as a reliable, representative indicator of inflammatory cell activation, and is considered to be a hallmark of MyD88-dependent activation.

Administration of recombinant TNFα to animals and humans results in many of the same effects as administration of endotoxin; supporting the notion that TNFα constitutes one of the most prominent mediators induced by LPS.[170] Blockade of TNFα with specific antibodies greatly inhibits the detrimental effects of experimental endotoxin administration in laboratory animals.[172,173] However, blockade of TNFα in some sepsis models failed to improve survival,[174]

and anti-TNFα antibodies have not proven useful in reducing lethality in clinical cases of human sepsis.[175]

Several studies have evaluated the importance of TNFα in equine endotoxemia. Early *in vitro* studies have documented the ability of equine alveolar[176] and peritoneal macrophages[177] to produce TNFα upon LPS stimulation. In these studies, production of TNFα in response to LPS occurred in a dose-dependent manner. *In vivo* studies of equine experimental endotoxemia, in adults and foals, showed that infusion of LPS induces significant increases in serum TNFα activity 1 to 4 hours after onset of endotoxemia.[178,179] Moreover, in these experiments, significant correlation of TNFα activity with clinical signs of endotoxemia, including tachycardia, depression, onset of fever, and neutropenia were demonstrated. Increased serum TNFα activity has been also documented in naturally occurring cases of equine acute abdominal disease in adults and in Gram-negative septicemia in neonates.[180,181] In adults with colic, elevated concentrations of TNFα were detected in 20% of 289 horses. Furthermore, marked increases in TNFα activity were correlated with increased mortality in adult horses and with increased disease severity in neonate foals.[180,181] Barton and Collatos[15] measured TNFα and IL-6 activities in serum and peritoneal fluid in horses with acute abdominal disease, founding higher concentration of LPS and TNFα in peritoneal fluids when compared to serum. Also, peritoneal TNFα activity was positively correlated with mortality and disease type, whereas serum TNFα concentrations were not correlated with survival.[15]

More recently, studies looking at the effect of LPS on gene expression of cytokines in equine peripheral blood cells also demonstrated the early expression of TNFα after LPS stimulation. Nieto et al[182] showed that TNFα gene expression in whole blood was significantly upregulated by 30 min, peaking at 60 minutes following infusion of 30ng/kg of body weight in normal horses. In addition, the TNFα concentrations returned to baseline values by 3 hours after the LPS infusion. *In vitro* studies using equine peripheral blood leukocytes also demonstrated increased gene expression of TNFα after LPS stimulation. Sun et al,[183] incubated equine peripheral blood monocytes with 100 pg/ml of LPS, which resulted in peak expression of TNFα at 1 hour after incubation; and these values returned to baseline by 4 hours. A similar finding was described in a different study where cells were stimulated with 10 ng/ml of LPS resulting in peak TNFα gene expression by 2 hours after LPS stimulation.[184]

IL-1 and TNFα have similar functions and biological effects, and may act synergistically, despite having different receptors and utilizing different intracellular signaling pathways. The main difference between them being that TNFα is cytotoxic to some transformed cell lines, whereas IL-1is generally not.[169] There are two different types of membrane receptors for IL-1, both of which are members of the immunoglobulin superfamily. The type I receptor is expressed on almost all cell types and is the major receptor for IL-1-mediated responses. The type II receptor is expressed on B cells, but may be induced on other cell types.[185]

Similar to TNFα, serum concentrations of IL-1 have been shown to increase during endotoxemia and have been correlated with severity of disease.[186] Treatment with an IL-1 receptor antagonist has resulted in improved survival in animals but not humans.[187] Additionally, the administration of IL-1 reproduces some of the signs of endotoxemia, including fever, neutropenia, pulmonary edema and sequestration of granulocytes.[188]

IL-6 is synthesized by mononuclear phagocytes, vascular endothelial cells and fibroblasts in response to TNFα and IL-1. The active form of IL-6 is a homodimer that binds to the type I cytokine receptor family. The main function of IL-6 is to stimulate the synthesis of acute phase proteins. These proteins activate complement and opsonize pathogens for phagocytosis by macrophages and neutrophils. In addition to the stimulation of acute phase protein synthesis by the liver, IL-6 acts as a growth factor for mature B cells and induces their final maturation into antibody-producing plasma cells. It is involved in T cell activation and differentiation, and participates in the induction of IL-2 and IL-2 receptor expression. Some of the regulatory effects of IL-6 involve inhibition of TNF production, providing negative feedback for limiting the acute inflammatory response.[189] In horses, IL-6 concentrations have predictive value for unfavorable outcome and the simultaneous presence of increased LPS and TNFα. Additionally, increased concentrations of IL-6 have been associated with a poor clinical condition and outcome in horses.[190]

During inflammatory responses, a counter-regulatory set of anti-inflammatory cytokines is concurrently produced whose function is to restore tissue homeostasis. Interleukin-10, the principal anti-inflammatory cytokine, is primarily produced by macrophages, and binds to type II cytokine receptors.[191] Interleukin-10 is an important regulator of the innate and adaptive immune system, exerting its effects by inhibiting further secretion of pro-inflammatory cytokines by activated neutrophils, monocytes and macrophages.[191] Additionally, IL-10 regulates the adaptive

immune system by inhibiting the expression of MHC proteins and costimulatory molecules on antigen-presenting cells. Furthermore, IL-10 inhibits T-cell proliferation, differentiation of monocyte-derived dendritic cells and alters the migration of dendritic cells by interfering with surface expression of chemokine receptors.[191]

Therapeutic strategies in endotoxemia

While there are different therapeutic approaches to endotoxemia, success comes from rapidly determining the underlying cause and subsequent implementation of appropriate therapy. Therapeutic strategies that should be considered in the treatment of endotoxemia include prevention of movement of endotoxin into the circulation; neutralization of endotoxin before it interacts with inflammatory cells; prevention of the synthesis, release, or action of inflammatory mediators and prevention of endotoxin-induced cellular activation.[2]

Elimination of the source of endotoxin or prevention of movement of endotoxin into the circulation is probably the most difficult strategy to achieve, and complete prevention may not be possible. However, the time available for endotoxin translocation in the host can be minimized by identifying the underlying disease process and initiating therapy in a timely manner. In equine medicine, removing affected structures such as devitalized bowel or an infected umbilicus as rapidly as possible will increase the chances of success. LPS-effector cell interaction initiates a cascade that culminates in the production of inflammatory mediators associated with the clinical signs of endotoxemia. In order to reduce morbidity, antiendotoxic therapy is targeted at prevention or reduction of synthesis, release or action of the inflammatory mediators. Therapeutic efficacy of nonsteroidal anti-inflammatory drugs, specifically flunixin meglumine, results from prevention of prostaglandin synthesis and thromboxane while reducing clinical signs associated with endotoxemia.[192,193] Pentoxifylline is a rheologic agent used in humans that has demonstrated the ability to reduce production of cytokines and thromboxane.[193-195] While the beneficial effects of pentoxifylline alone are limited, when combined with flunixin meglumine the protective hemodynamic effects were greatly enhanced.[193]

Additionally, general supportive care strategies with intravenous fluids such as crystalloids and colloids are commonly used.[2] Currently, investigations into therapeutic agents that would prevent or interfere with endotoxin induced cellular activation are being explored.

Given that no treatment options work completely and some therapeutics are toxic, novel therapeutic options are constantly being explored.

There are some strategies that will aid in the neutralization of endotoxin before it interacts with inflammatory cells. Anti-endotoxin antibodies targeted against the O-antigen of the LPS molecule represent one therapeutic option.[196-200] These antibodies are collected from horses vaccinated against the core regions of the Re *Salmonella* mutant or the rough strains of J5 *Escherichia coli*. However, these antibodies are specific for a bacterial strain and therefore do not afford much crossover protection. Additionally, hyperimmune plasma or serum, from horses immunized against Gram negative bacteria and their endotoxins, has been used in the treatment of endotoxemia in horses, and its clinical effects have been evaluated. Some studies have shown beneficial effects of the administration of anti-endotoxin antibodies in experimental endotoxemia, horses with colic and critically ill and septic neonatal foals.[199,201] In contrast, the results of other studies failed to reveal beneficial effects.[197,202,203] The exact reason for this discrepancy is not fully understood, but may potentially be explained by different timing of administration of the antibodies in the course of the disease among studies or the fact that the anti-endotoxin titer may have been insufficient to effectively neutralize the endotoxin challenge.[2] Since the interactions between endotoxin and the horse's inflammatory cells occur rapidly, the antibodies may have been administered too late in some of the studies. This form of treatment may have its best opportunity to be successful when administered early in the course of the disease, but controlled clinical trials to evaluate this are lacking.

As an alternative approach, polymyxin B (PMB) has been used in an attempt to prevent endotoxin from interacting with the horse's inflammatory cells. Polymyxin B is a cationic amphiphilic cyclic decapeptide antibiotic isolated from *Bacillus polymyxa* that has long been recognized to bind lipid A and neutralize its toxicity *in vitro* in animal models of endotoxemia.[204] While not used in humans or in horses at standard antimicrobial dosages due to life-threatening toxicity, low dose PMB is a recognized beneficial form of therapy for endotoxemia in horses and foals.[202,205-207] Polymyxin B exhibits beneficial effects, such as lessening fever, tachycardia and tachypnea, when administered to endotoxemic foals by reducing TNFα and IL-6 production.[202] However, studies have shown PMB to exhibit toxic side effects such as ataxia when given intravenously every 6 hours at high dosages such as 36,000 IU/kg in horses.[208] In a more recent *in vivo* study, the use of PMB at a dose of 1000-5000 IU/kg given intravenously every 12 hours

for 3 days duration did not demonstrate toxic side effects.[206] Another recent study, where polymyxin B was administered at a slightly higher dosage at 6,000 IU/kg intravenously every 8 hours for five doses, showed that the treatment was safe and efficacious in ameliorating the clinical signs associated with endotoxin infusion in healthy horses.[205]

Lipid A receptor antagonists such as the unusual lipid A derived from *Rhodobacter sphaeroides* is a strategy that has produced mixed results as in the case with Polymyxin B. Diphosphoryl lipid A from *R. sphaeroides* (RsDPLA) and the TLR4 antagonist E5531 inhibited binding of enteric LPS, cytokine release, and activity of LPS induced gene expression in human and murine models.[209,210] However, in horses, RsDPLA and E5531 behave as potent agonists.[209-211] This receptor-ligand interaction is suggested to result from the composition of equine TLR4.

In vitro and preclinical studies have demonstrated that lipoproteins, notably high-density lipoprotein, can bind and neutralize endotoxin. Protein-free phospolipid emulsion (PLE) has been shown to bind and neutralize endotoxin in humans.[212] Clinical studies in humans revealed poor outcomes in critically ill patients, including septic patients, with low serum lipoprotein concentrations.[213] Exogenous lipoprotein administration showed a survival benefit in animal studies.[214] Two studies have investigated the administration of PLE in horses receiving endotoxin infusions. Winchell et al[27] found beneficial results of PLE administration such as lower rectal temperature, heart rate and TNFα concentrations, however, hemolysis was observed in treated horses. A more recent study[215], using rapid infusion of PLE showed similar clinical benefits as the latter study, and significant hemolysis was not identified in the treated horses. Although the results are encouraging, further studies are needed before PLE infusion be considered as a treatment for endotoxemia. In a recent human Phase II clinical study (the Lipid Infusion and Patient Outcomes in Sepsis [LIPOS] study), the safety and efficacy of one PLE (GR270773) were tested in patients with severe sepsis. The LIPOS study failed to demonstrate any treatment benefit of the emulsion over placebo in the reduction of 28-day mortality and the prevention of organ failure in a population of patients with suspected or confirmed Gram-negative sepsis.[216]

As previously discussed, CD14 is essential for LPS recognition, binding and signaling on the effector cells. Therefore, CD14 is a valuable target in anti-endotoxin therapeutic strategies. The ability of anti CD14 monoclonal antibodies to prevent mortality and disease progression in endotoxin-induced animals models of sepsis have been demonstrated in few studies.[114,115]

Overall, results of these studies demonstrated that anti-CD14 attenuates the release of proinflammatory cytokines, reduces organ pathology and prevents septic shock and mortality. Several studies of Gram-negative infection models have indicated that anti-CD14 antibodies impair the host response to bacteria leading to reduced bacterial clearance and infection complications in the absence of antibiotic treatment.[116,217] In contrast, CD14 deficient mice appear to clear Gram-negative organisms without any sequel.[218]

In an effort to resolve these contradictions, the effect of an anti-CD14 antibody on bacterial clearance in the presence or absence of antimicrobial therapy was assessed using a rabbit bacterial clearance model.[219] The rabbits were treated with anti-CD14 intravenously, 30 minutes prior to administration of a highly virulent strain of *Escherichia coli*. Each animal was treated with either ceftazidime or placebo 30 minutes after *E. coli* challenge. The results showed that anti-CD14 antibody did not adversely affect bacterial clearance in the presence of appropriate antimicrobial treatment. In the animals that receive antibiotics and anti-CD14, colony counts in blood and organs were similar or even lower than control rabbits at all time points. However, animals treated with anti-CD14, but not receiving ceftadizime, had higher degree of bacteremia, confirming previous reports of anti-CD14 strategies adversely affecting bacterial clearance. As reported previously, clinical studies in humans have been done to evaluate the safety, pharmacology and efficacy of a chimeric monoclonal antibody directed against human CD14, namely IC14.[220-222] The results of such studies showed that IC14 administered at single intravenous doses ranging from 0.03 to 4 mg/kg is well tolerated[222] and pretreatment with 1mg/kg of IC14 significantly attenuated the release of cytokines (TNFα, IL-6) and markers of endothelial cell activation (von Willebrandt Factor, E-selectin) in healthy humans in response to LPS.[220] Moreover, a phase I clinical trial was conducted in humans with severe sepsis. A total of 40 patients were randomized to receive 3 different dose regimens of IC14 or placebo. Eligible patients had clinical evidence of infection, evidence of systemic inflammatory response, and evidence of either sustained hypotension or organ dysfunction and were evaluated for 28 days. No differences in mortality were observed in any IC14 treatment groups compared to placebo, but patients receiving single doses of 1mg/kg or 4mg/kg of IC14 had significant decreases of IL-6 and procalcitonin compared to baseline and improved multiple organ dysfunction scores. In the same clinical trial, serum concentrations of soluble CD14 isoform were measured and noted to increase in response to IC14 treatment in a dose-related manner. In

patients with severe sepsis, this increase occurred earlier than in healthy subjects (by 24 hours compared to 72 hours, respectively). The implications of increased sCD14 concentrations in patients with severe sepsis are uncertain. Soluble CD14 concentrations have been reported to correlate with increased disease severity in septic patients[223], however, exogenously administered sCD14 has been shown to prevent endotoxin mediated shock in mice.[126] In addition, the study by Gluck et al[224] suggested that increased sCD14 may be associated with greater survival in patients with severe sepsis.

The use of corticosteroids for the treatment of endotoxemia and sepsis remains controversial. Glucocorticoids (GC) are lipophilic and readily cross the cell membrane into the cell cytoplasm. Binding of GC to its receptor, GCR, results in translocation of the GC-GCR complex to the cell nucleus, where it has a variety of actions. Binding with GC response elements (GRE) on the promoter region of specific GC responsive genes results in activation of transcription and expression of several anti-inflammatory proteins.[225] The GC-GCR complex can also down-regulate inflammatory mediators such as cytokines, adhesion molecules, and enzymes through effects on transcription factors such as nuclear factor kappa B (NFκB) and activator protein 1 (AP-1). Steroids have been shown to alter the production of TNFα and IL-6 by a specific effect at the mRNA level.[226]

In septic or endotoxic shock, GCs are thought to have effects on the immune responses and on vascular responsiveness to cathecolamines. The effect on the immune response is by a reduction in pro-inflammatory cytokine production and a reduction in the inflammatory response without causing immunosupression. This is the result of effects at the transcriptional level.[225] Beneficial effects of GCs have been shown in the treatment of inflammatory diseases, including endotoxic shock in horses.[36,227,228] Despite the results of multiple clinical trials and meta-analyses in human medicine, the use of corticosteroids in sepsis and septic shock remains controversial. Current consensus guidelines limit the role of corticosteroids in patients with septic shock, suggesting an intravenous regimen of hydrocortisone (<300 mg daily, tapered once vasopressors have been stopped).[229]

Anti-cytokine therapies have also been evaluated for the treatment of endotoxemia and sepsis. Since the 1990s numerous studies have been performed to evaluate the effect of monoclonal antibodies to TNFα and soluble receptor fusion proteins on cytokine concentrations and survival of septic human patients.[229] Fourteen clinical trials including a total of 3552 patients

with septic syndrome have been performed to test the safety, efficacy and effect on the inflammatory cascade of various anti-TNF monoclonal antibodies. No survival benefit was documented in any of these trials.[229] More recently, an anti-human TNFα polyclonal antibody, purified by affinity against ovine fragment antigen-binding fragments, has been developed and tested in septic patients.[230,231] It has been administered in clinical trials of efficacy, safety and pharmacology, with the rationale that polyclonality allows interactions with more than one domain of TNFα, probably resulting in more effective neutralization of the cytokine. In the trials, no difference in 28 day mortality was observed between treated and placebo groups. However, administration of the polyclonal antibody resulted in decreased serum TNFα and IL-6 concentrations, which was accompanied by an increased number of ventilator-free and ICU-free days compared to the placebo patients.[230,231] Monoclonal and polyclonal antibodies to TNFα have been evaluated in experimentally induced endotoxemia in horses.[232,233] Pretreatment with monoclonal antibodies against equine TNFα reduced the severity of clinical signs and synthesis of TNFα and IL-6 in ponies administered LPS.[233] However, administration of rabbit polyclonal recombinant human TNFα antibody after the LPS infusion did not alter the response to experimental endotoxemia in light breed horses, even though the antibody was capable of inhibiting native equine TNFα activity in vitro.[232]

Other therapies targeting cytokines have been evaluated in humans, including recombinant IL-10, IL-1 receptor antagonist and platelet-activation factor antagonists, but studies have not been conducted in equine models.[229]

Chapter 2: Manuscript

The role of CD14 in equine endotoxemia

ABSTRACT

Objectives – To evaluate the effects of equine sCD14 and monoclonal antibodies (mAbs) to equine CD14 on LPS-induced tumor necrosis factor α (TNFα) expression of equine peripheral blood mononuclear cells. To determine serum concentrations of soluble CD14 (sCD14) in a population of horses with gastrointestinal diseases or other illnesses likely to result in endotoxemia and to identify relationships between this protein and clinical data.

Animals – Part 1; 10 healthy horses. Part 2; 55 clinical cases and 23 healthy control horses.

Procedure – Part 1; equine monocytes were incubated with *Escherichia coli* LPS, CD14 mAb, sCD14, CD14 mAb plus *E coli* LPS or sCD14 plus *E coli* LPS. Supernatants were collected at 6 hours and assayed for TNFα activity. Part 2; Serum sCD14 was measured at admission and then at 24 and 48 hours after admission using a bead-based multiplex assay.

Results – Part 1; Pre-incubation with CD14 mAb did not inhibit LPS-induced TNFα protein production in isolated equine monocytes. Use of sCD14 inhibited LPS-induced TNFα protein production in isolated monocytes in a concentration-dependent manner. Part 2; Serum concentration of sCD14 was positively related to duration of clinical signs ($P = 0.007$), respiratory rate ($P=0.04$) and band neutrophil count ($P = 0.0002$). There was no correlation between serum concentration of sCD14 and heart rate, temperature, hematocrit, lactate, white blood cell count, fibrinogen, creatinine, urea nitrogen, glucose and anion gap values. Although serum sCD14 was significantly increased in the clinical cases compared to healthy horses, sCD14 did not correlate with outcome.

Conclusions and Relevance – Results of this study indicate that release of sCD14 is increased in ill horses and that TNFα production by PBMCs is decreased when cells are pre-treated with sCD14. These findings provide the basis for future studies to more fully characterize the role of sCD14 in equine endotoxemia and encourage us to consider sCD14 as a potential therapeutic agent for endotoxemia.

Introduction

Endotoxemia is an important clinical problem in horses and is implicated in the pathogenesis of conditions such as acute gastrointestinal disease and Gram-negative neonatal sepsis.[2,234] Endotoxemia has been associated with the development of life-threatening complications, such as laminitis, disseminated intravascular coagulopathy and organ failure; it is also responsible for major economic losses due to treatment cost, long-term complications and death.[235] Endotoxin, the lipopolysaccharide (LPS) component of the outer cell envelope of enteric Gram-negative bacteria, is liberated during cell activation or death.[43] Upon entering the circulation, LPS is transferred by LPS-binding protein in the plasma to CD14, a LPS receptor expressed predominantly on mononuclear phagocytes. After LPS becomes concentrated on the cell surface by CD14, LPS interacts with the Toll-like receptor 4/MD-2 complex. These interactions lead to activation of intracellular signaling cascades that culminate in the synthesis and secretion of the proinflammatory mediators (e.g. TNF) that are responsible for many of the deleterious systemic effects associated with endotoxemia. Thus, it is reasonable to hypothesize that agents that interfere with these signaling cascades would reduce endotoxin induced cytokine production.

CD14 is a 55-kDa serum/cell-surface glycoprotein that acts as a pattern recognition receptor. It is expressed on the surface of most cells of hematopoietic origin as well as endothelial cells.[85] The importance of CD14 in LPS-induced inflammation has been documented extensively, best exemplified by the demonstration that CD14-deficient mice are resistant to LPS-induced lethality.[111] Anti-CD14 antibodies, administered prophylactically or as long as 4 hours after LPS injection, protected rabbits from death, and prevented hypotension and leucopenia.[115] Pretreatment with anti-CD14 mAbs prevented LPS-induced hypotension and reduced plasma cytokine concentrations in cynomolgus monkeys[114] and inhibited endotoxin-mediated symptoms, proinflammatory cytokine release and leukocyte activation in response to endotoxin in healthy humans.[236] IC14, a recombinant chimeric monoclonal antibody that recognizes the human membrane bound and the soluble forms of CD14, was administered to human patients with severe sepsis.[117] Results of this therapeutic trial showed that IC14 administration was well tolerated and decreased the concentrations of several proinflammatory cytokines (including TNFα, IL-1 and IL-6).[117]

The soluble form of CD14 (sCD14) also plays a crucial role in the LPS response and can participate in cell activation by transferring LPS to membrane bound CD14 or by transferring LPS directly to the MD-2/TLR-4 complex on cells with low mCD14 expression, such as endothelial and epithelial cells.[121-123] Elevated serum concentration of sCD14 has been reported in human patients with various diseases, and sCD14 concentrations increase significantly during acute inflammation and sepsis in humans.[124] Moreover, sCD14 has been associated with poor prognosis for survival in humans during different clinical situations, including gram-negative sepsis.[139,141,142] Although elevated serum sCD14 has been associated with high mortality in human patients, studies *in vitro* and in laboratory animals have demonstrated a beneficial effect of sCD14 in LPS mediated conditions. It was established that elevated concentrations of sCD14 (within the range of those found in plasma of septic patients) markedly inhibit monocyte responses to cell-bound LPS in whole blood or undiluted serum.[124] Moreover, recombinant human sCD14 was able to prevent LPS induced septic shock and TNF-α production in mice.[125-127] Administration of recombinant bovine sCD14 (rbosCD14) decreased mortality of mice challenged intraperitoneally with LPS, and reduced severity of mastitis in mice after intramammary challenge with *Escherichia coli*.[128]

To the authors' knowledge, sCD14 serum concentrations have not been evaluated in sick horses and the same is true regarding the effects of sCD14 and monoclonal antibodies (mAbs) to equine CD14 on TNFα production by equine peripheral blood mononuclear cells. As the magnitude of response to LPS displays both interspecies and interindividual variability, species-specific research into the mechanisms involved in LPS-mediated diseases is indicated. Therefore, the purposes of the study reported here were to determine serum concentrations of sCD14 in a population of horses with gastrointestinal diseases or other illnesses likely to result in endotoxemia, and to determine whether sCD14 levels were correlated to clinical data and outcome. A further aim was to evaluate the effects of equine sCD14 and mAbs to equine CD14 on LPS-induced TNFα expression of equine peripheral blood mononuclear cells. We hypothesized that horses suffering from endotoxemia would have an increased sCD14 compared to normal horses and that higher sCD14 concentrations would correlate with a poor prognosis for survival. Moreover, we hypothesized that co-incubation of equine PBMCs with sCD14 would decrease the LPS-mediated TNFα response, while application of antiCD14 mAbs would inhibit the response.

Material and Methods

The study comprised 2 parts. In part 1, the *in vitro* effects of equine sCD14 and mAbs to equine CD14 on LPS-induced TNFα activity of equine mononuclear cells were investigated. In part 2, blood samples from healthy horses and clinical cases were collected for quantification of serum sCD14 levels.

Part 1: In vitro study

Subjects

Ten clinically healthy horses from Virginia Tech Mare Center were used in this part of the study (9 mares and one gelding; mean age 7 years). Their general health was determined the day of blood collection by clinical examination and routine hematological examination, including total white cell count, differential count and determination of fibrinogen concentration. The hair over the left jugular vein was clipped and aseptically prepared and ninety milliliters of blood collected into lithium heparin tubes[a] by venipuncture.

LPS, eqCD14 mAbs and equine sCD14

Lipopolysaccharide (*E. coli* O55:B5 LPS)[b] was reconstituted in RPMI-1640[c] to 1 mg/ml and stored in 1 ml aliquots at -80°C. Prior to use, LPS was further diluted in RPMI 1640 and mixed by vortexing. Equine anti-CD14 monoclonal antibodies (clone 174-2) and equine recombinant soluble CD14 (CD14/IgG1)[146] were kindly provided by Dr. B. Wagner (Department of Population Medicine and Diagnostic Sciences College of Veterinary Medicine Cornell University). All experiments were conducted in triplicate for each horse.

Isolation and treatment of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated as previously described.[237] In brief; the heparinized blood was centrifuged at 600 g for 10 minutes at 20°C and the buffy coat removed and suspended in RPMI 1640 incomplete medium. The cell suspension was then layered onto Lymphoprep[d] in sterile 15 ml tubes at room temperature and centrifuged at 350 g for 30 minutes at 20°C. This was repeated to use the entire sample. The mononuclear cells were recovered from the density gradient media interface by gentle suction, resuspended in incomplete RPMI 1640 medium and centrifuged at 600 g for 5 minutes. Cells were washed in incomplete RPMI 1640 medium three times then resuspended in complete RPMI 1640[e] medium containing

l-glutamine, Hepes Buffer, 10% heat inactivated fetal bovine serum and penicillin (50 IU/ml)/streptomycin (50 μg/ml). Absolute cell counts were determined and cell suspensions were diluted in complete RPMI 1640 medium to a concentration of 1 x 10^6/300μl viable cells, which were added to each well of a 48-well polysterene plate[f] for subsequent determination of TNFα production.

Control samples used in the study contained PBMCs with medium only (negative control sample), *E.coli* LPS (10 ng/ml) in RPMI 1640 (positive control sample), c174-2 (1 μg/ml) in RPMI1640 (antibody control sample) and sCD14 (10ng/ml) in RPMI 1640 (sCD14 control sample). To test for antagonistic activity of equine CD14 monoclonal antibody (c174-2), PBMC's from the 10 horses were first pretreated for 1 hour (37°C, 5% CO_2) with different concentrations of c174-2, after which the stimulus (10ng of LPS) was added. To investigate the effect of sCD14, LPS was first incubated with increasing concentrations of sCD14 (5-60 ng/ml) for 1 hour (37°C, 5% CO_2) and then PBMCs from the 10 horses were added to the suspension. The final volume of each well was 1 ml and the final concentration of LPS and PBMC's were 10ng/ml and 1 x 10^6 cells/ml; respectively.

After a final incubation of 6 hours at 37°C in 5% CO_2, the supernatants were collected and stored frozen (-80°C) until assayed for TNFα as described below.

TNFα assay

Cell culture supernatants were assayed for TNFα concentration with a commercially available equine TNFα ELISA assay[g]. The assay was performed following the manufacture's recommendations. In brief, TNFα antibodies were reconstituted in carbonate/bicarbonate buffer and 100 μl were added to each well of a 96-well ELISA plate and incubated overnight at room temperature (22-25°C). The following day, the plates were washed 3 times, and incubated for 1 hour with 300 μl of blocking buffer with 4% bovine serum albumin in Dulbecco's PBS. Next, 100 μl of each sample or standard was added to triplicate wells, and plates were incubated for 1 hour at room temperature. After washing 3 times, 100 μl of detection antibody was added and the plates incubated for 1 hour at room temperature, after which they were washed three times with wash buffer. Next, 100 μl of Streptavidin-Horseradish Peroxidase (SA-HRP) was added to each well and incubated for 30 minutes at room temperature. After washing, 100 μl of TMB Substrate solution was added to each well and the plates were incubated in the dark for 20 minutes at room

temperature. After the addition of 100 µl of Stop Solution the absorbance was measured at 405nm using a microplate reader.

Briefly, beads coupled with anti-CD14 mAb 105[146] were sonicated, mixed and diluted in blocking buffer to a final concentration of 1 x 10^5 beads/ml. For the assay, 5 x 10^3 beads were used per microtiter well. The standard used for the assay was CD14/IgG1 fusion protein[146] starting at a concentration of 100 ng/ml, followed by seven 5-fold dilutions (lowest standard concentration was 1.28 pg/ml). Milipore Multiscreen HTS plates were soaked with PBS-T for 2 min. The solution was aspirated from the plates and 50µl of each diluted standard concentration or the samples (serum diluted at 1:200) were applied to the plates. Then, 50µl of bead solutions was added to each well and incubated for 30 min at room temperature. The plate was then washed with PBS-T, 50 µl of the primary detection antibody diluted in blocking buffer was added to each well and incubated for 30 min at room temperature. The primary detection antibody used was biotinylated anti-CD14 mAb 59.[146] After washing, 50 µl of streptavidin-phycoerythrin and a phycoerythrin-conjugated donkey anti-goat antibody was added. Plates were incubated for 30 min and washed. The beads were resuspended on 100 µl of blocking buffer and the plate was placed on the shaker for 15 min. The assay was analyzed in a Luminex IS 100 instrument[h].

Part 2: Serum levels of sCD14

Subjects

In this prospective study, blood samples were obtained on admission (and every 24 hours for up to 2 days afterwards) from adult horses admitted to the Marion DuPont Scott Equine Medical Center from March through September of 2011for evaluation of acute gastrointestinal disease or other illnesses likely to result in endotoxemia (e.g. metritis, pleuropneumonia).

Blood was likewise obtained from 23 healthy adult horses randomly selected from the Virginia Tech teaching herd (22 mares and one gelding; mean age of 13 years; range 4-22 years). These samples were used to establish reference ranges for serum concentrations of sCD14. All 23 horses were in good body condition, had no history of illness for ≥ 6 months and were determined to be healthy on the basis of normal physical examination findings, CBC and plasma

fibrinogen concentration. The study protocol was approved by the Virginia Tech Institutional Animal Care and Use Committee.

Sample collection

Peripheral blood samples were obtained from healthy horses and clinical cases via venipuncture after sterile preparation of the skin over a jugular vein. Blood was placed into endotoxin-free plastic tubes[i] without anticoagulant and allowed to clot for 60-90 minutes at room temperature. The tubes were then centrifuged at 3000rpm for 10 minutes and serum was collected, aliquoted and stored frozen at -80°C until assayed for sCD14.

Clinical data for clinical cases

Clinical data were collected prospectively. Demographic data were also obtained for each horse, including age, sex and breed. Clinical data collected upon admission included duration of signs before presentation, rectal temperature, heart and respiratory rates, hematocrit, total protein, white blood cell count, band neutrophil count, fibrinogen, lactate, blood urea nitrogen, creatinine, glucose and anion gap. Final diagnosis was based on surgical or postmortem findings where applicable or on the basis of the most prominent clinical signs.

Outcome was categorized as survival (i.e. discharged alive from the hospital) or nonsurvival (dead or euthanized). Horses euthanized for financial reasons alone were excluded; however, animals euthanized on the basis of a poor prognosis as assessed by the attending veterinarian were included for outcome analysis.

Quantification of sCD14

Serum concentrations of sCD14 were quantified with a bead-based multiplex assay.[238] This analysis was performed by an external laboratory[j]. The frozen serum samples were submitted to the laboratory on dry ice.

Data analysis

Results were entered into a standard desktop PC using commercial statistical analysis software[k]. After data entry was validated, data were summarized and descriptive statistics produced. For the *in vitro* part of the study, the effect of c174-2 and sCD14 treatment and LPS

stimulation on TNFα activity was analyzed using one way ANOVA after confirmation of appropriate statistical assumptions for this test by examining a plot of residuals. Data not meeting the assumptions of the ANOVA were transformed as necessary. Correlations between sCD14 serum concentration and clinical variables was evaluated using Spearman's rank correlation coefficient. For a comparison between the sCD14 concentrations of survivors and nonsurvivors a Student's *t* test was used. To test for an effect of time on serum concentration of sCD14 in clinical cases, concentrations were compared by a repeated measures analysis of variance, with multiple comparisons conducted by Tukey's test. All continuous data were evaluated by observation of a plot of the residuals to ensure that the data met the assumptions of the ANOVA. Those data not meeting the assumptions of the ANOVA were transformed as necessary prior to final analysis. Statistical significance was set at $P \leq 0.05$.

Results

Part 1

To define a suitable concentration of LPS to use in the rest of the study, we first measured supernatant concentrations of TNFα after equine peripheral blood monocytes were incubated with a range of concentrations (0.1 ng/ml to 100 ng/ml) of *E. coli* LPS. The lowest concentration tested that yielded the maximal increase in TNFα production was 10 ng/ml LPS (data not shown); thus this concentration of LPS was used in subsequent experiments.

Equine monocyte production of TNFα protein was increased by incubation with *E. coli* O55:B5 LPS. Pre-incubation with mAb c174-2 did not inhibit LPS-induced TNFα protein production in isolated equine PBMCs (Figure 3). However, pre-incubation with sCD14 inhibited LPS-induced TNFα protein production in isolated monocytes in a concentration-dependent manner (Figure 4). Tumor necrosis factor α production was significantly less than the control (759.6 ± 375 pg/ml) in the cells treated with 30 and 60 ng/ml sCD14 (381.9 ± 201.6 pg/ml [P=0.027], 342.1 ± 169.6 pg/ml [P=0.01] respectively). (Figure 4)

Neither c174-2 mAb nor sCD14 induced TNFα release by equine PBMCs in absence of LPS (data not shown).

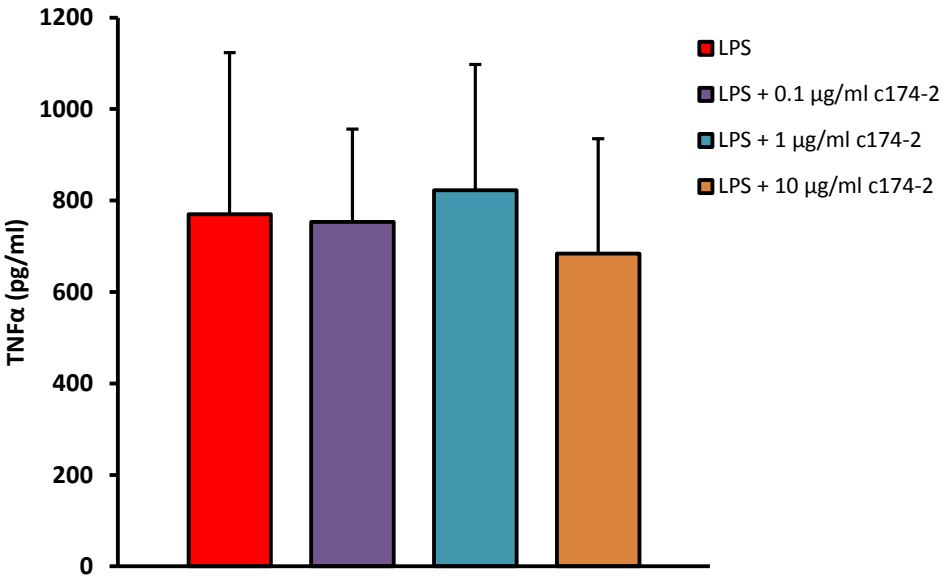

Figure 3. Production of TNFα protein in supernatants of monocytes incubated with *E coli* 055:B5 LPS (10 ng/ml) and c174-2 mAb (0.1 µg/ml to 10 µg/ml) for 6 hours. Results are expressed as the mean ± SD for samples obtained from 10 horses.

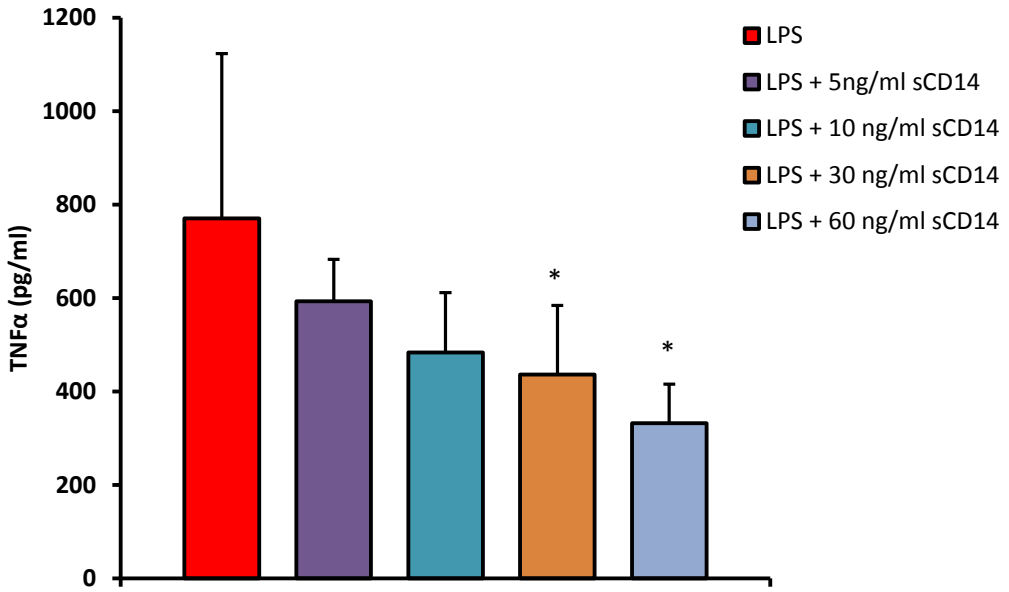

Figure 4. Production of TNFα protein in supernatants of monocytes incubated with *E coli* 055:B5 LPS (10 ng/ml) and sCD14 (10 ng/ml to 60 ng/ml) for 6 hours. Results are expressed as the mean ± SD for samples obtained from 10 horses. * The value differs significantly ($P < 0.05$) from the value for LPS.

Part 2

Clinical population and outcome– A total of 55 horses were included in the study. There were twenty two females (40%), 31 (56%) geldings and 2 (4%) intact males. Horses were between 1 and 27 years of age (mean 12.9 ± 6.7). Twenty-four were Thoroughbreds, 12 were warmbloods, 8 were Quarter Horses, 7 were draft breeds and 4 were pony breeds.

The duration of clinical signs at the time of arrival at the clinic varied between 3 and 168 hours with a mean of 29.8 ± 43.8 hours. Baseline patient characteristics are presented in Table 1. The majority of cases (45/55 [82%]) were presented for assessment of colic. Of these 45 horses, 11 (24%) received a final diagnosis of strangulating small intestinal lesion; 9 (20%) had a nonstrangulating large intestinal lesion; 7 (15%) had colic of unknown etiology responding to medical therapy; 5 (11%) had small intestinal ileus; 4 (9%) had a final diagnosis of colitis; 3 (6%) had strangulating large intestinal lesion; 2 (4%) had postpartum metritis and one (2%) peritonitis. Of the remaining 3 horses evaluated for colic, one had a final diagnosis of gastric impaction, one had multicentric lymphoma and the last had a ruptured viscus. Eight (14%) horses were presented with a primary complaint of fever and two (3%) with a primary complaint of diarrhea. The diagnoses for the eight horses presented for further evaluation of fever, included pleuropneumonia (2), postpartum metritis (1), colitis (1), peritonitis (1), acute renal failure (1), muscular abscess (1) and fever of unknown origin (1). The two horses presented for treatment of diarrhea received a final diagnosis of colitis.

Forty-one horses (75%) were discharged alive and 14 (25%) did not survive (were euthanized). Of the 14 horses euthanized, 12 (86%) had gastrointestinal disease, one had metritis and one had pleuropneumonia. Surgery was recommended for 11/12 horses with gastrointestinal illnesses, with only 6 undergoing surgical intervention. Intraoperative euthanasia was performed in two horses due to poor prognosis (1 with rupture viscus and 1 with large colon volvulus necessitating resection); the other 4 were euthanized post operatively due to complications (time to euthanasia after surgery ranged from 2 to17 days). The remaining 5 horses were euthanized shortly after admission due to poor prognosis. The one gastrointestinal case treated medically had a diagnosis of peritonitis and was euthanized due to poor prognosis, 48 hours after initiation of medical therapy. The other 2 horses that were euthanized included a mare with postpartum metritis that developed acute laminitis of all four limbs and a horse with pleuropneumonia, which was euthanized due to poor prognosis after 14 days of treatment.

Table 1. Clinical and Clinicopathologic data of the ill horses

Variable	Survivors (n=41) Mean ± SD	Non-survivors (n=14) Mean ± SD
Age (years)	11.7 ± 6.2	16.4 ± 7
Heart rate (beats/min)	53.2 ± 13.8	72.3 ± 14.8
Respiratory rate (breaths/min)	18 ± 5.5	35 ± 25.8
Temperature (F)	100.2 ± 1	100.7 ± 1
Hematocrit (%)	40.4 ± 7.1	50.1 ± 12.8
Total protein (g/dl)	7.06 ± 0.94	7.66 ± 1.66
WBC (cells/µl)	9,032 ± 4,055	10,143 ± 7,280
Bands (cells/µl)	894.3 ± 1,288	2,562 ± 3,267
Fibrinogen (mg/dl)	256 ± 133	286 ± 183
Lactate (mmol/l)	1.6 ± 1.2	5.7 ± 3.7
BUN (mg/dl)	17.4 ± 5.6	22.9 ± 8.9
Creatinine (mg/dl)	1.4 ± 0.4	2.3 ± 1
Glucose (mg/dl)	142.2 ± 46.3	188.4 ± 101.8
Anion Gap	10 ± 3	13 ± 4
Duration of signs (hours)	29.7 ± 47.2	21.8 ± 21.9
sCD14 on admission (µg/ml)	2.48 ± 1.99	3.0 ± 2.62

Serum levels of sCD14 – serum samples collected from 23 healthy horses were used to determine a reference range. Median concentration for these horses was 1.45 µg/ml, with a range of 0.62 to 3.4 µg/ml. Mean and standard deviation was 1.64 ± 0.83 µg/ml; thus 2 SDs above the mean was 3.2 µg/ml.

Although clinical cases could provide up to 3 samples, many horses provided fewer samples, with some providing only one. All 55 horses provided admission samples. Fourteen horses (25%) had only a single sample (admission sample) collected for sCD14 measurement. Forty horses (73%) had admission and 24h samples and 33 (60%) had all three samples (admission, 24h and 48h) collected. In the group of horses where only a single or two samples were collected, horses were either euthanized shortly after admission (7/20) or subsequent samples were missed (13/20).

Mean serum concentrations in sera of the clinical cases at time point admission were significantly higher than in 23 healthy controls (mean 2.62 vs. 1.64 μg/ml; P=0.038). The mean and standard deviation sCD14 concentration of clinical cases at time point admission was 2.62 ± 2.15 μg/ml (median 2.07 μg/ml; range 0.70-11.08 μg/ml); 3.34 ± 1.55 μg/ml (median 3.02 μg/ml; range 1.35-7.72 μg/ml) at time point 24h; and 3.39 ± 1.43 μg/ml (median 3.39 μg/ml; range 0.95-8.22 μg/ml) at time point 48h. The sCD14 concentration was significantly different between time points admission and 24h (P = 0.038), but did not differ significantly between time points admission and 48h (P = 0.08); and 24h and 48h (P = 0.98).

sCD14 and outcome – the sCD14 concentration did not vary significantly for outcome (P = 0.5). There was no significant difference in sCD14 concentrations between survivors (mean 2.48 ± 1.98 μg/ml; range 0.7-11.08 μg/ml) and non survivors (mean 3 ± 2.6 μg/ml; range 0.87-10.73 μg/ml).

sCD14 and clinical data – Correlation analysis indicated that serum sCD14 concentration was positively related to duration of clinical signs (P = 0.007), respiratory rate (P=0.04) and band neutrophil count (P = 0.0002). There was no correlation between serum concentration of sCD14 and heart rate, temperature, hematocrit, lactate, white blood cell count, fibrinogen, creatinine, urea nitrogen, glucose and anion gap values (Table 2).

Differences in serum concentrations of sCD14 between diagnostic categories were not evaluated due to the small numbers of horses within each category. sCD14 concentrations for different diagnoses are depicted in Table 3.

Table 2. Correlation summary of serum sCD14 concentration with clinical parameters at admission in ill horses

Parameter	**N**	**Spearman's Correlation**	
		Coeff	*P*
Temperature	55	0.21	0.16
Heart rate	55	0.24	0.08
Respiratory rate	51	0.29	0.04
Hematocrit	55	0.25	0.06
WBC	54	-0.04	0.76
Bands	54	0.49	0.0002
Fibrinogen	53	0.06	0.67
Lactate	50	0.10	0.49
BUN	54	0.10	0.47
Creatinine	54	0.14	0.32
Glucose	53	0.06	0.68
Anion Gap	52	0.06	0.65
Duration of clinical signs	54	0.43	0.007

Table 3. Concentrations of sCD14 by diagnosis.

Diagnosis (n)	**sCD14 admission (mg/dl)**	**sCD14 24h (mg/dl)**	**sCD14 48h (mg/dl)**
Small intestine obstruction (16)	2.43 ± 1.27	3.28 ± 1.01	4.25 ± 1.8
Colitis (7)	2.54 ± 1.6	3.06 ± 1.64	3.01 ± 1.1
Idiopathic colic (7)	2.46 ± 0.98	2.95 ± 0.46	2.72 ± 1.36
Large colon obstruction (9)	1.36 ± 0.66	2.56 ± 0.89	2.91 ± 0.88
Large colon volvulus (3)	1.21 ± 0.23	2.5 ± 0.95	3.42 ± 0.04
Peritonitis (3)	7.76 ± 5.44	5.65 ± 3.46	4.31 ± 1.94
Post-partum metritis (2)	3.05 ± 0.18	3.3 ± 0.87	2.01 ± 0.56
Pleuropneumonia (2)	1.82 ± 0.34	3.87 ± 0.95	4.08 ± 1.6

Discussion

In this study we report that sCD14 is found in equine serum in physiologic and pathophysiological conditions. Soluble CD14 concentrations in the serum of healthy horses are similar to those found in healthy adult human subjects (1.5-5μg/ml).[118,119] Concentrations of sCD14 were significantly elevated in the sick horses compared to healthy controls. However, sCD14 concentrations were not associated with outcome. We hypothesized that serum sCD14 concentrations would be increased in horses with diseases commonly characterized by clinical signs of endotoxemia and would correlate with a poor prognosis for survival. We based this hypothesis on the fact that endotoxemia is a potent stimulus for sCD14 release or shedding in other species. However, serum concentrations of sCD14 were not consistently increased in horses with disease processes commonly associated with the release of endotoxin in the circulation (eg, intestinal ischemia), nor were serum concentrations associated with a poor prognosis for survival. Studies in other species indicate that serum concentrations of sCD14 are increased in patients with gram-negative sepsis, systemic inflammatory response syndrome and septic or hypovolemic shock.[118,124,223] Results of a study[139] in humans provides evidence that increased serum concentrations of sCD14 in patients with gram-negative shock are associated with significantly higher mortality rates, suggesting an association between sCD14 and severity of disease. It is important to note that the population in the latter study was well characterized, comprised of patients with documented gram-negative septic shock and therefore severe disease; with overall mortality rate of 54%. In contrast, Gluck et al[224] found significantly higher concentrations of sCD14 at study entry among survivors as compared to non-survivors. In that study, human patients in early phase of sepsis (duration of symptoms not exceeding 24hours) were evaluated and stratified according to disease severity. Patients with severe disease and APACHE II scores > 20 that survived had significantly higher sCD14 at study entry.[224] In our study, the population was heterogeneous and severity of the disease was not assessed or controlled for. The criteria for inclusion in this study was suspected or confirmed diagnosis of gastrointestinal disease or other illnesses commonly associated with endotoxemia, but did not include more specific entry criteria such as enrolling only horses that had signs consistent with systemic inflammatory response syndrome (SIRS). The abnormalities associated with the clinical syndrome of endotoxemia result from a nonspecific innate inflammatory response. SIRS, which represents a common terminal phase of the inflammatory response characterized by

malignant global activation of multiple pro-inflammatory pathways, is defined by the presence of two or more of the following abnormalities: fever or hypothermia, tachycardia (>60 beats per minute), tachypnea (>30 breaths per minute) or hypocapnia ($PaCO_2$ < 32 mmHg), leukocytosis or leucopenia, or left shift (greater than 10% band neutrophils).[239] An attempt was made to further classify the horses in two groups; the ones with SIRS versus the ones without; however, meaningful comparisons were hindered by the small numbers of nonsurvivors and lack of significant difference in sCD14 concentrations among groups. Follow-up studies with larger number of cases and more uniform population and study entry criteria may provide further information and insight into the importance of sCD14 concentrations in equine patients.

Moreover, because the study involved clinical cases, we were unable to control for the possible effect of treatments received before referral. The majority of the horses received nonsteroidal anti-inflammatory drugs, but none received anti-endotoxin antibodies or polymyxin B, both of which bind endotoxin, before referral. Considering the mechanisms of action of nonsteroidal anti-inflammatory drugs, it seems unlikely that prior treatment significantly affected the analyses of sCD14 in this study, but it may have affected the clinical variables (eg, heart rate, temperature) observed on admission. Furthermore, because endotoxin concentrations were not measured in the samples, we were unable to assess any potential correlations between circulating concentrations of endotoxin and sCD14 or clinical signs and diagnoses.

Concentrations of sCD14 at admission were above the reference range (3.2 μg/ml) in only 18% of the clinical cases in our study; 70% of which were presented for colic and 40% were euthanized due to a poor prognosis. We found a correlation between duration of clinical signs and sCD14 concentrations. Two cases of peritonitis (one survivor and one nonsurvivor) provided the highest serum concentrations of sCD14 (10.7 and 11.1 μg/ml) and the two horses had prolonged course of disease, being admitted to the hospital days after (3 and 7 days; respectively) the onset of clinical signs. Although some studies have detected significantly higher sCD14 in human patients at study entry, a study[118] evaluating patients with polytrauma and severe burns demonstrated that sCD14 was elevated above reference range only after 48h of injury, peaked at 7 days post injury and remained elevated for 4-14 days afterwards. It is possible that considerable variability exists in horses regarding the rate at which concentrations of sCD14 increase and then return to control values. Thus, the duration of the disease process prior to

admission, the type of disease process and the time at which samples were obtained for measurement of sCD14 in this study, may have influenced the sCD14 concentrations.

In the *in vitro* part of the study, pre-incubation with the monoclonal antibody to equine CD14 clone 174-2 did not block LPS-induced TNFα release by equine PBMCs. Several studies have focused on identifying the LPS-binding domain of human CD14. Separate studies demonstrated that four CD14 deletion mutation proteins representing removal of the most hydrophilic regions (amino acid residues 1–66 of human CD14) were independently necessary for LPS ligand binding. Subsequently, protease protection experiments, an additional deletion CD14 mutation protein, and mAb epitope mapping identified region 57-64 as required for *E. coli* LPS binding.[240,241] Another study confirmed that region 39-44 is involved in *E. coli* LPS binding with the use of multiple alanine replacement mutations and monoclonal epitope mapping.[242] Additional mutations and monoclonal epitope mapping indicated that region 7-10 may be involved in LPS signaling, but not binding.[243] These data indicate that there are several different regions located throughout the amino-terminal region of CD14 that could be involved in LPS binding. Most likely, clone 174-2 recognizes an epitope of CD14 not required for LPS binding and signaling and testing using other monoclonal antibodies is needed to indentify the region of equine CD14 required to interact with LPS. In contrast to clone 174-2 mAb, equine rsCD14 was able to inhibit TNFα release by equine PBMCs in a concentration dependent manner. This is comparable to similar studies in other species.[124,125] Furthermore, due to limited availability, the concentrations of sCD14 used in our study were significantly lower (5-60 ng/ml) than that used in the referenced studies (1-100 μg/ml), whereas the concentration of LPS used was higher (10 ng/ml vs. 0.5 ng/ml, respectively). These findings may suggest that equine rsCD14 binds LPS with more affinity and is able to inhibit TNFα activity at a lower concentration than that needed in other species. Further studies are needed to elucidate these questions.

There are a number of shortcomings in this study. In part 1, the first shortcoming is that, although a relatively large number of patients were included, the population was diverse and the number of animals in many of the final diagnosis groups was relatively small. The second limitation is the low mortality rate; overall survival was 75%. In addition, more than half of those animals that died did so shortly after admission. In part 2, only one type of monoclonal antibody to equine CD14 was tested and proved to have no inhibitory effects, limiting the assessment of potential beneficial effects of blocking the CD14 receptor in TNFα release by equine PBMCs. It

would have been valuable to use other types of monoclonal antibodies to equine CD14 as well as anti-human CD14 monoclonal antibodies against CD14 to assess if blockage of TNFα would occur as it is observed in other species.

The preliminary data presented here are certainly not sufficient to advocate therapies for endotoxemia targeting sCD14. In other species, sCD14-based therapeutic concepts have thus far only proved to be beneficial *in vitro* and in laboratory animals.[74,125-127] Further studies are needed to more thoroughly evaluate the role of the membrane bound and soluble CD14 molecule in equine medicine.

In conclusion, the findings here indicate that sCD14 is present in serum of healthy and ill horses, but sCD14 concentrations measured during the first 3 days of hospitalization in sick horses were of limited value and did not correlate with outcome. In addition, we showed that sCD14 is able to inhibit TNFα production by equine monocytes in a concentration dependent manner. These findings provide the basis for future studies designed to more fully characterize the role of sCD14 in equine endotoxemia.

FOOTNOTES

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[a]Vacutainer, Becton Dickinson VACUTAINER systems, Franklin Lakes, NJ.

[b]*Escherichia coli* 055:B5 LPS, Sigma-Aldritch Corp, St. Louis, MO

[c]RPMI 1640 incomplete medium, Sigma-Aldrich Inc, St Louis, MO.

[d]Lymphoprep™, Accurate Chemical & Scientific Corp., Westbury, NY

[e]RPMI 1640 complete medium, Sigma-Aldrich Inc, St Louis, MO.

[f]48 Well cell culture cluster, Corning Inc, Corning, NY.

[g]Equine TNF-α screening set, Thermo Scoentific, Rockford, IL

[h]Luminex Corp., http://www.luminexcorp.com

[i]Polystyrene Tubes, VWR International, LLC, NJ.

[j]Wagner Laboratory of Immunology, Cornell University, Ithaca, NY.

[k]SAS statistical software, SAS Institute Inc, Cary, NC.

REFERENCES

1. Park SH, Kim ND, Jung JK, et al. Myeloid differentiation 2 as a therapeutic target of inflammatory disorders. *Pharmacol Ther* 2012;133:291-298.
2. Moore JN, Barton MH. Treatment of endotoxemia. *Vet Clin North Am Equine Pract* 2003;19:681-695.
3. van der Poll T, Opal SM. Host-pathogen interactions in sepsis. *Lancet Infect Dis* 2008;8:32-43.
4. Opal SM, Yu RL, Jr. Antiendotoxin strategies for the prevention and treatment of septic shock. New approaches and future directions. *Drugs* 1998;55:497-508.
5. Weiss DJ, Evanson OA. Evaluation of lipopolysaccharide-induced activation of equine neutrophils. *Am J Vet Res* 2002;63:811-815.
6. Wurfel MM, Park WY, Radella F, et al. Identification of high and low responders to lipopolysaccharide in normal subjects: an unbiased approach to identify modulators of innate immunity. *J Immunol* 2005;175:2570-2578.
7. Alexander JW, Boyce ST, Babcock GF, et al. The process of microbial translocation. *Ann Surg* 1990;212:496-510; discussion 511-492.
8. Opal SM, Scannon PJ, Vincent JL, et al. Relationship between plasma levels of lipopolysaccharide (LPS) and LPS-binding protein in patients with severe sepsis and septic shock. *J Infect Dis* 1999;180:1584-1589.
9. Herndon DN, Zeigler ST. Bacterial translocation after thermal injury. *Crit Care Med* 1993;21:S50-54.
10. Barton MH, Williamson L, Jacks S, et al. Effects on plasma endotoxin and eicosanoid concentrations and serum cytokine activities in horses competing in a 48-, 83-, or 159-km endurance ride under similar terrain and weather conditions. *Am J Vet Res* 2003;64:754-761.
11. Jeukendrup AE, Vet-Joop K, Sturk A, et al. Relationship between gastro-intestinal complaints and endotoxaemia, cytokine release and the acute-phase reaction during and after a long-distance triathlon in highly trained men. *Clin Sci (Lond)* 2000;98:47-55.
12. Moore JN, Garner HE, Berg JN, et al. Intracecal endotoxin and lactate during the onset of equine laminitis: a preliminary report. *Am J Vet Res* 1979;40:722-723.
13. King JN, Gerring EL. Detection of endotoxin in cases of equine colic. *Vet Rec* 1988;123:269-271.
14. Garrett LA, Brown R, Poxton IR. A comparative study of the intestinal microbiota of healthy horses and those suffering from equine grass sickness. *Vet Microbiol* 2002;87:81-88.
15. Barton MH, Collatos C. Tumor necrosis factor and interleukin-6 activity and endotoxin concentration in peritoneal fluid and blood of horses with acute abdominal disease. *J Vet Intern Med* 1999;13:457-464.
16. McGorum BC, Ellison J, Cullen RT. Total and respirable airborne dust endotoxin concentrations in three equine management systems. *Equine Vet J* 1998;30:430-434.
17. Pirie RS, Collie DD, Dixon PM, et al. Inhaled endotoxin and organic dust particulates have synergistic proinflammatory effects in equine heaves (organic dust-induced asthma). *Clin Exp Allergy* 2003;33:676-683.
18. Senior JM, Proudman CJ, Leuwer M, et al. Plasma endotoxin in horses presented to an equine referral hospital: Correlation to selected clinical parameters and outcomes. *Equine Vet J* 2011.
19. Fessler JF, Bottoms GD, Coppoc GL, et al. Plasma endotoxin concentrations in experimental and clinical equine subjects. *Equine Vet J Suppl* 1989:24-28.
20. Barton MH, Morris DD, Norton N, et al. Hemostatic and fibrinolytic indices in neonatal foals with presumed septicemia. *J Vet Intern Med* 1998;12:26-35.

21. Eades SC, Moore JN. Blockade of endotoxin-induced cecal hypoperfusion and ileus with an alpha 2 antagonist in horses. *Am J Vet Res* 1993;54:586-590.

22. Moore JN, White NA, Berg JN, et al. Endotoxemia following experimental intestinal strangulation obstruction in ponies. *Can J Comp Med* 1981;45:330-332.

23. Menzies-Gow NJ, Bailey SR, Stevens K, et al. Digital blood flow and plasma endothelin concentration in clinically endotoxemic horses. *Am J Vet Res* 2005;66:630-636.

24. Lavoie JP, Madigan JE, Cullor JS, et al. Haemodynamic, pathological, haematological and behavioural changes during endotoxin infusion in equine neonates. *Equine Vet J* 1990;22:23-29.

25. Morris DD, Moore JN, Crowe N, et al. Effect of experimentally induced endotoxemia on serum interleukin-6 activity in horses. *Am J Vet Res* 1992;53:753-756.

26. Kiku Y, Kusano K, Miyake H, et al. Flow cytometric analysis of peripheral blood mononuclear cells induced by experimental endotoxemia in horse. *J Vet Med Sci* 2003;65:857-863.

27. Winchell WW, Hardy J, Levine DM, et al. Effect of administration of a phospholipid emulsion on the initial response of horses administered endotoxin. *Am J Vet Res* 2002;63:1370-1378.

28. Burrows GE. Dose-response of ponies to parenteral Escherichia coli endotoxin. *Can J Comp Med* 1981;45:207-210.

29. Oikawa M, Ohnami Y, Koike M, et al. Endotoxin-induced injury of the central, autonomic and enteric nervous systems and intestinal muscularis in Thoroughbred horses. *J Comp Pathol* 2007;136:127-132.

30. Clark ES, Collatos C. Hypoperfusion of the small intestine during slow infusion of a low dosage of endotoxin in anesthetized horses. *Cornell Vet* 1990;80:163-172.

31. King JN, Gerring EL. The action of low dose endotoxin on equine bowel motility. *Equine Vet J* 1991;23:11-17.

32. Valk N, Doherty TJ, Blackford JT, et al. Phenylbutazone prevents the endotoxin-induced delay in gastric emptying in horses. *Can J Vet Res* 1998;62:214-217.

33. Toth F, Frank N, Chameroy KA, et al. Effects of endotoxaemia and carbohydrate overload on glucose and insulin dynamics and the development of laminitis in horses. *Equine Vet J* 2009;41:852-858.

34. Sprouse RF, Garner HE, Green EM. Plasma endotoxin levels in horses subjected to carbohydrate induced laminitis. *Equine Vet J* 1987;19:25-28.

35. Parsons CS, Orsini JA, Krafty R, et al. Risk factors for development of acute laminitis in horses during hospitalization: 73 cases (1997-2004). *J Am Vet Med Assoc* 2007;230:885-889.

36. Werners AH, Bull S, Fink-Gremmels J. Endotoxaemia: a review with implications for the horse. *Equine Vet J* 2005;37:371-383.

37. Van Amersfoort ES, Van Berkel TJ, Kuiper J. Receptors, mediators, and mechanisms involved in bacterial sepsis and septic shock. *Clin Microbiol Rev* 2003;16:379-414.

38. Opal SM. The host response to endotoxin, antilipopolysaccharide strategies, and the management of severe sepsis. *Int J Med Microbiol* 2007;297:365-377.

39. Volk HD, Reinke P, Docke WD. Clinical aspects: from systemic inflammation to 'immunoparalysis'. *Chem Immunol* 2000;74:162-177.

40. Bone RC. Sir Isaac Newton, sepsis, SIRS, and CARS. *Crit Care Med* 1996;24:1125-1128.

41. Beutler B, Rietschel ET. Innate immune sensing and its roots: the story of endotoxin. *Nat Rev Immunol* 2003;3:169-176.

42. Seydel U, Schromm AB, Blunck R, et al. Chemical structure, molecular conformation, and bioactivity of endotoxins. *Chem Immunol* 2000;74:5-24.

43. Rietschel ET, Brade H, Holst O, et al. Bacterial endotoxin: Chemical constitution, biological recognition, host response, and immunological detoxification. *Curr Top Microbiol Immunol* 1996;216:39-81.

44. Lepper PM, Held TK, Schneider EM, et al. Clinical implications of antibiotic-induced endotoxin release in septic shock. *Intensive Care Med* 2002;28:824-833.

45. Atherton R, Furr M. Endotoxin release after antimicrobial treatment in sick foals is mediated by antimicrobial class. *Journal of Equine Veterinary Science* 2006;26:356-363.

46. Alexander C, Rietschel ET. Bacterial lipopolysaccharides and innate immunity. *J Endotoxin Res* 2001;7:167-202.

47. Gioannini TL, Weiss JP. Regulation of interactions of Gram-negative bacterial endotoxins with mammalian cells. *Immunol Res* 2007;39:249-260.

48. Trent MS, Stead CM, Tran AX, et al. Diversity of endotoxin and its impact on pathogenesis. *J Endotoxin Res* 2006;12:205-223.

49. Gioannini TL, Teghanemt A, Zhang D, et al. Isolation of an endotoxin-MD-2 complex that produces Toll-like receptor 4-dependent cell activation at picomolar concentrations. *Proc Natl Acad Sci U S A* 2004;101:4186-4191.

50. Kono H, Rock KL. How dying cells alert the immune system to danger. *Nat Rev Immunol* 2008;8:279-289.

51. Janeway CA, Jr., Medzhitov R. Innate immune recognition. *Annu Rev Immunol* 2002;20:197-216.

52. Aird WC. Endothelial cell dynamics and complexity theory. *Crit Care Med* 2002;30:S180-185.

53. Mathison JC, Tobias PS, Wolfson E, et al. Plasma lipopolysaccharide (LPS)-binding protein. A key component in macrophage recognition of gram-negative LPS. *J Immunol* 1992;149:200-206.

54. Schumann RR, Leong SR, Flaggs GW, et al. Structure and function of lipopolysaccharide binding protein. *Science* 1990;249:1429-1431.

55. Ulevitch RJ, Tobias PS. Recognition of gram-negative bacteria and endotoxin by the innate immune system. *Curr Opin Immunol* 1999;11:19-22.

56. Schumann RR. Old and new findings on lipopolysaccharide-binding protein: a soluble pattern-recognition molecule. *Biochem Soc Trans* 2011;39:989-993.

57. Zweigner J, Gramm HJ, Singer OC, et al. High concentrations of lipopolysaccharide-binding protein in serum of patients with severe sepsis or septic shock inhibit the lipopolysaccharide response in human monocytes. *Blood* 2001;98:3800-3808.

58. Schumann RR, Latz E. Lipopolysaccharide-binding protein. *Chem Immunol* 2000;74:42-60.

59. Schumann RR, Kirschning CJ, Unbehaun A, et al. The lipopolysaccharide-binding protein is a secretory class 1 acute-phase protein whose gene is transcriptionally activated by APRF/STAT/3 and other cytokine-inducible nuclear proteins. *Mol Cell Biol* 1996;16:3490-3503.

60. Zweigner J, Schumann RR, Weber JR. The role of lipopolysaccharide-binding protein in modulating the innate immune response. *Microbes Infect* 2006;8:946-952.

61. Hallatschek W, Fiedler G, Kirschning CJ, et al. Inhibition of hepatic transcriptional induction of lipopolysaccharide-binding protein by transforming-growth-factor beta 1. *Eur J Immunol* 2004;34:1441-1450.

62. Schiff DE, Kline L, Soldau K, et al. Phagocytosis of gram-negative bacteria by a unique CD14-dependent mechanism. *J Leukoc Biol* 1997;62:786-794.

63. Gegner JA, Ulevitch RJ, Tobias PS. Lipopolysaccharide (LPS) signal transduction and clearance. Dual roles for LPS binding protein and membrane CD14. *J Biol Chem* 1995;270:5320-5325.

64. Hailman E, Lichenstein HS, Wurfel MM, et al. Lipopolysaccharide (LPS)-binding protein accelerates the binding of LPS to CD14. *J Exp Med* 1994;179:269-277.

65. Gallay P, Heumann D, Le Roy D, et al. Mode of action of anti-lipopolysaccharide-binding protein antibodies for prevention of endotoxemic shock in mice. *Proc Natl Acad Sci U S A* 1994;91:7922-7926.

66. Le Roy D, Di Padova F, Tees R, et al. Monoclonal antibodies to murine lipopolysaccharide (LPS)-binding protein (LBP) protect mice from lethal endotoxemia by blocking either the binding of LPS to LBP or the presentation of LPS/LBP complexes to CD14. *J Immunol* 1999;162:7454-7460.

67. Wurfel MM, Monks BG, Ingalls RR, et al. Targeted deletion of the lipopolysaccharide (LPS)-binding protein gene leads to profound suppression of LPS responses ex vivo, whereas in vivo responses remain intact. *J Exp Med* 1997;186:2051-2056.

68. Jack RS, Fan X, Bernheiden M, et al. Lipopolysaccharide-binding protein is required to combat a murine gram-negative bacterial infection. *Nature* 1997;389:742-745.

69. Yang KK, Dorner BG, Merkel U, et al. Neutrophil influx in response to a peritoneal infection with Salmonella is delayed in lipopolysaccharide-binding protein or CD14-deficient mice. *J Immunol* 2002;169:4475-4480.

70. Knapp S, de Vos AF, Florquin S, et al. Lipopolysaccharide binding protein is an essential component of the innate immune response to Escherichia coli peritonitis in mice. *Infect Immun* 2003;71:6747-6753.

71. Fan MH, Klein RD, Steinstraesser L, et al. An essential role for lipopolysaccharide-binding protein in pulmonary innate immune responses. *Shock* 2002;18:248-254.

72. Heumann D, Lauener R, Ryffel B. The dual role of LBP and CD14 in response to Gram-negative bacteria or Gram-negative compounds. *J Endotoxin Res* 2003;9:381-384.

73. Lamping N, Dettmer R, Schroder NW, et al. LPS-binding protein protects mice from septic shock caused by LPS or gram-negative bacteria. *J Clin Invest* 1998;101:2065-2071.

74. Wurfel MM, Hailman E, Wright SD. Soluble CD14 acts as a shuttle in the neutralization of lipopolysaccharide (LPS) by LPS-binding protein and reconstituted high density lipoprotein. *J Exp Med* 1995;181:1743-1754.

75. Gioannini TL, Teghanemt A, Zarember KA, et al. Regulation of interactions of endotoxin with host cells. *J Endotoxin Res* 2003;9:401-408.

76. Wurfel MM, Wright SD. Lipopolysaccharide (LPS) binding protein catalyzes binding of LPS to lipoproteins. *Prog Clin Biol Res* 1995;392:287-295.

77. Feingold KR, Funk JL, Moser AH, et al. Role for circulating lipoproteins in protection from endotoxin toxicity. *Infect Immun* 1995;63:2041-2046.

78. Vandenplas ML, Moore JN, Barton MH, et al. Concentrations of serum amyloid A and lipopolysaccharide-binding protein in horses with colic. *Am J Vet Res* 2005;66:1509-1516.

79. Marra MN, Wilde CG, Collins MS, et al. The role of bactericidal/permeability-increasing protein as a natural inhibitor of bacterial endotoxin. *J Immunol* 1992;148:532-537.

80. Weiss J. Bactericidal/permeability-increasing protein (BPI) and lipopolysaccharide-binding protein (LBP): structure, function and regulation in host defence against Gram-negative bacteria. *Biochem Soc Trans* 2003;31:785-790.

81. Fisher CJ, Jr., Marra MN, Palardy JE, et al. Human neutrophil bactericidal/permeability-increasing protein reduces mortality rate from endotoxin challenge: a placebo-controlled study. *Crit Care Med* 1994;22:553-558.

82. Levin M, Quint PA, Goldstein B, et al. Recombinant bactericidal/permeability-increasing protein (rBPI21) as adjunctive treatment for children with severe meningococcal sepsis: a randomised trial. rBPI21 Meningococcal Sepsis Study Group. *Lancet* 2000;356:961-967.

83. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. *Cell* 2010;140:805-820.

84. Stelter F. Structure/function relationships of CD14. *Chem Immunol* 2000;74:25-41.

85. Wright SD, Ramos RA, Tobias PS, et al. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science* 1990;249:1431-1433.

86. Wright SD. CD14 and innate recognition of bacteria. *J Immunol* 1995;155:6-8.

87. Schutt C. Cd14. *Int J Biochem Cell Biol* 1999;31:545-549.

88. Ferrero E, Goyert SM. Nucleotide sequence of the gene encoding the monocyte differentiation antigen, CD14. *Nucleic Acids Res* 1988;16:4173.

89. Ikeda A, Takata M, Taniguchi T, et al. Molecular cloning of bovine CD14 gene. *J Vet Med Sci* 1997;59:715-719.

90. Qiu XT, Li YH, Li H, et al. Molecular cloning, mapping, and tissue expression of the porcine cluster of differentiation 14 (CD14) gene. *Biochem Genet* 2007;45:459-468.

91. Vychodilova-Krenkova L, Matiasovic J, Horin P. Single nucleotide polymorphisms in four functionally related immune response genes in the horse: CD14,TLR4, Cepsilon, andFcepsilon R1 alpha. *Int J Immunogenet* 2005;32:277-283.

92. Pasquier LD. Germline and somatic diversification of immune recognition elements in Metazoa. *Immunol Lett* 2006;104:2-17.

93. Kim JI, Lee CJ, Jin MS, et al. Crystal structure of CD14 and its implications for lipopolysaccharide signaling. *J Biol Chem* 2005;280:11347-11351.

94. Jersmann HP, Hii CS, Hodge GL, et al. Synthesis and surface expression of CD14 by human endothelial cells. *Infect Immun* 2001;69:479-485.

95. Gong JP, Dai LL, Liu CA, et al. Expression of CD14 protein and its gene in liver sinusoidal endothelial cells during endotoxemia. *World J Gastroenterol* 2002;8:551-554.

96. Funda DP, Tuckova L, Farre MA, et al. CD14 is expressed and released as soluble CD14 by human intestinal epithelial cells in vitro: lipopolysaccharide activation of epithelial cells revisited. *Infect Immun* 2001;69:3772-3781.

97. Sugawara S, Arakaki R, Rikiishi H, et al. Lipoteichoic acid acts as an antagonist and an agonist of lipopolysaccharide on human gingival fibroblasts and monocytes in a CD14-dependent manner. *Infect Immun* 1999;67:1623-1632.

98. Antal-Szalmas P, Strijp JA, Weersink AJ, et al. Quantitation of surface CD14 on human monocytes and neutrophils. *J Leukoc Biol* 1997;61:721-728.

99. Haziot A, Chen S, Ferrero E, et al. The monocyte differentiation antigen, CD14, is anchored to the cell membrane by a phosphatidylinositol linkage. *J Immunol* 1988;141:547-552.

100. Simons K, Toomre D. Lipid rafts and signal transduction. *Nat Rev Mol Cell Biol* 2000;1:31-39.

101. Arroyo-Espliguero R, Avanzas P, Jeffery S, et al. CD14 and toll-like receptor 4: a link between infection and acute coronary events? *Heart* 2004;90:983-988.

102. Labeta MO, Durieux JJ, Fernandez N, et al. Release from a human monocyte-like cell line of two different soluble forms of the lipopolysaccharide receptor, CD14. *Eur J Immunol* 1993;23:2144-2151.

103. Bazil V, Horejsi V, Baudys M, et al. Biochemical characterization of a soluble form of the 53-kDa monocyte surface antigen. *Eur J Immunol* 1986;16:1583-1589.

104. Bazil V, Baudys M, Hilgert I, et al. Structural relationship between the soluble and membrane-bound forms of human monocyte surface glycoprotein CD14. *Mol Immunol* 1989;26:657-662.

105. Durieux JJ, Vita N, Popescu O, et al. The two soluble forms of the lipopolysaccharide receptor, CD14: characterization and release by normal human monocytes. *Eur J Immunol* 1994;24:2006-2012.

106. Labeta MO, Vidal K, Nores JE, et al. Innate recognition of bacteria in human milk is mediated by a milk-derived highly expressed pattern recognition receptor, soluble CD14. *J Exp Med* 2000;191:1807-1812.

107. Kusunoki T, Hailman E, Juan TS, et al. Molecules from Staphylococcus aureus that bind CD14 and stimulate innate immune responses. *J Exp Med* 1995;182:1673-1682.

108. Dziarski R, Gupta D. Function of CD14 as a peptidoglycan receptor: differences and similarities with LPS. *Journal of Endotoxin Research* 1999;5:56-61.

109. Wang JE, Warris A, Ellingsen EA, et al. Involvement of CD14 and toll-like receptors in activation of human monocytes by Aspergillus fumigatus hyphae. *Infect Immun* 2001;69:2402-2406.

110. Weidemann B, Schletter J, Dziarski R, et al. Specific binding of soluble peptidoglycan and muramyldipeptide to CD14 on human monocytes. *Infect Immun* 1997;65:858-864.

111. Haziot A, Ferrero E, Kontgen F, et al. Resistance to endotoxin shock and reduced dissemination of gram-negative bacteria in CD14-deficient mice. *Immunity* 1996;4:407-414.

112. Grunwald U, Fan X, Jack RS, et al. Monocytes can phagocytose Gram-negative bacteria by a CD14-dependent mechanism. *J Immunol* 1996;157:4119-4125.

113. Tobias PS, Soldau K, Gegner JA, et al. Lipopolysaccharide binding protein-mediated complexation of lipopolysaccharide with soluble CD14. *J Biol Chem* 1995;270:10482-10488.

114. Leturcq DJ, Moriarty AM, Talbott G, et al. Antibodies against CD14 protect primates from endotoxin-induced shock. *J Clin Invest* 1996;98:1533-1538.

115. Schimke J, Mathison J, Morgiewicz J, et al. Anti-CD14 mAb treatment provides therapeutic benefit after in vivo exposure to endotoxin. *Proc Natl Acad Sci U S A* 1998;95:13875-13880.

116. Frevert CW, Matute-Bello G, Skerrett SJ, et al. Effect of CD14 blockade in rabbits with Escherichia coli pneumonia and sepsis. *J Immunol* 2000;164:5439-5445.

117. Reinhart K, Glück T, Ligtenberg J, et al. CD14 receptor occupancy in severe sepsis: Results of a phase I clinical trial with a recombinant chimeric CD14 monoclonal antibody (IC14)*. *Critical Care Medicine* 2004;32:1100-1108.

118. Kruger C, Schutt C, Obertacke U, et al. Serum CD14 levels in polytraumatized and severely burned patients. *Clin Exp Immunol* 1991;85:297-301.

119. Grunwald U, Kruger C, Westermann J, et al. An enzyme-linked immunosorbent assay for the quantification of solubilized CD14 in biological fluids. *J Immunol Methods* 1992;155:225-232.

120. Berner R, Furll B, Stelter F, et al. Elevated Levels of Lipopolysaccharide-Binding Protein and Soluble CD14 in Plasma in Neonatal Early-Onset Sepsis. *Clinical and Vaccine Immunology* 2002;9:440-445.

121. Frey EA, Miller DS, Jahr TG, et al. Soluble CD14 participates in the response of cells to lipopolysaccharide. *J Exp Med* 1992;176:1665-1671.

122. Pugin J, Schurer-Maly CC, Leturcq D, et al. Lipopolysaccharide activation of human endothelial and epithelial cells is mediated by lipopolysaccharide-binding protein and soluble CD14. *Proc Natl Acad Sci U S A* 1993;90:2744-2748.

123. Hailman E, Vasselon T, Kelley M, et al. Stimulation of macrophages and neutrophils by complexes of lipopolysaccharide and soluble CD14. *J Immunol* 1996;156:4384-4390.

124. Kitchens RL. Plasma CD14 decreases monocyte responses to LPS by transferring cell-bound LPS to plasma lipoproteins. *Journal of Clinical Investigation* 2001;108:485-493.

125. Haziot A, Rong GW, Bazil V, et al. Recombinant soluble CD14 inhibits LPS-induced tumor necrosis factor-alpha production by cells in whole blood. *J Immunol* 1994;152:5868-5876.

126. Haziot A, Rong GW, Lin XY, et al. Recombinant soluble CD14 prevents mortality in mice treated with endotoxin (lipopolysaccharide). *J Immunol* 1995;154:6529-6532.

127. Stelter F, Witt S, Furll B, et al. Different efficacy of soluble CD14 treatment in high- and low-dose LPS models. *Eur J Clin Invest* 1998;28:205-213.

128. Lee JW, Paape MJ, Zhao X. Recombinant bovine soluble CD14 reduces severity of experimental Escherichia coli mastitis in mice. *Vet Res* 2003;34:307-316.

129. Lee JW, Paape MJ, Elsasser TH, et al. Recombinant soluble CD14 reduces severity of intramammary infection by Escherichia coli. *Infect Immun* 2003;71:4034-4039.

130. Filipp D, Alizadeh-Khiavi K, Richardson C, et al. Soluble CD14 enriched in colostrum and milk induces B cell growth and differentiation. *Proc Natl Acad Sci U S A* 2001;98:603-608.

131. Schopf RE, Dobmeyer J, Dobmeyer T, et al. Soluble CD14 monocyte antigen in suction blister fluid and serum of patients with psoriasis. *Dermatology* 1993;186:45-49.

132. Pforte A, Schiessler A, Gais P, et al. Expression of CD14 correlates with lung function impairment in pulmonary sarcoidosis. *Chest* 1994;105:349-354.

133. Nockher WA, Wigand R, Schoeppe W, et al. Elevated levels of soluble CD14 in serum of patients with systemic lupus erythematosus. *Clin Exp Immunol* 1994;96:15-19.

134. Wenisch C, Wenisch H, Parschalk B, et al. Elevated levels of soluble CD14 in serum of patients with acute Plasmodium falciparum malaria. *Clin Exp Immunol* 1996;105:74-78.

135. Juffermans NP, Verbon A, van Deventer SJ, et al. Serum concentrations of lipopolysaccharide activity-modulating proteins during tuberculosis. *J Infect Dis* 1998;178:1839-1842.

136. Martin TR, Rubenfeld GD, Ruzinski JT, et al. Relationship between soluble CD14, lipopolysaccharide binding protein, and the alveolar inflammatory response in patients with acute respiratory distress syndrome. *Am J Respir Crit Care Med* 1997;155:937-944.

137. Cauwels A, Frei K, Sansano S, et al. The origin and function of soluble CD14 in experimental bacterial meningitis. *J Immunol* 1999;162:4762-4772.

138. Endo S, Inada K, Kasai T, et al. Soluble CD14 (sCD14) levels in patients with multiple organ failure (MOF). *Res Commun Chem Pathol Pharmacol* 1994;84:17-25.

139. Landmann R, Zimmerli W, Sansano S, et al. Increased circulating soluble CD14 is associated with high mortality in gram-negative septic shock. *J Infect Dis* 1995;171:639-644.

140. Hiki N, Berger D, Prigl C, et al. Endotoxin binding and elimination by monocytes: secretion of soluble CD14 represents an inducible mechanism counteracting reduced expression of membrane CD14 in patients with sepsis and in a patient with paroxysmal nocturnal hemoglobinuria. *Infect Immun* 1998;66:1135-1141.

141. Sandler NG, Wand H, Roque A, et al. Plasma levels of soluble CD14 independently predict mortality in HIV infection. *J Infect Dis* 2011;203:780-790.

142. Raj DS, Carrero JJ, Shah VO, et al. Soluble CD14 levels, interleukin 6, and mortality among prevalent hemodialysis patients. *Am J Kidney Dis* 2009;54:1072-1080.

143. Marcos V, Latzin P, Hector A, et al. Expression, regulation and clinical significance of soluble and membrane CD14 receptors in pediatric inflammatory lung diseases. *Respir Res* 2010;11:32.

144. Lakatos PL, Kiss LS, Palatka K, et al. Serum lipopolysaccharide-binding protein and soluble CD14 are markers of disease activity in patients with Crohn's disease. *Inflamm Bowel Dis* 2011;17:767-777.

145. Barton MH, Williams J, Henson S, et al. COLOSTRAL CD14: MORE THAN A RECEPTOR FOR ENDOTOXIN. Dorothy Russell Havemeyer Foundation Neonatal Septicemia Workshop 2001.

146. Kabithe E, Hillegas J, Stokol T, et al. Monoclonal antibodies to equine CD14. *Vet Immunol Immunopathol* 2010;138:149-153.

147. da Silva Correia J, Soldau K, Christen U, et al. Lipopolysaccharide is in close proximity to each of the proteins in its membrane receptor complex. transfer from CD14 to TLR4 and MD-2. *J Biol Chem* 2001;276:21129-21135.

148. Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. *Nat Immunol* 2004;5:987-995.

149. Brikos C, O'Neill LA. Signalling of toll-like receptors. *Handb Exp Pharmacol* 2008:21-50.

150. Jungi TW, Farhat K, Burgener IA, et al. Toll-like receptors in domestic animals. *Cell Tissue Res* 2011;343:107-120.

151. Kobe B, Kajava AV. The leucine-rich repeat as a protein recognition motif. *Curr Opin Struct Biol* 2001;11:725-732.

152. O'Neill LA, Bowie AG. The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. *Nat Rev Immunol* 2007;7:353-364.

153. Opal SM, Huber CE. Bench-to-bedside review: Toll-like receptors and their role in septic shock. *Crit Care* 2002;6:125-136.

154. Poltorak A, He X, Smirnova I, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* 1998;282:2085-2088.

155. Hoshino K, Takeuchi O, Kawai T, et al. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J Immunol* 1999;162:3749-3752.

156. Qureshi ST, Lariviere L, Leveque G, et al. Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4). *J Exp Med* 1999;189:615-625.

157. Arbour NC, Lorenz E, Schutte BC, et al. TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. *Nat Genet* 2000;25:187-191.

158. Werners AH, Bull S, Vendrig JC, et al. Genotyping of Toll-like receptor 4, myeloid differentiation factor 2 and CD-14 in the horse: an investigation into the influence of genetic polymorphisms on the LPS induced TNF-alpha response in equine whole blood. *Vet Immunol Immunopathol* 2006;111:165-173.

159. Miyake K. Innate recognition of lipopolysaccharide by CD14 and toll-like receptor 4-MD-2: unique roles for MD-2. *Int Immunopharmacol* 2003;3:119-128.

160. Ohnishi T, Muroi M, Tanamoto K. MD-2 is necessary for the toll-like receptor 4 protein to undergo glycosylation essential for its translocation to the cell surface. *Clin Diagn Lab Immunol* 2003;10:405-410.

161. Medvedev AE, Flo T, Ingalls RR, et al. Involvement of CD14 and complement receptors CR3 and CR4 in nuclear factor-kappaB activation and TNF production induced by lipopolysaccharide and group B streptococcal cell walls. *J Immunol* 1998;160:4535-4542.

162. Couturier C, Haeffner-Cavaillon N, Caroff M, et al. Binding sites for endotoxins (lipopolysaccharides) on human monocytes. *J Immunol* 1991;147:1899-1904.

163. Malhotra R, Bird MI. L-Selectin--a signalling receptor for lipopolysaccharide. *Chem Biol* 1997;4:543-547.

164. Chen Y, Wermeling F, Sundqvist J, et al. A regulatory role for macrophage class A scavenger receptors in TLR4-mediated LPS responses. *Eur J Immunol* 2010;40:1451-1460.

165. van Oosten M, van de Bilt E, van Berkel TJ, et al. New scavenger receptor-like receptors for the binding of lipopolysaccharide to liver endothelial and Kupffer cells. *Infect Immun* 1998;66:5107-5112.

166. Inohara, Chamaillard, McDonald C, et al. NOD-LRR proteins: role in host-microbial interactions and inflammatory disease. *Annu Rev Biochem* 2005;74:355-383.

167. Cuschieri J, Maier RV. Mitogen-activated protein kinase (MAPK). *Crit Care Med* 2005;33:S417-419.

168. Liu SF, Malik AB. NF-kappa B activation as a pathological mechanism of septic shock and inflammation. *Am J Physiol Lung Cell Mol Physiol* 2006;290:L622-L645.

169. Feghali CA, Wright TM. Cytokines in acute and chronic inflammation. *Front Biosci* 1997;2:d12-26.

170. Mannel DN, Echtenacher B. TNF in the inflammatory response. *Chem Immunol* 2000;74:141-161.

171. Bradley JR. TNF-mediated inflammatory disease. *J Pathol* 2008;214:149-160.

172. Opal SM, Cross AS, Kelly NM, et al. Efficacy of a monoclonal antibody directed against tumor necrosis factor in protecting neutropenic rats from lethal infection with Pseudomonas aeruginosa. *J Infect Dis* 1990;161:1148-1152.

173. Fiedler VB, Loof I, Sander E, et al. Monoclonal antibody to tumor necrosis factor--alpha prevents lethal endotoxin sepsis in adult rhesus monkeys. *J Lab Clin Med* 1992;120:574-588.

174. Eskandari MK, Bolgos G, Miller C, et al. Anti-tumor necrosis factor antibody therapy fails to prevent lethality after cecal ligation and puncture or endotoxemia. *J Immunol* 1992;148:2724-2730.

175. Marshall JC. Clinical trials of mediator-directed therapy in sepsis: what have we learned? *Intensive Care Med* 2000;26 Suppl 1:S75-83.

176. MacKay RJ, King RR, Dankert JR, et al. Cytotoxic tumor necrosis factor activity produced by equine alveolar macrophages: preliminary characterization. *Vet Immunol Immunopathol* 1991;29:15-30.

177. Morris DD, Moore JN, Fischer K, et al. Endotoxin-induced tumor necrosis factor activity production by equine peritoneal macrophages. *Circ Shock* 1990;30:229-236.

178. Morris DD, Crowe N, Moore JN. Correlation of clinical and laboratory data with serum tumor necrosis factor activity in horses with experimentally induced endotoxemia. *Am J Vet Res* 1990;51:1935-1940.

179. MacKay RJ, Merritt AM, Zertuche JM, et al. Tumor necrosis factor activity in the circulation of horses given endotoxin. *Am J Vet Res* 1991;52:533-538.

180. Morris DD, Moore JN, Crowe N. Serum tumor necrosis factor activity in horses with colic attributable to gastrointestinal tract disease. *Am J Vet Res* 1991;52:1565-1569.

181. Morris DD, Moore JN. Tumor necrosis factor activity in serum from neonatal foals with presumed septicemia. *J Am Vet Med Assoc* 1991;199:1584-1589.

182. Nieto JE, Macdonald MH, Braim AEP, et al. Effect of lipopolysaccharide infusion on gene expression of inflammatory cytokines in normal horses in vivo. *Equine Veterinary Journal* 2009;41:717-719.

183. Sun WC, Moore JN, Hurley DJ, et al. Differential modulation of lipopolysaccharide-induced expression of inflammatory genes in equine monocytes through activation of adenosine A2A receptors. *Vet Immunol Immunopathol* 2010;134:169-177.

184. Neuder LE, Keener JM, Eckert RE, et al. Role of p38 MAPK in LPS induced pro-inflammatory cytokine and chemokine gene expression in equine leukocytes. *Vet Immunol Immunopathol* 2009;129:192-199.

185. Dinarello CA. Cytokines as endogenous pyrogens. *J Infect Dis* 1999;179 Suppl 2:S294-304.

186. Casey LC, Balk RA, Bone RC. Plasma cytokine and endotoxin levels correlate with survival in patients with the sepsis syndrome. *Ann Intern Med* 1993;119:771-778.

187. Fisher CJ, Jr., Dhainaut JF, Opal SM, et al. Recombinant human interleukin 1 receptor antagonist in the treatment of patients with sepsis syndrome. Results from a randomized, double-blind, placebo-controlled trial. Phase III rhIL-1ra Sepsis Syndrome Study Group. *JAMA* 1994;271:1836-1843.

188. Lynn WA, Cohen J. Adjunctive therapy for septic shock: a review of experimental approaches. *Clin Infect Dis* 1995;20:143-158.

189. Kruttgen A, Rose-John S. Interleukin-6 in sepsis and capillary leakage syndrome. *J Interferon Cytokine Res* 2012;32:60-65.

190. Steverink PJGM, Sturk A, Rutten VPMG, et al. Endotoxin, interleukin-6 and tumor necrosis factor concentrations in equine acute abdominal disease: relation to clinical outcome *Innate Immunity* 1995;2:289-299

191. Moore KW, O'Garra A, de Waal Malefyt R, et al. Interleukin-10. *Annu Rev Immunol* 1993;11:165-190.

192. Kelmer G. Update on treatments for endotoxemia. *Vet Clin North Am Equine Pract* 2009;25:259-270.

193. Baskett A, Barton MH, Norton N, et al. Effect of pentoxifylline, flunixin meglumine, and their combination on a model of endotoxemia in horses. *Am J Vet Res* 1997;58:1291-1299.

194. Barton MH, Moore JN. Pentoxifylline inhibits mediator synthesis in an equine in vitro whole blood model of endotoxemia. *Circ Shock* 1994;44:216-220.

195. Barton MH, Moore JN, Norton N. Effects of pentoxifylline infusion on response of horses to in vivo challenge exposure with endotoxin. *Am J Vet Res* 1997;58:1300-1307.

196. Gaffin SL, Wells MT. A morphological study of the action of equine anti-lipopolysaccharide plasma on gram-negative bacteria. *J Med Microbiol* 1987;24:165-168.

197. Morris DD, Whitlock RH. Therapy of suspected septicemia in neonatal foals using plasma-containing antibodies to core lipopolysaccharide (LPS). *J Vet Intern Med* 1987;1:175-182.

198. Wells MT, Gaffin SL, Gregory M, et al. Properties of equine anti-lipopolysaccharide hyperimmune plasma: binding to lipopolysaccharide and bactericidal activity against gram-negative bacteria. *J Med Microbiol* 1987;24:187-196.

199. Spier SJ, Lavoie JP, Cullor JS, et al. Protection against clinical endotoxemia in horses by using plasma containing antibody to an Rc mutant E. coli (J5). *Circ Shock* 1989;28:235-248.

200. BonenClark GD, MacKay RJ, Ward RE, et al. Effect of vaccination of ponies with A4 anti-idiotypic antibody on serum idiotype (1C9) and antilipid A concentration. *Am J Vet Res* 1996;57:655-658.

201. Peek SF, Semrad S, McGuirk SM, et al. Prognostic value of clinicopathologic variables obtained at admission and effect of antiendotoxin plasma on survival in septic and critically ill foals. *J Vet Intern Med* 2006;20:569-574.

202. Durando MM, MacKay RJ, Linda S, et al. Effects of polymyxin B and Salmonella typhimurium antiserum on horses given endotoxin intravenously. *Am J Vet Res* 1994;55:921-927.

203. Morris DD, Moore JN. The effect of immunity to core lipopolysaccharides (LPS) on the production of thromboxane and prostacyclin by equine peritoneal macrophages. *Cornell Vet* 1989;79:231-247.

204. Falagas ME, Michalopoulos A. Polymyxins: old antibiotics are back. *Lancet* 2006;367:633-634.

205. Morresey PR, Mackay RJ. Endotoxin-neutralizing activity of polymyxin B in blood after IV administration in horses. *Am J Vet Res* 2006;67:642-647.

206. Barton MH, Parviainen A, Norton N. Polymyxin B protects horses against induced endotoxaemia in vivo. *Equine Vet J* 2004;36:397-401.

207. MacKay RJ, Clark CK, Logdberg L, et al. Effect of a conjugate of polymyxin B-dextran 70 in horses with experimentally induced endotoxemia. *Am J Vet Res* 1999;60:68-75.

208. Raisbeck MF, Garner HE, Osweiler GD. Effects of polymyxin B on selected features of equine carbohydrate overload. *Vet Hum Toxicol* 1989;31:422-426.

209. Lohmann KL, Vandenplas M, Barton MH, et al. Lipopolysaccharide from Rhodobacter sphaeroides is an agonist in equine cells. *J Endotoxin Res* 2003;9:33-37.

210. Bryant CE, Ouellette A, Lohmann K, et al. The cellular Toll-like receptor 4 antagonist E5531 can act as an agonist in horse whole blood. *Vet Immunol Immunopathol* 2007;116:182-189.

211. Lohmann KL, Vandenplas ML, Barton MH, et al. The equine TLR4/MD-2 complex mediates recognition of lipopolysaccharide from Rhodobacter sphaeroides as an agonist. *J Endotoxin Res* 2007;13:235-242.

212. Gordon BR, Parker TS, Levine DM, et al. Neutralization of endotoxin by a phospholipid emulsion in healthy volunteers. *J Infect Dis* 2005;191:1515-1522.

213. Chien JY, Jerng JS, Yu CJ, et al. Low serum level of high-density lipoprotein cholesterol is a poor prognostic factor for severe sepsis. *Crit Care Med* 2005;33:1688-1693.

214. Goldfarb RD, Parker TS, Levine DM, et al. Protein-free phospholipid emulsion treatment improved cardiopulmonary function and survival in porcine sepsis. *Am J Physiol Regul Integr Comp Physiol* 2003;284:R550-557.

215. Moore JN, Norton N, Barton MH, et al. Rapid infusion of a phospholipid emulsion attenuates the effects of endotoxaemia in horses. *Equine Veterinary Journal* 2007;39:243-248.

216. Dellinger RP, Tomayko JF, Angus DC, et al. Efficacy and safety of a phospholipid emulsion (GR270773) in Gram-negative severe sepsis: results of a phase II multicenter, randomized, placebo-controlled, dose-finding clinical trial. *Crit Care Med* 2009;37:2929-2938.

217. Wenneras C, Ave P, Huerre M, et al. Blockade of CD14 aggravates experimental shigellosis. *J Endotoxin Res* 2001;7:442-446.

218. Haziot A, Hijiya N, Gangloff SC, et al. Induction of a novel mechanism of accelerated bacterial clearance by lipopolysaccharide in CD14-deficient and Toll-like receptor 4-deficient mice. *J Immunol* 2001;166:1075-1078.

219. Opal SM, Palardy JE, Parejo N, et al. Effect of anti-CD14 monoclonal antibody on clearance of Escherichia coli bacteremia and endotoxemia. *Crit Care Med* 2003;31:929-932.

220. Verbon A, Dekkers PE, ten Hove T, et al. IC14, an anti-CD14 antibody, inhibits endotoxin-mediated symptoms and inflammatory responses in humans. *J Immunol* 2001;166:3599-3605.

221. Verbon A, Meijers JC, Spek CA, et al. Effects of IC14, an anti-CD14 antibody, on coagulation and fibrinolysis during low-grade endotoxemia in humans. *J Infect Dis* 2003;187:55-61.

222. Axtelle T, Pribble J. IC14, a CD14 specific monoclonal antibody, is a potential treatment for patients with severe sepsis. *J Endotoxin Res* 2001;7:310-314.

223. Endo S, Inada K, Takakuwa T, et al. [Levels of soluble CD14 in patients with septic multiple organ failure (MOF): preliminary report]. *Nihon Geka Gakkai Zasshi* 1994;95:129.

224. Gluck T, Silver J, Epstein M, et al. Parameters influencing membrane CD14 expression and soluble CD14 levels in sepsis. *Eur J Med Res* 2001;6:351-358.

225. Barnes PJ. How corticosteroids control inflammation: Quintiles Prize Lecture 2005. *Br J Pharmacol* 2006;148:245-254.

226. Quante T, Ng YC, Ramsay EE, et al. Corticosteroids reduce IL-6 in ASM cells via up-regulation of MKP-1. *Am J Respir Cell Mol Biol* 2008;39:208-217.

227. Frauenfelder HC, Fessler JF, Moore AB, et al. Effects of dexamethasone on endotoxin shock in the anesthetized pony: hematologic, blood gas, and coagulation changes. *Am J Vet Res* 1982;43:405-411.

228. Morris DD, Moore JN, Crowe N, et al. Dexamethasone reduces endotoxin-induced tumor necrosis factor activity production in vitro by equine peritoneal macrophages. *Cornell Vet* 1991;81:267-276.

229. Antonopoulou A, Giamarellos-Bourboulis EJ. Immunomodulation in sepsis: state of the art and future perspective. *Immunotherapy* 2011;3:117-128.

230. Rice TW, Wheeler AP, Morris PE, et al. Safety and efficacy of affinity-purified, anti-tumor necrosis factor-alpha, ovine fab for injection (CytoFab) in severe sepsis. *Crit Care Med* 2006;34:2271-2281.

231. Morris PE, Zeno B, Bernard AC, et al. A placebo-controlled, double-blind, dose-escalation study to assess the safety, tolerability and pharmacokinetics/pharmacodynamics of single and multiple intravenous infusions of AZD9773 in patients with severe sepsis and septic shock. *Crit Care* 2012;16:R31.

232. Barton MH, Bruce EH, Moore JN, et al. Effect of tumor necrosis factor antibody given to horses during early experimentally induced endotoxemia. *Am J Vet Res* 1998;59:792-797.

233. Cargile JL, MacKay RJ, Dankert JR, et al. Effect of treatment with a monoclonal antibody against equine tumor necrosis factor (TNF) on clinical, hematologic, and circulating TNF responses of miniature horses given endotoxin. *Am J Vet Res* 1995;56:1451-1459.

234. Morris DD. Endotoxemia in horses. A review of cellular and humoral mediators involved in its pathogenesis. *J Vet Intern Med* 1991;5:167-181.

235. Sykes BW, Furr MO. Equine endotoxaemia--a state-of-the-art review of therapy. *Aust Vet J* 2005;83:45-50.

236. Reinhart K, Gluck T, Ligtenberg J, et al. CD14 receptor occupancy in severe sepsis: results of a phase I clinical trial with a recombinant chimeric CD14 monoclonal antibody (IC14). *Crit Care Med* 2004;32:1100-1108.

237. Sykes BW, Furr M, Giguere S. In vivo pretreatment with PGG-glucan fails to alter cytokine mRNA expression of equine peripheral blood mononuclear cells exposed to endotoxin ex vivo. *Vet Ther* 2005;6:67-76.

238. Wagner B, Freer H. Development of a bead-based multiplex assay for simultaneous quantification of cytokines in horses. *Vet Immunol Immunopathol* 2009;127:242-248.

239. McKenzie HC, Furr MO. Equine Neonatal Sepsis: The Pathophysiology of Severe Inflammation and Infection. *Compendium Continuing Education for Veterinarians: Equine Edition* 2001;23:661-672.

240. McGinley MD, Narhi LO, Kelley MJ, et al. CD14: physical properties and identification of an exposed site that is protected by lipopolysaccharide. *J Biol Chem* 1995;270:5213-5218.

241. Juan TS, Hailman E, Kelley MJ, et al. Identification of a lipopolysaccharide binding domain in CD14 between amino acids 57 and 64. *J Biol Chem* 1995;270:5219-5224.

242. Stelter F, Bernheiden M, Menzel R, et al. Mutation of amino acids 39-44 of human CD14 abrogates binding of lipopolysaccharide and Escherichia coli. *Eur J Biochem* 1997;243:100-109.

243. Juan TS, Hailman E, Kelley MJ, et al. Identification of a domain in soluble CD14 essential for lipopolysaccharide (LPS) signaling but not LPS binding. *J Biol Chem* 1995;270:17237-17242.