DIFFERENTIAL GENE EXPRESSION INDUCED BY

TOLL-LIKE RECEPTOR LIGANDS IN EQUINE MONOCYTES

by

MONICA DIAS FIGUEIREDO

(Under the Direction of James N. Moore)

ABSTRACT

Toll–like receptors (TLRs) recognize conserved molecular motifs of microbes and elicit inflammatory and immune responses through specific intracellular adaptor molecules, particularly MyD88 and TRIF. Initiation of inflammatory responses is an important component of the host response to microbial pathogens and for the development of immunity.

The first two studies reporting this dissertation project established the basis for the subsequent three studies. Firstly, an optimal source of lipopolysaccharide-binding protein (LBP) for eliciting responses by equine monocytes to lipopolysaccharide (LPS) was identified. Secondly, twenty-nine equine primer pairs to measure gene expression by real-time quantitative RT-PCR in equine monocytes were optimized and validated. The remaining three studies investigated the response of equine monocytes to different TLR agonists and a specific TLR4 antagonist.

Based on the results of the initial study, pooled commercial and autologous equine sera are optimal sources of LBP activity for studies elucidating the effects of LPS on equine monocytes. A reliable set of equine primer pairs were used to determine that similar gene expression profiles were induced by the TLR4 (*E. coli* LPS) and TLR2 (Pam_3CSK_4) ligands that signal via MyD88. In contrast, the TLR3 (Poly I:C) ligand that signals through TRIF induced

significantly higher expression of interferon-ß, IFN-γ inducible protein 10 and CCL5 than either the TLR2 or TLR4 ligands. Natural and synthetic lipid A compounds derived from *E. coli* strongly induced signaling through the MyD88-dependent pathway. Furthermore, the presence of the KDO moiety, resulted in significantly greater expression of inflammatory cytokines than did lipid A compounds lacking KDO. Differences in the length of the fatty acid chains attached to the lipid A backbone induced different levels of biological responses from equine monocytes. Finally, the second-generation synthetic lipid A analagoue, E5564, induced minimal pro-inflammatory effects in equine whole blood or monocytes. Most importantly, E5564 inhibited LPS-induced expression of procoagulant activity and tumor necrosis factor-α (TNF-α) production and mRNA expression of TNF-α, interleukin-1ß (IL-1ß) and IL-10.

INDEX WORDS: Equine, Monocytes, Toll-like receptors, Lypopolysaccharide, Lipid A, Gene expression, Procoagulant activity, TNF-α, TLR4 antagonist

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DEDICATION

To my parents Alberto and Neuza for their never-ending support, and to my husband Dmitry for his love and friendship.

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CHAPTER 1

INTRODUCTION

The studies contained in this report were undertaken to contribute our knowledge regarding the responses of equine monocytes to Toll-like receptor (TLR) ligands. Chapter 2 contains a review of the literature and is divided into three sections. Section I contains an introduction to TLRs and their role in innate and adaptive immunity. In addition, this section also describes the TLR structure, intracellular signaling pathways and downstream inflammatory mediators. Section II describes the TLR4 receptor complex and section III contains a description of synthetic lipid A compounds serving as adjuvants or antagonists. Chapters 3 through 7 each consist of manuscripts describing the results of studies comprising this dissertation research. Chapter 3 summarizes studies performed comparing equine and bovine sera as sources of lipopolysaccharide binding protein activity in equine monocytes incubated with lipopolysaccharide. Chapter 4 contains a report describing the validation of a reliable set of primer pairs for measuring gene expression by real-time quantitative RT-PCR in equine monocytes, and Chapter 5 summarizes studies performed in which different TLR agonists were used to differentially activate the MyD88- and TRIF-dependent signaling pathways in equine monocytes. Chapter 6 contains a report describing the responses of equine monocytes to four structurally distinct *E. coli* lipid A compounds, and Chapter 7 summarizes the effects of a second-generation synthetic lipid A analagoue, E5564, on responses of equine monocytes and whole blood to lipopolysaccharide. Finally, Appendix A describes the inhibition of TRIF-dependent gene expression by 2-aminopurine.

CHAPTER 2

LITERATURE REVIEW

SECTION I: TOLL-LIKE RECEPTORS

Introduction

The mammalian immune system can be divided in adaptive and innate immunity. Adaptive immunity detects antigen through molecularly defined receptors expressed on the surface of B and T cells. This process is driven by extensive proliferation and clonal expansion of these cells. The resulting response represents an amplification of a few selected clones from a highly diverse repertoire of cells bearing different antigen receptors.[1] This system provides specific recognition of dangerous antigens and the development of memory.[2] However, for the response to infection to occur, the effector cells must expand and differentiate, which delays the action of the adaptive component of the host's immune response for 4 to 7 days.[3] In contrast, the innate immune system can provide prompt recognition of invading microorganisms, by utilizing a microbial sensing system of pattern-recognition receptors that identify specific microbial patterns (also known as Pathogen Associated Molecular Patterns). These microbial patterns are broadly conserved among microbes, and are essential for their survival and multiplication.[4-6] Innate immune cells also play a central role in directing the adaptive immune system as to which type of specific response should develop to optimally eradicate infectious agents. By sensing danger or damage by infectious agents, cells of the innate immune system are capable of "communicating" with the adaptive immune system by expressing membrane-bound

costimulatory molecules and producing soluble mediators, such as cytokines and chemokines. Thus, recognition of these patterns present in the microbial structure allows the immune system to rapidly identify dangerous invaders and to later effectively direct the adaptive immune response.[7,8]

The main functions of the pattern recognition receptors include opsonization, activation of complement and coagulation cascades, phagocytosis, activation of proinflammatory signaling cascades, and induction of apoptosis.[9] These receptors can be divided according to their function and cellular localization. Mannan-binding lectin, C-reactive protein and serum amyloid protein are secreted pattern recognition receptors that are produced by the liver as part of the acute phase response component of inflammation. The function of these secreted receptors is to bind and clear microorganisms by opsonization and subsequently, activate the complement and the phagocytic systems.[10] Another family of detection proteins is the nucleotide-binding site/leucine-rich repeat (NBS/LRR) family.[11] The best characterized members of this family are Nod1 and Nod2. These proteins are located in the cytoplasm and are involved in the detection of specific motifs in bacteria, leading to the induction of pro-inflammatory mediators.[12] The other group of pattern recognition receptors are present in the membranes of phagocytes and include the scavenger receptors, C-type lectins and the Toll-like receptors (TLR).[13] The first two of these receptors mediate phagocytosis of microorganisms by enhancing antigen uptake.[14] The TLRs are signaling receptors that activate signaling pathways, inducing antimicrobial effector responses, and initiate inflammation upon recognition of the microbial patterns. The TLR family is unique primarily because of its capacity for initiating an adaptive immune response through activation of professional antigen-presenting cells.[15]

The first member of the Toll family identified was a Drosophila protein, involved in the dorso-ventral formation of the fly embryo.[16] Studies performed in the early 1990's described the similarities between that Drosophila Toll intracellular domain and the mammalian interleukin-1 (IL-1) receptor domain.[17] In 1995, Lemaitre et al. demonstrated that the Drosophila Toll was also involved in the fly's immune response.[18] After the role of Toll in the fly's host immune defense was discovered, a mammalian homologue of the drosophila Toll was identified.[19]

The mammalian TLR family consists of 11 members, 10 identified in humans and an additional one in mice. These receptors differ from each other in ligand specificities, expression patterns and gene expression induced (Tables 1-3 and Fig 2.1). [4,5,20-22] The TLRs are a type I transmembrane protein, composed of N-terminal leucine-rich repeats and a cytoplasmic Toll-IL-1 receptor (TIR) domain (Fig 2.2).[19] The leucine-rich repeats are involved in ligand recognition and signal transduction, and are present in both cytoplasmic and transmembrane proteins.[23] The TIR intracellular domain is present in all TLRs and is essential for cellular signaling, by recruiting a group of adaptor proteins based on specific protein interactions.[24] The 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).

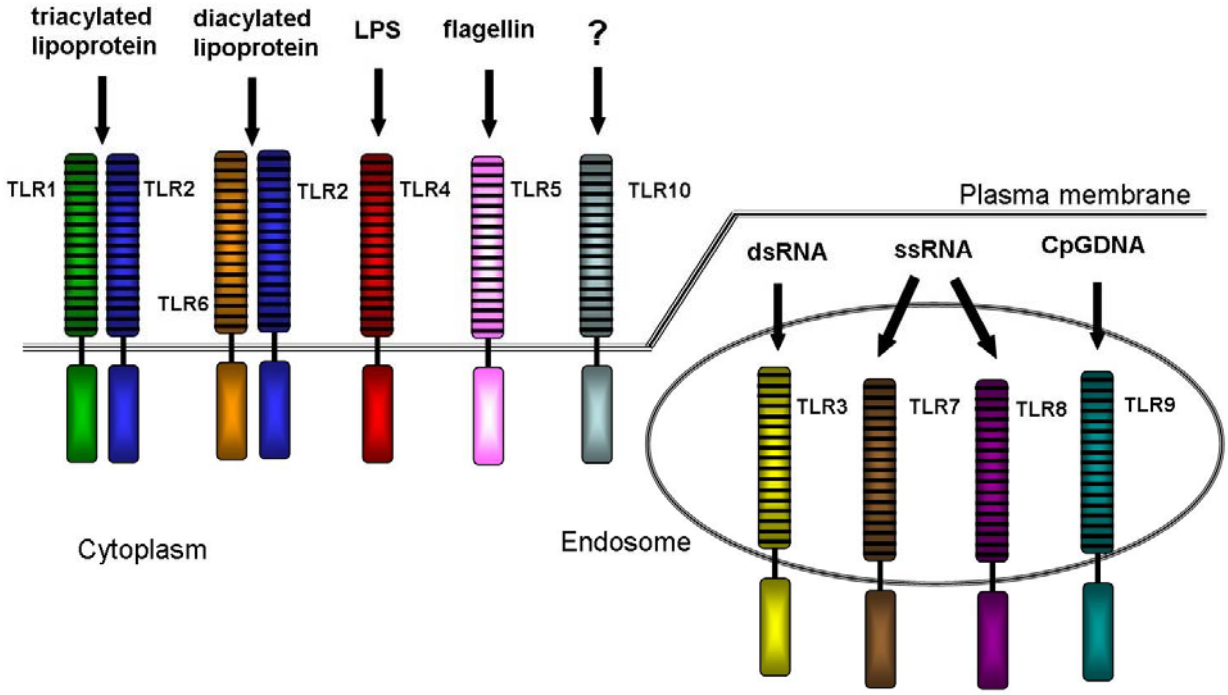

Figure 2.1 TLR cell localization and microbial ligands

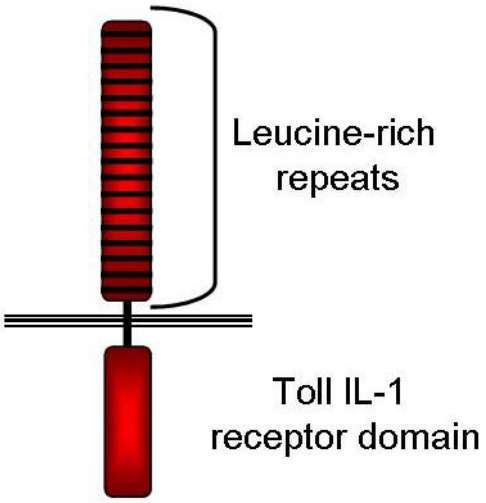

Figure 2.2 TLR structure

TLRs mediate innate and adaptive immunity

Much importance has been placed on TLRs and their role linking innate and adaptive immune responses.[25,26] Ligand recognition by TLRs activates monocyte-derived dendritic cells, and other antigen-presenting cells, to secrete the proinflammatory cytokines that promote dendritic cell maturation, antigen uptake and presentation.[27] TLR activation of dendritic cells induces specific patterns of expression of chemokine receptors and adhesion molecules that allow dendritic cells to enter the lymphatic systems and migrate towards the lymph nodes.[25] These dendritic cells are responsible for providing the signals that activate naïve T cells. The first of these signals is an antigen-specific signal relayed in the context of major histocompatibility complex class I or II (MHC). The second signal is delivered by costimulatory molecules expressed on the surface of the dendritic cell that trigger expression of other molecules on naïve T cells. It is the combination of the antigen peptides presented, in the context of MHC proteins, the expression of costimulatory molecules, and the secretion of specific cytokines by the dendritic cells that induce the differentiation of $CD4^+$ cells into either Th1 or Th2 cells, and leads to the activation of $CD8^+$ T cells.[28]

Intracellular signaling pathways

A distinctive feature of the intracellular signal transduction pathways utilized by the TLRs is their reliance on adaptor molecules. The most widely studied of these adaptor molecules are MyD88 and TRIF.[29] In mammalian species studied to date, TLR1, 2, 5, 6, 7, 8 and 9 signal only through MyD88, TLR3 utilizes only TRIF, and TLR4 has been reported to recruit both MyD88 and TRIF. The importance of these two adaptor molecules lies in the finding that each leads to a distinct profile of immune mediators. [30]

MyD88 adaptor protein

MyD88 was the first adaptor molecule to be described and has been identified as a key adaptor for TLR signaling.[31] MyD88 contains a TIR domain in its C-terminal portion and a death domain in N-terminal portion. The adaptor molecule is recruited to the TIR domain of the TLRs after ligand binding, leading to the recruitment of IL-1R-associated kinase (IRAK4 and IRAK1) (Figure 2.3).[32] The IRAKs then enable the recruitment and activation of tumor necrosis factor (TNF) receptor associated factor (TRAF) 6, which activates the transforming growth factor (TGF)-β activated kinase (TAK1) through polyubiquitination.[33] TAK1 in turn activates the inhibitor of nuclear factor-B (IκB) kinase (IKK) complex, leading to its ubiquitination and degradation.[34] This latter effect allows nuclear transcription factor kappa B (NF-kB) to be translocated into the nucleus.[35] The activation of TAK1 also results in the activation of mitogen activated protein (MAP) kinases (c-Jun N-terminal kinase/p38), followed by the activation of the transcription factor AP-1.[36] The pathway described above is central to the development of a strong pro-inflammatory response. Many of the genes activated by NFκB are also upstream activators of NFκB, further amplifying the host response.

Mal adaptor protein

The adaptor protein Mal has been described as a 235 amino acid protein, homologous to MyD88 and containing a TIR domain at the carboxyl-terminal. Although Mal has similarities to MyD88, it lacks a death domain and is 75 amino acids shorter.[37] Previously, Mal was believed to interact specifically with TLR4, however recent studies have demonstrated that Mal is essential for TLR4 and TLR2-mediated MyD88-dependent signaling.[38]

TRIF adaptor protein

TRIF is a TIR containing adaptor protein that mediates MyD88-independent signaling. With the assistance of the bridging adaptor TRIF-related adaptor molecule (TRAM), TRIF can activate NFκB in either a TRAF6 dependent manner or a TRAF6-independent manner involving the receptor-interacting protein 1 (RIP1) (Figure 2.3).[39] Another protein involved in the TRIF signaling pathway is TRAF1, a member of the TRAF family of adapter proteins involved in signal transduction for the TNF receptor and TLR/IL-1R superfamilies.[40] TRAF1 is a TRIF interacting protein that is induced by Epstein-Barr virus infection in humans.[41] TRIF also interacts with the TRAF family member-associated NFκB activator (TANK)-binding kinase 1 (TBK1) and inducible IKB kinase (IKK-i). These interactions result in activation of the transcription factors IRF-3 and IRF-7, leading to the induction of interferon-inducible genes.[42-44] Induction of these genes is important for anti-viral and anti-bacterial responses,[45,46] and has a profound impact on dendritic cell maturation, an important link between innate and adaptive immune responses.[25,26,30,47] TRIF-dependant signaling can be inhibited by 2-aminopurine, a specific inhibitor of double stranded RNA dependent protein kinase (PKR).[48]

TRAM adaptor protein

Like TRIF, TRAM activates both IRF3 and NFκB.[49] Overexpression of TRIF and TRAM in HEK293 cells suggests that TRAM facilitates activation of TRIF.[50] Studies using TRAM-deficient mice are phenotypically similar to TRIF-deficient mice.[49]

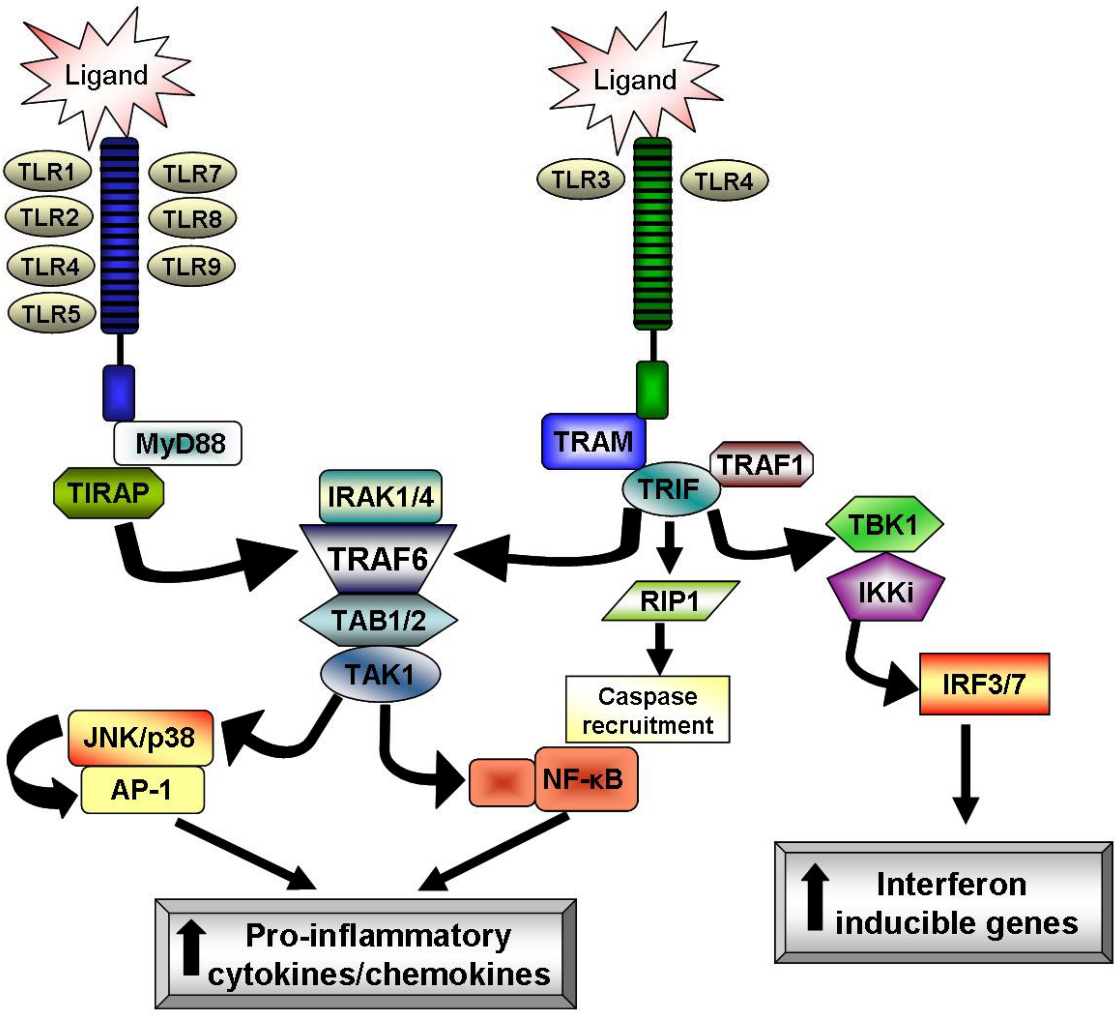

Figure 2.3 TLR intracellular signaling

Differential induction of the MyD88 and TRIF-dependent pathway

Studies performed using specific knock-out (KO) mice have elucidated how different TLRs are activated by individual microbial ligands.[51] For example, mice deficient in the TLR4 gene are hyporesponsive to LPS, an integral component of the outer membrane of gram-negative bacteria.[52] Similarly, studies performed with TLR2 KO mice demonstrated high susceptibility of these animals to *S. aureus* infection, indicating that peptidoglycan or other components of gram positive organisms are recognized by TLR2.[53-55] It was subsequently determined that these bacterial components are recognized by heterodimers comprised of TLR2 and other TLR family members. For example, the TLR2/TLR1 heterodimer recognizes bacterial lipopeptide and the TLR2/TLR6 heterodimer recognizes mycoplasmal lipopeptides.[56,57] Studies performed with TLR3-deficient mice showed reduced responses to viral RNA and Poly I:C, indicating the role of TLR3 in viral recognition.[58] Furthermore, mice lacking TLR9 failed to produce pro-inflammatory cytokines, or have evidence of proliferation of B cells or maturation of dendritic cells in response to unmethylated CpG DNA.[59]

In addition, the development of MyD88-deficient mice ($MyD88^{-/-}$) provided an important tool for investigating the critical role of MyD88 in TLR signaling. $MyD88^{-/-}$ mice lack responsiveness to LPS and are resistant to endotoxic shock typically induced by high doses of LPS.[60] The finding, however, that LPS activates the MAP kinases and NFκB signaling cascades in $MyD88^{-/-}$ mice demonstrated the presence of a MyD88-independent pathway, hereafter referred to as the TRIF-dependent pathway.[61]

The interplay between pathways utilizing MyD88 and TRIF has been illustrated by microarray studies that examined LPS-induced changes in gene expression in macrophages from wild-type, $MyD88^{-/-}$, $TRIF^{-/-}$ and $MyD88^{-/-}$ $TRIF^{-/-}$ mice. Results from those studies

demonstrated that many genes are redundantly regulated by both MyD88 and TRIF, but no genes are up-regulated in macrophages lacking both MyD88 and TRIF.[62,63]

NFκB and downstream inflammatory mediators

A major pathway activated by all TLRs is the one that culminates in the activation of the transcription factor NFκB. This transcription factor is considered to be a master switch for inflammation, regulating the expression of many genes that encode proteins involved in immunity and inflammation.[64-67]

NFκB is a dimeric protein of either identical or structurally homologous protein subunits of about 50 to 75 kD. The common structural motif shared by these proteins is called a Rel homology domain. The mammalian proteins that share this motif are p50, p52, p65, c-Rel and RelB. The heterodimer p50/p65 is the most important inducible complex in mammals.[68] 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.[65]

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.[69]

Tumor necrosis factor-α (TNF) is the principal mediator of the acute inflammatory response and is responsible for many of the systemic complications during severe infections.

Although activated mononuclear cells are the major source of TNF-α, T cells, NK cells and mast cells can also secrete this cytokine.[70] The TLR4 ligand, LPS, is a potent inducer of TNF-α production.[71] TNF-α is synthesized as a nonglycosylated type II membrane protein, that contains an intracellular amino terminus and a large extracellular carboxyl terminus. The membrane bound form of TNF-α is expressed as a homotrimer and binds to the type II TNF receptor (TNF-RII). The membrane form can also be cleaved by a membrane-associated metalloproteinase, after the release of a 17-kD polypeptide.[72] The combined polypeptide trimer forms the 51kD circulating TNF-α protein that can bind to two distinct receptors, TNF-RI and TNF-RII. Both of these receptors are present on almost all cell types. Binding to TNF-RI leads to apoptosis, whereas binding to TNF-RII leads to the recruitment of TRAF proteins and activation of the transcription factors NFκB and AP-1.[73]

TNF-α 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 infection and to activate these cells to eliminate invading microorganisms. TNF-α also stimulates the secretion of chemokines and the production of acute phase proteins, such as, serum amyloid A and fibrinogen, by hepatocytes.[74] Therefore, TNF-α is often used as a reliable, representative indicator of inflammatory cell activation, and is considered to be a hallmark of MyD88-dependent activation.

TNF-α also stimulates endothelial cell expression of tissue factor, a potent activator of coagulation.[75] Tissue factor is a transmembrane glycoprotein that contains three portions; a large extracellular domain, a transmembrane domain and a cytoplasmic tail.[76] This protein initiates blood coagulation by interacting with plasma factor VII which then activates factors X and IX. This leads to the generation of thrombin, fibrin deposition and platelet activation.[77,78] Tissue

factor is normally expressed by extravascular cells and in an encrypted form in the circulation that limits the inappropriate activation of the blood coagulation cascade. During inflammation, tissue factor is expressed by monocytes and endothelial cells. Although tissue factor is often considered for its role in development of disseminated intravascular coagulation, a beneficial effect of its synthesis is the development of fibrin thrombi that help prevent the spread of microorganisms during tissue infections. Expression of the gene encoding tissue factor is regulated by NFκB and AP-1.[79]

IL-1β (interleukin) and TNF-α have similar functions and biological effects despite having different receptors and utilizing different intracellular signaling pathways.[80] IL-1β polypeptides are synthesized as 33kD precursors, and are proteolytically cleaved by a protease, called IL-1β-converting enzyme, to generate the biologically active 17 kD proteins that are secreted. There are two different types of membrane receptors for IL-1β, 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-1β-mediated responses. The type II receptor is expressed on B cells, but may be induced on other cell types.[81]

IL-6 is synthesized by mononuclear phagocytes, vascular endothelial cells and fibroblasts in response to TNF-α and IL-1β. 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.

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.[82] 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.[83] 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.[84]

IRF-3/7 and downstream inflammatory mediators

Activation of TLR3 leads to the nuclear localization of the transcription factors IRF-3 and IRF-7. IRF-3 is constitutively expressed in growing cells, whereas the expression of IRF-7 is induced by IFN-α/ß.[44,85] Both of these transcription factors are present in the cytoplasm as inactive monomers until their serine residues are phosphorylated by two kinases; TBK1 and IKKe/IKKi.[42,43] After they are activated, the IRF-3 and 7 monomers form dimers, which translocate into the nucleus and binds to the IFN-stimulated response element leading to the expression of type IFN-ß. In turn, secreted IFN-ß induce the expression of CCL5 [previously known as RANTES (regulated on activation normal T cell expressed and secreted)] and IFN-γ inducible protein 10 (IP-10).[86] The critical role of IRFs-3 and 7 in the production of IFNs in response to viral infection has been demonstrated convincingly in IRF-$3^{-/-}$, IRF-$7^{-/-}$and IRF-3/IRF-$7^{-/-}$ mice.[87,88]

Type I IFNs are responsible for mediating the early innate immune responses to all intracellular infections, including viral infection. This group consists of two distinct proteins; IFN-α and IFN-β, both of which induce similar biological responses and bind to the same cell

surface receptor. The type I IFN receptor is a member of the type II cytokine receptor family. This receptor has a heterodimeric structure, in which one portion binds to the cytokine and the other transduces signals through the JAK/STAT pathway. Type I IFNs inhibit viral replication by inducing the synthesis of enzymes, such as 2'5' oligoadenylate synthase, which interfere with the transcription of viral RNA or DNA.[89] Type I IFN production is induced primarily by two pathways: first, by intracellular sensors of infection and second by TLR3, TLR7 and TLR9. The first pathway is ubiquitous and occurs in virally infected cells, resulting in autocrine or paracrine IFN-mediated signaling that establishes an antiviral state in both infected and neighboring cells. In contrast, the second pathway involves ligand recognition through the TLRs (3, 7 and 9), leading to type-I-IFN-producing specialized cells.[90] There is evidence that type I IFNs also may be produced in response to cellular stimulation by TLR4 ligands.[91]

CCL5 and IP-10 (or CXCL10) are chemokines that are responsible for attracting leukocytes into tissues. They exert their biological effects by interacting with specific G protein-linked transmembrane receptors on the surfaces of their target cells. Chemokines can be subdivided into four groups according to the position of their first two cysteine residues. This subdivision includes CXC (α-chemokine), CC (ß-chemokine), C (γ-chemokine) and CXsC (δ-chemokine). CCL5 is a member of the CC subfamily and IP-10 is an important member of CXC subfamily.[92] Increased expression of CCL5 has been associated with inflammatory disorders, such as arthritis, inflammatory airway disease, glomerulonephritis and neurologic disorders.[93] Furthermore, a role has been proposed for CCL5 as a potent inhibitor of human immuno-deficiency virus replication *in vitro*.[94] The chemokine IP-10 also is induced by IFN-γ and promotes adhesion of T cells to endothelial cells, exerts antitumor effect in vivo, and inhibits both human bone marrow hematopoietic cells and angiogenesis.[95-98]

Table 2.1. Cell expression pattern of Toll-like receptors

TLR	Species	Location in the cell	Cell type distribution
TLR1	H/M	Cell surface	Ubiquitous
TLR2	H/M		NK, monocytes, PMN
TLR3	E/H/M	Intracellular compartment	DC and NK cells
TLR4	E/H/M	Cell surface	DC, NK, macrophages, monocytes, PMN
TLR5	H/M		DC, NK, macrophages, monocytes, T cells, PMN
TLR6	H/M		B cells, PMN, low expression in monocytes and NK cells
TLR7	H/M	Intracellular compartment	B cells, DC, PMN
TLR8	H/M		Monocytes, PMN, low in T and NK cells
TLR9	E/H/M		B cells, DC, macrophage, PMN, NK and microglial cells
TLR10	H	Cell surface	B cells, low in DC, PMN
TLR11	M		Mice cells

H: human; M: murine; E: equine; DC: dentritic cells; PMN: polymorphonuclear cells; NK: natural killer cells

Table 2.2. Toll-like receptor and their ligands

TLR	Natural Ligands	Synthetic ligands
TLR1	Mycobacteria	Triacyl lipopeptide (Pam_3CSK_4)
TLR2	Lipoprotein, peptidoglycan, lipotheic acid, Hsp, porins, zymosan	Triacylated lipoproteins (Pam_3CSK_4)
TLR3	ds RNA	Poly I:C
TLR4	LPS, Taxol, fusion and envelope protein, Hsp, oligossacharides of HA	Synthetic Lipid A molecules and HA fragments
TLR5	Bacteria with flagella	Flagellin
TLR6	Fungi, mycoplasma, gram-positive bacteria	Zymosan, dyacyl lipopeptides, lipotheic acid
TLR7	Single-stranded RNA	Imiquimod
TLR8	Single-stranded RNA	Resquimod
TLR9	Bacterial/viral DNA	Unmethylated CpG DNA
TLR10	Unknown	Unknown
TLR11	Uropathogenic bacteria	Unknown

Table 2.3. Toll-like receptor signaling pathways

TLR	Cytosolic Adapters	Main transcription factors	Genes induced
TLR1/2 and TLR2/6	MyD88/TIRAP	AP-1 and NFκB	Inflammatory cytokines
TLR3	TRIF	IRF-3 and NFκB	IFN-ß inducible genes, Inflammatory cytokines
TLR4	MyD88, MAL, TRAM, TRIF	AP-1, NFκB and IRF-3	Inflammatory cytokines IFN-ß inducible genes
TLR5	MyD88	NFκB	Inflammatory cytokines
TLR7 and TLR9	MyD88	NFκB IRF-7	Inflammatory cytokines Interferon-α
TLR 8, 10 and 11	Unknown		

SECTION II: TLR4 RECEPTOR COMPLEX

Lipopolysaccharide molecule

Lipopolysaccharide is a major component of the outer membrane of Gram-negative bacteria and its composition is different depending upon the bacterial strain from which it was isolated.[99] LPS from *Escherichia coli* consists of three regions: the O-chain, the core and the lipid A portion (Fig 2.4). The O-chain and core form the polysaccharide component of the molecule. The polysaccharide component is bound to the lipid A portion through a unique sugar called 2-keto-3-deoxyoctulosonic acid (KDO). The outermost layer O-chain, which consists of many repeating units of a branched tetrasaccharide, is highly variable among bacteria and determines the antigenic specificity of LPS. The inner most layer, lipid A, which is responsible for the toxicity associated with LPS, consists of six fatty acyl chains linked to two phosphorylated glucosamine residues (Fig 2.5).[100]

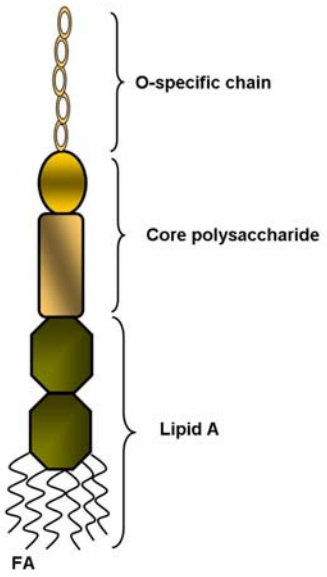

Fig. 2.4 Structure of *E. coli* lipopolysaccharide

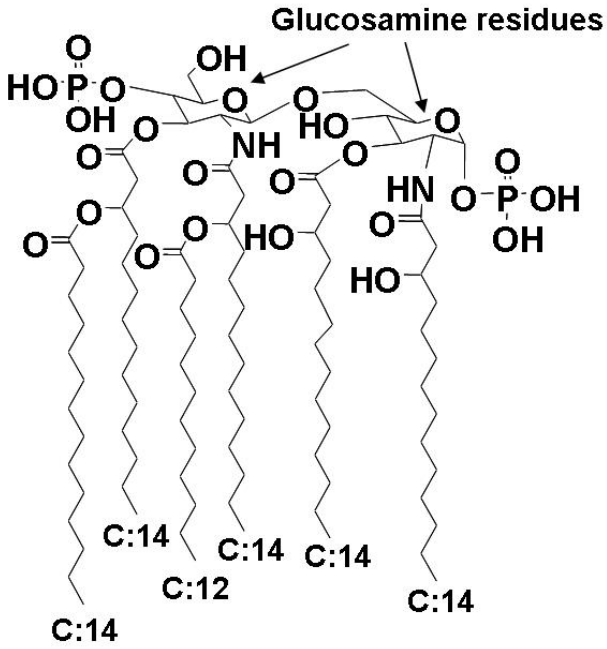

Fig 2.5 Structure of *E. coli* lipid A

TLR4 receptor complex

Toll-like receptor 4 was the first human homolog of *Drosophila* Toll to be identified.[19] The recognition of LPS by TLR4 was elucidated when positional cloning analysis of the LPS-nonresponsive mouse strain, C3H/HeJ, revealed a mutation that replaces proline with histidine in the signaling domain of TLR4.[52] Shortly thereafter, other strains of mice with targeted deletions of the TLR4 gene were reported to be unresponsive to LPS.[101,102] Collectively, the results of these studies demonstrated the essential role for TLR4 in recognition of LPS from gram-negative bacteria.

However, TLR4 is not the sole receptor involved in LPS recognition, as LPS binding protein (LBP), cluster differentiation antigen 14 (CD14) and myeloid differentiation protein 2 (MD-2) interact with TLR4 to form the TLR4 receptor complex (Figure 2.5). Due to the

amphiphilic structure of LPS, the molecule forms aggregates after it is released from Gram-negative bacteria and enters aqueous environments. In order for LPS be successfully transported in the host, it must bind to transport molecules. Examples of these molecules include LBP, high-density lipoproteins (HDL) and bactericidal-increasing protein (BPI). LBP is a 60 kDa serum glycoprotein produced in the liver and lung, that has the properties of an acute phase protein.[103-105] LBP interacts with the lipid A region of LPS, and is a lipid transferase that catalyzes the transfer of LPS to CD14 or HDL. Transfer of LPS to CD14 results in cell activation via the TLR4 receptor complex, whereas transfer to HDL results in neutralization of LPS and its clearance by phagocytic cells in the liver. Thus, the rate of both transfer processes will determine the response of the host to LPS.[106,107] Cells isolated from LBP KO mice are hyporesponsive to LPS, and the addition of LBP enhances the sensitivity of these mice to LPS by approximately 300-fold.[108] Another protein that interacts with LPS is BPI, which has both binding and bactericidal functions against Gram-negative bacteria. BPI is a 57 kDa cationic antimicrobial protein that is present in the azurophilic granules of polymorphonuclear leukocytes. The presence of inflammatory mediators strongly induces upregulation of BPI.[109] BPI is capable of binding to heparin and LPS, and it shares approximately 44% sequence homology with LBP.[110] Other proteins that bind LPS include serum amyloid P, hemoglobin, apoliprotein apo E, lactoferrin, heparin-binding protein, lysozyme and proline-rich peptides.[111]

CD14 is a 50 kDa glycoprotein that is either present in a soluble form (sCD14) or as a glycosylphosphatidylinositol (GDI)-linked form (mCD14) present on myeloid lineage cells.[112] Soluble CD14 can interact directly with LPS, a process that is catalyzed by LBP. The sCD14/LPS complex can bind to CD14 negative cells, such as endothelial cells or some epithelial cells. The membrane form of CD14 has no intrinsic signaling capabilities, as it lacks

transmembrane and cytoplasmic domains. The importance of CD14 in responses induced by LPS has been demonstrated in studies using CD14 KO mice. These mice are insensitive to low concentrations of LPS and the addition of CD14 enhances the LPS response by 1000-fold.[113] Because mice that lack CD14 are still able to respond to high concentrations of LPS, this protein has more of an amplifying role in the response to LPS than an essential role.[114] CD14 also is a major receptor for other bacterial components, including peptidoglycan, lipotheichoic acid, lipoprotein and others.[115]

MD-2 is an 18-25 kDa protein that is secreted into the circulation in dimeric subunits. The results of recent studies indicate that LPS binds to MD-2 in the absence of LBP, and is physically associated with MD2 and TLR4 in the presence of CD14.[116,117] TLR4 and MD2 become associated in the Golgi with the assistance of the endoplasmic reticulum chaperone gp96.[118] MD2 has also been implicated in the glycosylation of TLR4, which is an essential step in the migration of TLR4 to the cell surface.[119] Although it is still not clear if LPS directly binds to TLR4, there is strong evidence to support an interaction between TLR4 and MD2. Studies with human and murine MD2 mutants indicate that binding of MD2 to TLR4 is dependent on the presence of amino acid residues Cys^{95}, Tyr^{102} and Cys^{105}, and that this association between MD2 and TLR4 is essential for LPS recognition.[120,121] Furthermore, 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.[122,123]

Upon LPS recognition by the TLR4 receptor complex, TLR4 undergoes oligomerization and recruits the adaptor molecules, MyD88, MAL, TRAM and TRIF. Recruitment of these

molecules is followed by activation of MAPK and the transcription factor NFκB, and selective activation of the transcription factor IRF3/7 through TRIF. Activation of the MyD88- and TRIF-dependent pathways distal to TLR4 leads to time-dependent differences in activation of NFκB, with activation of the MyD88-dependent pathway resulting in rapid activation of NFκB and delayed activation of NFκB when the TRIF-dependent pathway is used.[86]

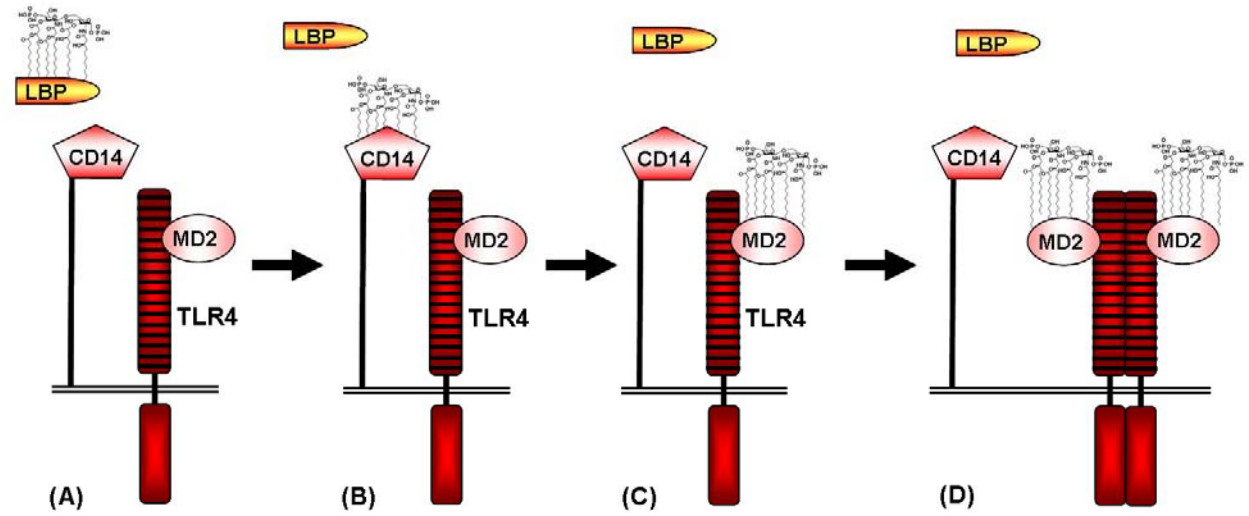

Fig 2.5 Schematic representation of lipid A recognition by the TLR4 receptor complex. (A) The lipid A molecule is transported by LBP in the circulation. (B) LBP facilitates the binding of lipid A to membrane bound CD14, which brings lipid A into close proximity to the membrane. (C) Lipid A is transferred to TLR4/MD2. (D) Recognition of LPS by the TLR4 receptor complex leads to TLR4 oligomerization and initiation of signal transduction. Figure adapted from Fitzgerald K. A., et al., 2004.

SECTION III: SYNTHETIC LIPID A COMPOUNDS AS ADJUVANTS OR ANTAGONISTS

Synthetic lipid A molecules

Until recently, the structure of lipid A was assumed to be highly conserved among Gram-negative bacteria. However, the results of recent studies indicate that the fatty acid acylation pattern, carbohydrate backbone and degree of phosphorylation vary considerably among bacterial species.[124,125] Furthermore, alterations in the number or length of the fatty acids attached to the backbone or in the charge of lipid A affect the magnitude of the cellular responses initiated by lipid A.[126-128]

To identify relationships between lipid A structure and cellular activation, many studies have utilized chemically synthesized and modified lipid As. The majority of the synthetic lipid A structures contain the typical ß-(1-6)-linked D-glucosamine dissacharide backbone, with differences in their patterns of acylation and degree of phosphorylation. For example, lipid A analogs with only one phosphate at either end have been shown to induce weak production of TNF-α and IL-1 ß in comparison to *E. coli* lipid A. Furthermore, lipid A compounds containing both phosphates, but with a reduced number of fatty acids did not induce TNF-α and IL-1ß production in murine cells.[129,130] Furthermore, synthetic compounds containing a glucosamine monosaccharide backbone that is phosphorylated and acylated in various positions induce little cellular activity.[131] Collectively, these results demonstrate that the diphosphorylated *E. coli*-like hexa-acyl lipid A containing two ß(1-6)-linked D-glucosamine components are crucial for the full biological activity of lipid A.

In the past few years, the bioactivity of lipid A molecules also has been related to their three-dimensional shapes.[126] For example, lipid A species with a conical shape, such as those from *E. coli,* are highly bioactive, whereas lipid A species with a cylindrical conformation, such as those from *R. capsulatum* or *C. violaceum,* are less bioactive. The differences in the three-dimensional conformations of these lipid A species are due to the length and number of acyl chains, asymmetry of acyl groups, and number and distribution of negative charges.[132]

Lypopolysaccharide has long been known to induce strong immunostimulatory adjuvant properties, but its extreme toxicity has precluded its therapeutic use. In an effort to avoid the toxic effects inherent to LPS, studies were performed using a detoxified, low pyrogenic, stable and safe immunomodulatory lipid A called monophosphoryl lipid A (MPLA).[133,134] This compound, isolated from *Salmonella minnesota* R595, has been advocated as a strong adjuvant candidate for vaccines.[135] Although MPLA interacts with TLR4 on immune cells and results in the synthesis of pro-inflammatory cytokines, the response is approximately 0.1% of that induced by LPS.[136,137] MPLA also induces the synthesis and release of IL-1 and IFN-γ, and promotes the generation of a Th1 response.[135,138] In a recent study using murine cells, MPLA induced the synthesis of the type I IFNs associated with TRIF-dependent cellular activation, while only weakly stimulating the synthesis of mediators associated with MyD88 signaling.[139]

Even though MPLA appears to be a strong and safe immunomodulatory agent, there is concern that lipid A preparations purified from bacterial cultures may suffer from lack of consistency, both in composition and performance.[140,141] Furthermore, due the heterogeneity of lipid A, one would expect to encounter significant variability in the composition of MPLA between batches, which could preclude its clinical use in the future. In contrast, synthetic lipid A analogues are pure, composed of a single molecule, therefore, achieve reproducibility and

consistency. Structure-function studies performed with MPLA provided the basis for new synthetic adjuvants based on aminoalkly glucosamine phosphate compounds.[142] These compounds have strong adjuvant activity in promoting T-cell proliferation, IFN-γ production and same level of immunostimulatory potency as MPLA.[140]

In keeping with the structural differences among lipid As from different bacteria, LPS molecules from different Gram-negative bacteria have different potencies or induce different patterns of cytokines. For example, different types of LPS from *E. coli*, *Salmonella* spp. and *Neisseria meningitides* are more potent than LPS from *Bordetella pertussis* or *Bacteroides fragilis*,[143,144] and LPS derived from *Rhodobacter sphaeroides* and *R. capsulatum* function as antagonists rather than agonists in human and murine cells.[145] Furthermore, the results of recent studies indicate that LPS from *E. coli* primarily induces the production of pro-inflammatory mediators (TNF-α, IL-1ß and IL-6), LPS from *Salmonella* primarily activates IFN-ß inducible genes, while LPS from *N. meningitides* induces both sets of genes.[128] These results suggest that LPS derived from different bacteria can differentially induce the MyD88-dependent pathway, the TRIF-dependent pathway or both. Collectively, these findings provide the basis for the use of specific lipid A molecules as potent vaccine adjuvants.

Synthetic TLR4 antagonists

Although the production of pro-inflammatory mediators by the host in response to LPS is an important innate immune response during Gram-negative infection, it may also contribute to the pathogenesis of organ injury and septic shock.[146,147] Therefore, competitive inhibition of the untoward effects of LPS by lipid A analogues has become an intriguing approach to limit the

production and release of inflammatory mediators during severe gram-negative bacterial sepsis.[148]

Synthetic TLR4 antagonists are based on the structures of natural lipid A and their biosynthetic precursors. The most studied synthetic TLR4 antagonists are the lipid A analogues; E5531 and E5564 (Figure 2.6). E5531, which was derived from the structure of the lipid A region of the non-toxic bacterium *Rhodobacter capsulatus*, acts as an LPS antagonist in isolated murine and human cells as well as in whole blood. It also protects mice from lethal injection with viable *E. coli* bacteria, and prevents the development of clinical signs and symptoms of sepsis in human volunteers administered LPS under controlled experimental conditions. [145,149-150] Although E5531 was a potent LPS antagonist, its inhibitory activity decreased over time, due to the interaction with plasma lipoproteins.[151,152]

E5564 (eritoran tetrasodium) is a second generation synthetic LPS analogue derived from the structure of *Rhodobacter sphaeroides*. E5564 is approximately 10 times more potent, has a longer duration of action, is structurally and synthetically less complex and is more stable than E5531.[153] Studies have shown that E5564 selectively inhibits LPS signaling via TLR4/MD2, but does not interfere with cell signaling initiated via TLR2 by *Staphylococcus aureus*.[154] Through crystal structure analysis, it has been determined that E5564 binds to the large internal pocket in MD2 (Figure 2.7).[155] Studies of LPS-induced chronic airway disease in mice indicate that treatment with E5564 decreases IL-6 levels in the bronchoalveolar fluid and infiltration of neutrophils into the airways.[156] E5564 blocks the action of LPS at the level of TLR4, thereby preventing *in vitro* and *in vivo* induction of cellular mediators in rodents and humans[148] and improving survival in LPS-challenged mice.[157] Furthermore, E5564 completely blocks the effects of experimental endotoxemia in humans while exhibiting no agonistic LPS-like activity in

human and animal model systems.[158] In contrast to the problems identified with E5531, the presence of serum components have minimal effects on antagonistic activity of E5564.[159] Results from a phase I clinical safety trial demonstrated that administration of E5564 was safe, well tolerated and antagonistic activity was retained for an additional 72 hours after discontinuation.[160] When considered together, these results indicate that E5564 is a potent antagonist of LPS and can be an effective therapeutic agent for treatment of diseases characterized by endotoxemia.

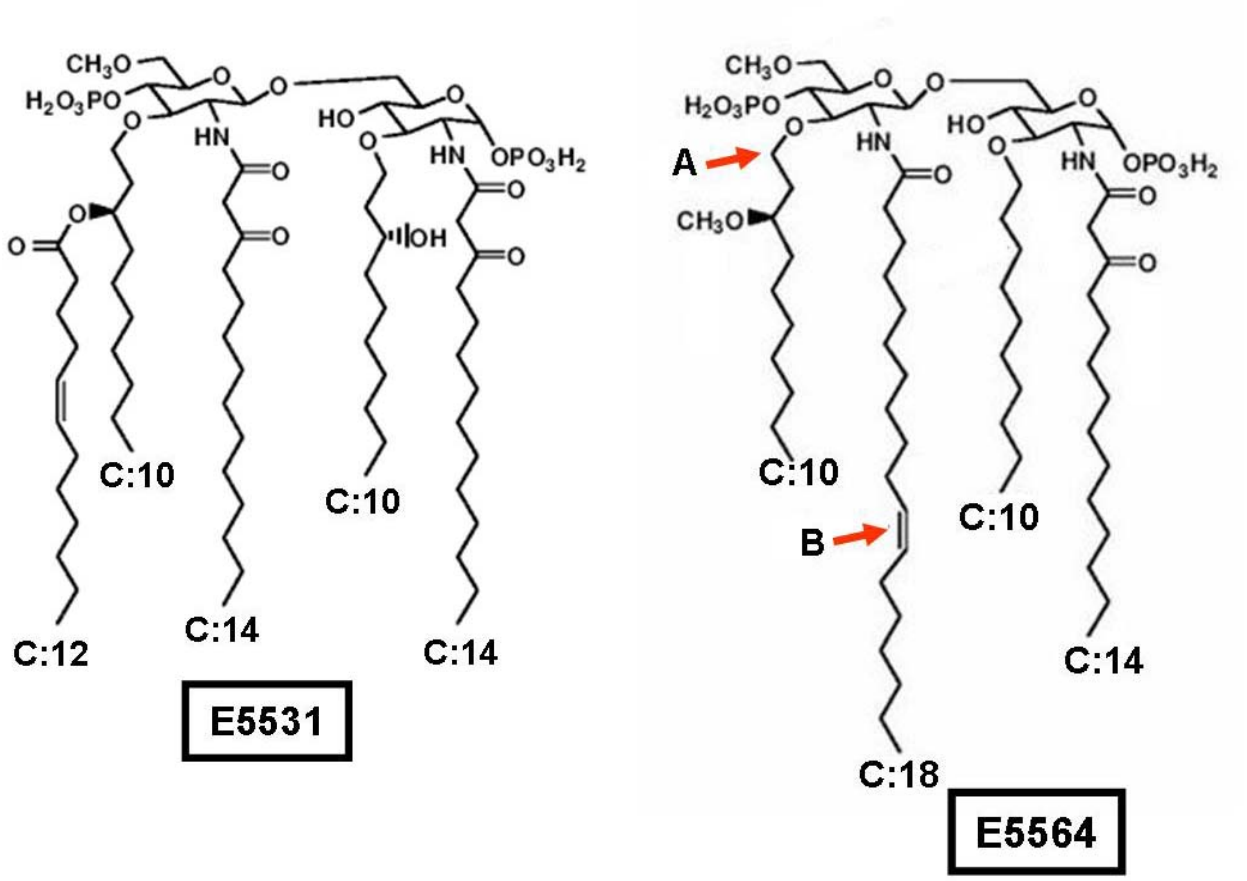

Figure 2.6 Chemical structure of E5531 and E5564. The main structural differences between E5531 and E5564 are: (A) position 3′ has an ether–linked C–10 side chain; (B) the side chain at position 2′ is acylated with cis–vaccenic acid. Figure adapted from Rossignol D.P. and Lynn M., 2002.

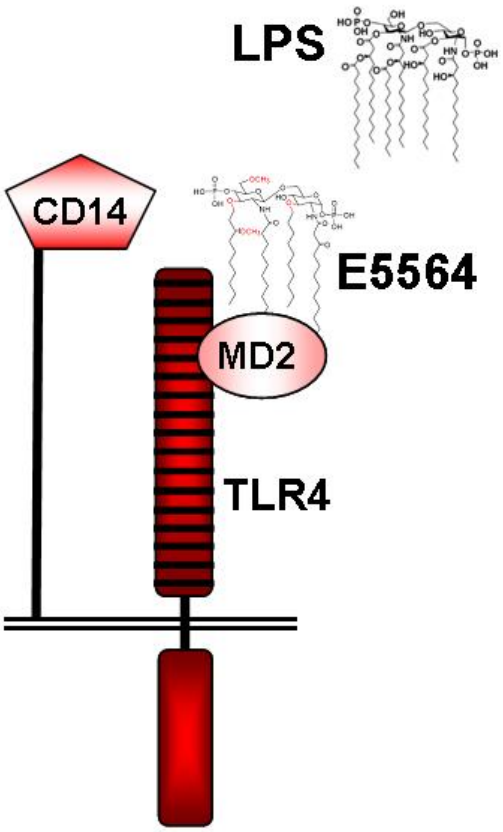

Fig. 2.7 Inhibitory mechanism of E5564. E5564 prevents the interaction between LPS and MD2 by binding to MD2.

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CHAPTER 3

A COMPARISON OF EQUINE AND BOVINE SERA AS SOURCES OF LIPOPOLYSACCHARIDE BINDING PROTEIN ACTIVITY IN EQUINE MONOCYTES INCUBATED WITH LIPOPOLYSACCHARIDE[1]

[1] M.D. Figueiredo, C.E. Salter, D.J. Hurley, J.N. Moore. 2008. *Veterinary Immunology and Immunopathology*. 121 (3-4) 275-80
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ABSTRACT

Lipopolysaccharide-binding protein (LBP) is an acute phase protein that binds the lipid A moiety of lipopolysaccharide (LPS) and transfers LPS monomers to soluble CD14 in plasma or membrane bound CD14 on mononuclear phagocytes. The result of these interactions is activation of the TLR4 receptor complex, and the synthesis and release of inflammatory mediators. Inclusion of LBP in cellular assays increases the sensitivity of cells expressing CD14 to LPS. Therefore, the objectives of this study were: (1) to compare differentially treated sera from cattle and horses as sources of LBP activity using LPS-induced expression of procoagulant activity (PCA) by equine monocytes as a readout, and (2) evaluate the use of commercial equine serum as a source of LBP activity using LPS concentration response and time course studies to validate the response.

Monocytes were isolated from eight horses and incubated with five different serum preparations in the presence or absence of *E. coli* LPS. The sera tested were: heat-inactivated fetal bovine serum (HI-FBS), pooled commercial equine serum (CES), heat-inactivated pooled commercial equine serum (HI-CES), autologous equine serum (AES), and heat-inactivated autologous equine serum (HI-AES). In the absence of LPS, monocytes from half of the horses in the study had increased expression of PCA when incubated with HI-FBS alone; PCA was unaffected by incubation with the other sera. There was a four-fold increase in PCA when monocytes were incubated with LPS in the presence of CES, HI-CES or AES compared to LPS without serum. The combination of HI-FBS and LPS increased PCA twenty-fold compared to LPS without serum. The HI-AES serum lacked significant LBP activity. Whereas maximal expression of PCA was induced by 1 ng/ml of LPS in the absence of serum, inclusion of 1%

CES reduced the LPS concentration required for maximal PCA to 30 pg/ml. Monocytes incubated with LPS in the presence of CES had increased PCA at 3 h and peaked at 6 h.

In conclusion, monocytes from many horses are directly stimulated by HI-FBS, suggesting that HI-FBS is not an optimal source of LBP for *in vitro* studies of LPS with equine monocytes. In contrast, CES and AES are effective sources of LBP activity for such studies, as they do not directly induce activation. Although the heat inactivation process did not affect the LBP activity in CES, it ablated LBP activity in AES. Consequently, investigators are advised to utilize either CES or AES in future studies, but not to heat inactivate AES.

INTRODUCTION

Lipopolysaccharide (LPS), a structural component of the outer cell wall of gram-negative bacteria, elicits the inflammatory responses that characterize sepsis and systemic inflammatory response syndrome. LPS may gain access to the circulation across a damaged intestinal mucosal barrier or from a focal area of infection. Once in the circulation, LPS can interact with a complex of cell receptors located on the surface of mononuclear phagocytes.[1] Activation of this receptor complex, which includes cluster differentiation antigen 14 (CD14), Toll-like receptor 4, and myeloid differentiation factor-2, leads to the production of inflammatory cytokines that can result in the development of septic shock.[2]

LPS binding protein (LBP) plays an integral role in the biological response to LPS by transferring LPS monomers to soluble CD14 in plasma and membrane bound CD14 on the surface of mononuclear phagocytes, thereby initiating intracellular signaling pathways that result in the production of inflammatory cytokines.[3] LBP is normally present in the plasma of

mammals and increases in concentration by more than ten-fold as part of the acute phase response to inflammation.[4] Inclusion of LBP purified from acute phase serum in cellular assays increases the sensitivity of cells expressing CD14 to LPS by more than 100-fold.[4,5] For studies involving cells from species for which neither recombinant nor purified LBP is available, a commonly used alternative is fetal bovine serum (FBS). A recent study performed in our laboratory determined that equine monocytes respond strongly to FBS by expressing PCA; however, this response can be circumvented in most situations by using heat-inactivated fetal bovine serum (HI-FBS).[6] Okano and colleagues also noted that a 1% concentration of HI-FBS provided optimal LBP activity for equine mononuclear cells incubated *in vitro* with *E. coli* LPS. Because HI-FBS alone activated cells from some horses, we hypothesized that a superior source for LBP activity for studies with equine cells might be heat-inactivated equine serum. Therefore, the objectives of the study reported here were to compare pooled commercial and autologous equine sera, as well as heat-inactivated preparations of these sera, with HI-FBS as sources of LBP activity using LPS-induced expression of PCA by equine monocytes as a sensitive readout. Concentration dependent response to LPS, and the time of the response were also assessed using pooled commercial equine serum as a source of LBP activity as part of this study.

MATERIALS AND METHODS

Preparation of Sera

Five serum preparations were tested in this study: heat-inactivated fetal bovine serum (HI-FBS, Hyclone Laboratories, Inc., Logan, UT, USA), pooled commercial equine serum (CES, Hyclone Laboratories, Inc., Logan, UT, USA), heat-inactivated pooled commercial equine serum

(HI-CES), autologous equine serum (AES), and heat-inactivated autologous equine serum (HI-AES). Autologous serum was obtained by collecting blood from each horse into a vacuum evacuated glass tube (BD, Franklin Lakes, NJ, USA). The blood was incubated at 37°C for 1 hour to allow clotting to occur, centrifuged, and the serum aliquoted into pyrogen-free tubes. To produce HI-FBS, HI-AES and HI-CES, aliquots of each serum were incubated for thirty minutes at 56°C in a water bath, and then transferred to pyrogen-free tubes for storage at -80°C until use.

Equine Monocyte Isolation and Stimulation

Peripheral blood from eight horses determined to be healthy by clinical exam was obtained by venipuncture after sterile skin preparation. The University Institutional Animal Care and Use Committee approved this experimental protocol. Mononuclear cells were isolated from leukocyte-rich plasma by single step density gradient centrifugation over Histopaque 1077 (Sigma-Aldrich, Inc., St. Louis, MO, USA). The mononuclear cells were collected at the Histopaque interface, washed with calcium and magnesium free Dulbecco's phosphate buffered saline (PBS, Mediatech, Herndon, VA, USA), counted, and assessed for viability in 0.04% trypan blue dye (Sigma-Aldrich, Inc., St. Louis, MO, USA) on a hemocytometer. The cells (> 98% viable) were suspended at 4 x 10^6 cells/ml in RPMI-1640 media containing 1% penicillin-streptomycin solution (10,000 IU penicillin, 10 ug/ml streptomycin, Mediatech, Inc., Herndon, VA, USA). After a two-hour incubation in pyrogen-free polystyrene tubes, the media and non-adherent cells were removed, and the adherent cells were washed once with warm RPMI.

Triplicate tubes containing adherent cells, primarily monocytes, were incubated with RPMI alone; 1%, 2%, and 5% of each serum in RPMI; and *E. coli* 0111:B4 LPS 100 pg/ml (List Biological Laboratories, Inc., Campbell, CA, USA) as controls. To assess each serum for LBP

activity, triplicate tubes of adherent cells were also incubated with a combination of *E. coli* 0111:B4 LPS 100 pg/ml and 1% of each serum in RPMI.

After a five-hour incubation (37°C, 5% CO_2), the supernatants were removed, and the cells were frozen at -80°C in 250 µl of a PBS solution (Mediatech, Inc., Herndon, VA, USA) containing 0.1% Tween (Fisher Scientific, Fair Lawn, NJ, USA) until cell lysates were assayed for PCA.

Measurement of PCA

Prior to the determination of PCA, cells were thawed and sonicated to ensure complete cell lysis. An automated one-stage clotting assay (ACL Coulter 1000, Instrumentation Laboratory, Lexington, MA, USA) was used to determine the effects of the cell lysates on calcium-induced clotting of pooled equine plasma. PCA was determined by comparing those results to a standard curve with equine brain thromboplastin.[6]

E. coli LPS Concentration Response in 1% CES

Monocytes were isolated as described above, and duplicate tubes were incubated with *E. coli* 0111:B4 LPS (0.1 pg/mL to 1 ng/mL) in RPMI alone or in RPMI containing 1% CES in RPMI and *E. coli* 0111:B4 LPS. Cells incubated with RPMI alone and 1% CES in RPMI were used as controls. After a five-hour incubation, supernatants were discarded, and cells were frozen at -80°C in 250 µl of a PBS solution containing 0.1% Tween until cell lysates were assayed for PCA.

Time Course of LPS-Induced PCA

The incubation time required for maximal LPS stimulation of monocytes with CES as a source of LBP was determined using monocytes isolated as described above. Duplicate tubes were incubated from one to eight hours with 100 pg/ml *E. coli* 0111:B4 LPS and 1% CES in RPMI. Cells incubated in 1% CES in RPMI alone were used as a control. At the end of the incubation, supernatants were removed, and the cells were frozen at -80°C in 250 µl of a PBS solution containing 0.1% Tween. Cell lysates were later assayed for PCA.

Data Analysis

Statistical analysis of all data was performed using the GraphPad Prism software. A one-way ANOVA followed by a post-hoc Bonferroni multiple-comparison test were used to compare the effects of the different sera at 1% and in the presence of LPS. An unpaired T test with Welch's correction was used to compare responses between the two subgroups of horses and between the different sera co-incubated with LPS or LPS alone. The time course data were analyzed using a second order regression analysis to determine the R^2 and fit of each subject. Only data that showed a clear second order fit were included in the time versus activity model, and a simple linear regression model of the means was used to analyze the best fit data. A value of $P < 0.05$ was considered significant, and all data are expressed as the mean ± standard error of the mean.

RESULTS

To validate the test system, monocytes were incubated in RPMI containing *E. coli* 0111:B4 LPS (100 pg/ml) in the absence of serum, or monocytes were incubated with medium containing 1%, 2% and 5% equine sera or the same concentration of heat-inactivated sera (commercial or autologous) in the absence of LPS. Less than 2 units of PCA activity were observed for monocytes incubated in RPMI alone, whereas the addition of LPS induced approximately 27 units. None of the equine sera tested, whether heat inactivated or not, induced significant expression of PCA activity (Figure 3.1).

An unexpected observation was that monocytes from 4 of the 8 horses strongly expressed PCA when incubated with all three concentrations (1%, 2% and 5%) of HI-FBS alone (only 1% data shown, Table 3.1). Therefore, the horses were subdivided into level responders and high responders (Table 3.1). Monocytes from these horses also expressed significantly more PCA when incubated with LPS in the presence of 1% HI-FBS than monocytes treated similarly from the other 4 horses (Table 3.1). Furthermore, the PCA induced by 1% HI-FBS was significantly greater than that induced by all concentrations of the four equine sera (Figure 3.1).

When monocytes were incubated with a combination of 100 pg/ml of *E. coli* 0111:B4 LPS and 1% of either CES, HI-CES, or AES, expression of PCA was increased approximately four-fold compared to values obtained for the same concentration of *E. coli* 0111:B4 LPS alone (Figure 3.2). Incubation of monocytes with the combination of 1% HI-FBS and *E. coli* 0111:B4 LPS (100 pg/ml) resulted in PCA nearly twenty-fold higher than that induced by the same concentration of *E. coli* 0111:B4 LPS alone (Figure 3.2).

In contrast to the other equine serum preparations, HI-AES lacked LBP activity, as the PCA of monocytes incubated with a combination of 1% HI-AES and *E. coli* 0111:B4 LPS (100 pg/ml) was indistinguishable from that induced in response to *E. coli* 0111:B4 LPS alone (Figure 3.2).

Co-incubation of monocytes with *E. coli* 0111:B4 LPS at concentrations exceeding 300 pg/ml in the absence of CES resulted in expression of PCA that was significantly greater than that detected for cells incubated in RPMI alone (Table 3.2); maximal expression of PCA (131.8 ± 36.9) induced by LPS in the absence of serum occurred at a concentration of 1 ng/ml. In contrast, incubation of monocytes with ≥ 30 pg/ml of *E. coli* 0111:B4 LPS in the presence of CES resulted in expression of PCA that was significantly greater than values obtained for cells incubated in RPMI alone. Maximal expression of PCA (>140 units) in the presence of CES was induced by 300 pg/ml of LPS.

No PCA was expressed by monocytes incubated with 100 pg/ml *E. coli* 0111:B4 LPS and 1% CES in RPMI for either one or two hours. Thereafter, PCA increased until a plateau was reached after four hours, with a modest peak in PCA being evident at six hours of incubation. The second order linear regression model demonstrated that five out of six horses fitted the expected model well. The linear regression model provided evidence of a direct linear relationship between incubation time and production of PCA (Figure 3.3).

DISCUSSION

The results of this study indicate that pooled commercial and autologous equine sera are effective sources of LBP activity for studies being performed to elucidate the effects of LPS on

equine monocytes. Furthermore, these sera do not stimulate equine cells in the absence of LPS, even when the sera are not heat-inactivated. The latter finding is in contradiction to the effects of FBS, which significantly increases expression of PCA by equine mononuclear cells when not heat-inactivated.[6]

While incubation of equine cells in the present study with 1% HI-FBS and 100 pg/ml *E. coli* 0111:B4 LPS resulted in PCA values greater than those produced by cells incubated with 1% CES, HI-CES, or AES and 100 pg/ml LPS, a substantial portion of this response was due to the activation of the monocytes by the HI-FBS itself. Furthermore, monocytes from half of the horses in the present study strongly expressed PCA when incubated with HI-FBS alone; this response was not noted when monocytes from any of the horses were incubated with either CES or AES in the absence of LPS. In addition, the magnitude of LPS-induced expression of PCA was indistinguishable among cells incubated with CES, HI-CES, or AES. Collectively, these findings indicate that equine sera provide a more predictable source of LBP activity than HI-FBS, and that inclusion of 1% of CES, HI-CES or AES in cell culture media provides comparable levels of LBP activity.

An unexpected finding in the present study was the effect of a commonly used heat inactivation procedure on AES. In contrast to CES and FBS, heat inactivation of AES completely eliminated its effectiveness as a source of LBP activity. While further study will be required to determine the reason for the pronounced reduction in LBP activity occurring exclusively with HI-AES, the more important conclusions are that neither pooled CES nor AES requires heat-inactivation prior to use, and that AES should not be heat inactivated for studies in which LBP activity is desired.

Once CES had been identified as the serum preparation of choice for providing LBP activity, we were then interested in identifying the lowest concentration of LPS at which this effect was evident. This was determined by comparing expression of PCA by cells incubated with a wide range of concentrations of LPS in the presence or absence of 1% CES. The results of that portion of the present study indicated that significant LBP activity was evident at concentrations of LPS as low as 30 pg/mL. This finding has direct relevancy to clinical situations as similar plasma concentrations of LPS have been detected in horses with naturally occurring gastrointestinal diseases.[7,8]

In the present study, we corroborated previous reports indicating that LPS-induced expression of PCA by equine monocytes is maximal after approximately six hours of incubation.[9] Because the previous studies had been performed either in the absence of a source of LBP activity or with HI-FBS, we performed the time course study using CES as the source of LBP activity. Although there was evidence of expression of PCA after three hours, the values obtained were approximately half those obtained after four hours. Based on the results of the present study, optimal expression of PCA can be expected when equine monocytes are incubated with LPS in RPMI containing 1% CES for a minimum of four hours.

In conclusion, although HI-FBS is an effective source of LBP activity for *in vitro* studies with equine monocytes stimulated by *E. coli* LPS, the results of the present study suggest that pooled CES and AES produce more consistent results among horses whose cells may respond to HI-FBS. Furthermore, neither of these sera requires heat inactivation for use in cellular assays.

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Table 3.1. Mean ± SEM procoagulant activity in equine monocytes in two subgroups after five hour incubation in RPMI media and 1% concentrations of various sera and LPS 100pg/ml.

Incubation condition	Subgroup 1 (level responders)	Subgroup 2 (high responders)
RPMI	1.5 ± 0.1	1.6 ± 0.2
RPMI and LPS	40.2 ± 27.5	14.7 ± 7.8
1% HI-FBS and LPS	333.0 ± 54.4	906.6 ± 112.0[a]
1% CES and LPS	227.7 ± 21.6	140.4 ± 31.2
1% HI-CES and LPS	241.3 ± 25.1	197.1 ± 47.1
1% AES and LPS	188.4 ± 25.7	162.2 ± 46.2
1% HI-AES and LPS	22.0 ± 6.5	35.2 ± 29.4

[a]Value is significantly different ($P < 0.001$) from subgroup 1, 1% HIFBS in the presence of LPS, as determined by an unpaired T test with Welch's correction. Heat-inactivated fetal bovine serum: HI-FBS, pooled commercial equine serum: CES, heat-inactivated pooled commercial equine serum: HI-CES, AES: autologous equine serum, HI-AES: heat-inactivated autologous equine serum.

Table 3.2. Mean ± SEM Procoagulant activity of equine monocytes after 5 hours of incubation with RPMI with various concentrations of LPS with or without 1% CES.

		Incubation condition	
LPS concentration	**n =**	**RPMI**	**RPMI and 1% CES**
0 pg/mL	10	2.0 ± 1.0	1.1 ± 0.3
0.1 pg/mL	4	1.8 ± 0.8	1.9 ± 0.5
1 pg/mL	10	1.8 ± 0.7	2.7 ± 1.2
3 pg/mL	6	0.9 ± 0.1	1.0 ± 0.2
10 pg/mL	10	4.9 ± 4.0	3.7 ± 1.0
30 pg/mL	6	1.6 ± 0.6	27.4 ± 9.5[c]
100 pg/mL	10	37.1 ± 16.9	142.5 ± 29.4[bc]
300 pg/mL	6	49.8 ± 10.2[a]	143.4 ± 33.6[bc]
1ng pg/mL	10	131.8 ± 36.9[a]	126.9 ± 33.9[b]

CES: Pooled commercial equine serum. [a] Value significantly ($P < 0.01$) different from the value for cells in RPMI with no LPS (0 pg/mL). [b] Value significantly ($P < 0.01$) different from the value for cells in RPMI and 1% CES with no LPS (0 pg/mL). [c] Value significantly ($P < 0.05$) different from the value for cells in RPMI with no CES at the same concentration of LPS.

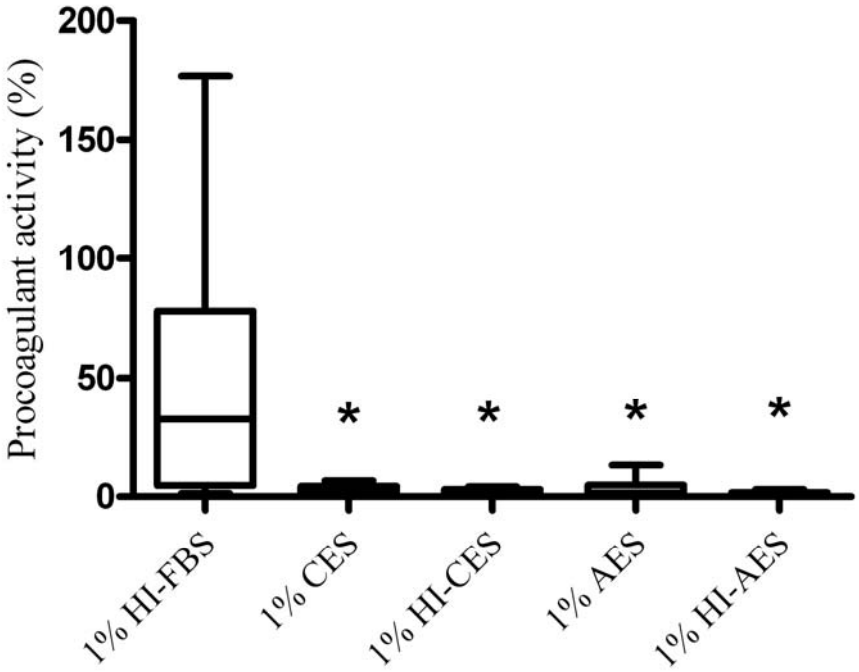

Figure 3.1 Box and Whiskers Plot of procoagulant activity in equine monocytes induced by 1% bovine or equine sera in RPMI in the absence of LPS. The horizontal bar within each box represents the median value, the bottom and top of the box represent the 1st and 3rd quartiles, respectively. The ‘‘whisker’’ represents the range of data. * Values are significantly different from 1% HI-FBS ($P < 0.05$) as determined by a 1-way ANOVA test followed by a post hoc Bonferroni multiple-comparison test. Heat-inactivated fetal bovine serum: HI-FBS, pooled commercial equine serum: CES, heat-inactivated pooled commercial equine serum: HI-CES, autologous equine serum:AES, heat-inactivated autologous equine serum: HI-AES.

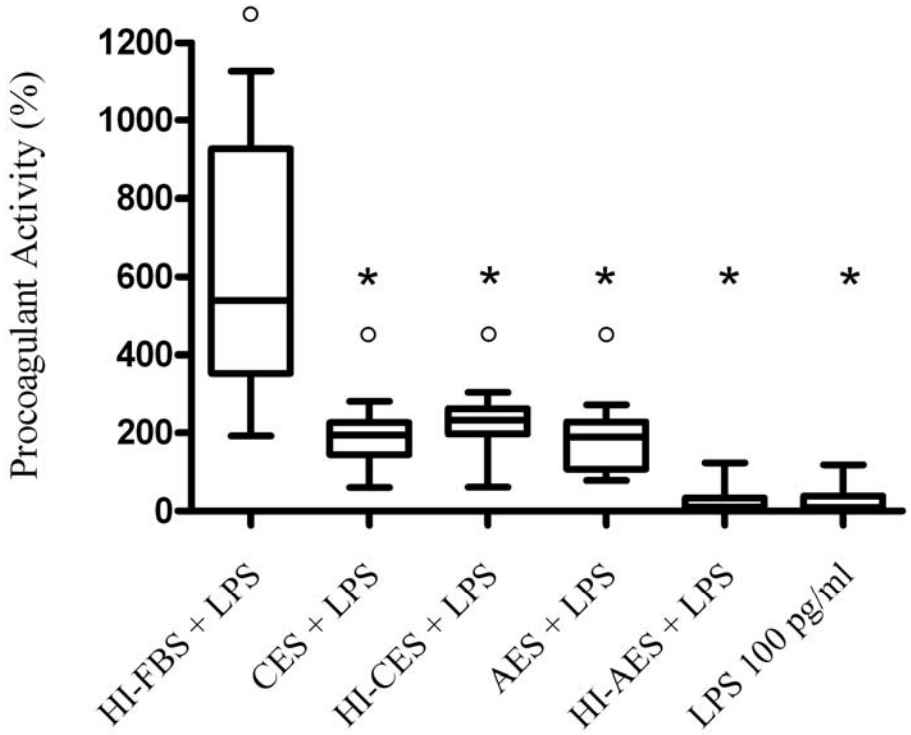

Figure 3.2 Box and Whiskers Plot of procoagulant activity in equine monocytes induced by LPS 100pg/ml alone, and 1% bovine and equine sera in RPMI in the presence of LPS 100 pg/ml. The horizontal bar within each box represents the median value, the bottom and top of the box represent the 1st and 3rd quartiles, respectively. The ‘‘whisker’’ represents the range of data. *Values are significantly different from 1% HIFBS in the presence of LPS 100 pg/ml. ($P < 0.001$) as determined by a 1-way ANOVA test followed by a post hoc Bonferroni multiple-comparison test. °Values are significantly different ($P < 0.001$) from LPS 100 pg/ml as determined by an unpaired T test with Welch’s correction. Heat-inactivated fetal bovine serum: HI-FBS, pooled commercial equine serum: CES, heat-inactivated pooled commercial equine serum: HI-CES, autologous equine serum: AES, heat-inactivated autologous equine serum: HI-AES, *E. coli* 0111:B4 LPS: LPS.

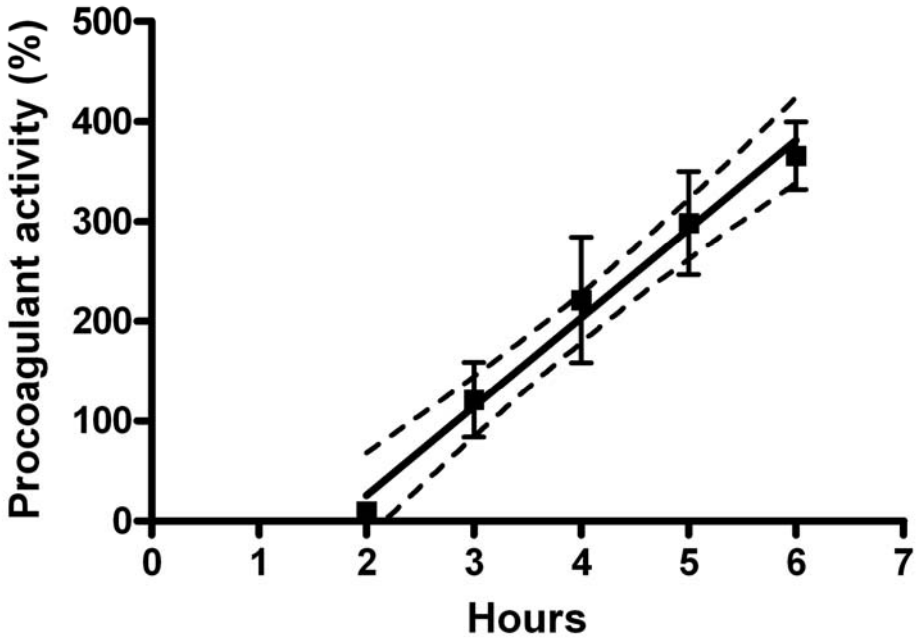

Figure 3.3 Simple linear regression line showing a positive correlation between hours of incubation (2 h – 6 h) and procoagulant activity in equine monocytes induced by LPS 100 pg/ml in the presence of 1% CES (r = 0.9888), (P = 0.0005). Ninety-five percent confidence intervals are shown between the dashed lines on either side of the regression line.

CHAPTER 4

VALIDATION OF A RELIABLE SET OF PRIMER PAIRS FOR MEASURING GENE EXPRESSION BY REAL-TIME QUANTITATIVE RT-PCR IN EQUINE LEUKOCYTES[1]

[1] M.D. Figueiredo, C.E. Salter, D.J. Hurley, J.N. Moore. 2008. Submitted to *Veterinary Immunology and Immunopathology*, 09/16/2008

ABSTRACT

Quantification of gene expression using real-time reverse transcription quantitative PCR (RT-qPCR) is a reliable method to monitor cellular responses to pro-inflammatory stimuli. The main objective of this study was to validate a set of equine primer pairs that can be routinely used to monitor expression of genes that are central to inflammatory and immune responses. This paper describes the steps used to optimize and validate twenty-nine equine primer pairs for RT-qPCR assays using SYBR Green detection. To validate these assays, monocytes were isolated from three horses and stimulated with *E. coli* LPS. Because four of the twenty-nine genes demonstrated poor amplification efficiency due to weak induction of gene expression by LPS, monocytes were stimulated with alternative agents (PMA and Poly I:C) known to induce gene expression in monocytes. These agents, acting through different pathways than LPS, improved the level of gene expression and yielded good amplification efficiency for these genes. PCR efficiency was based on a standard curve for each gene and the calculated efficiency was approximately 100% for all 29 genes. The PCR efficiencies for the majority of the target genes were equivalent to that of the housekeeping gene (18S rRNA) used in all experiments. Dissociation curve analysis and gel electrophoresis revealed a single product for each gene analyzed. The availability of the twenty-nine validated primer pairs reported herein will allow investigators to elucidate the response of horses to a variety of inflammatory stimuli and will further our understanding of disease pathogenesis in horses.

INTRODUCTION

Our understanding of the horse's immune system and responses to inflammatory stimuli is limited by the paucity of immunological and biological assays that accurately measure specific mediators at either the gene or protein levels. Currently there are relatively few antibodies validated for measuring equine cytokines.[1-4] Alternatively, cytokines can be measured using biological activity assays (e.g. WEHI assay for TNF-α activity).[5] However, these assays are generally labor intensive, costly, and the results are difficult to compare among laboratories. Furthermore, as the cell cultures used for these assays are carried through many passages, changes can occur in the phenotype and sensitivity of the cells, making the results of biological activity assays complex to interpret.[6,7]

Several methods are now available for measuring gene expression in horses, including reverse transcription competitive polymerase chain reaction (RT-cPCR), quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR), in situ hybridization, ribonuclease protection assay, and Nothern blot analysis.[8-16] Although the latter two techniques are quantitative, they require large amounts of RNA, which are often not practical to obtain. All of these techniques depend on the availability of specific equine gene sequences, which are far more widely available than are antibodies that recognize the corresponding gene products. Of these techniques, the most commonly used is RT-qPCR. The advantages of RT-qPCR are that it is relatively easy to perform, provides quick results, requires small amounts of template, and can produce quantitative data with an accurate dynamic range of 7 to 8 log orders of magnitude.[17-19] The main disadvantages associated with this technique are the costs of the equipment and reagents.

The majority of previous studies reporting measures of gene expression in horses via RT-qPCR have not included full descriptions of the validation processes used.[20-22] As a result, it is difficult for other investigators to implement these assays in their own laboratories and compare results across laboratories. Therefore, the primary aim of this study was to fully describe the steps used to optimize and validate twenty-nine equine primer pairs for RT-qPCR assays using SYBR Green as the detector. SYBR Green is a highly specific double-stranded intercalating DNA binding dye, that detects PCR products that accumulate during the PCR cycles. Due to the ability of SYBR Green to detect non-specific double-stranded DNA, primer pairs must be optimized and fully validated before this technique can yield data that are accurate and reproducible.

Based on our interest in monitoring responses of equine leukocytes to pro-inflammatory stimuli involved in the pathogenesis of equine gastrointestinal diseases, the main objective of this study was to validate reliable equine primer pairs required to monitor expression of genes that are central to these responses. These twenty-nine genes (Table 1) were selected for their roles in inflammatory and immune responses, and include proteins representing different functional groups, most notably antimicrobial peptides, cell adhesion molecules, cell surface receptors, chemokines, cytokines, enzymes, and mediators of signal transduction, among others. The availability of these validated primer pairs will allow other investigators to more fully understand the horse's response to a variety of inflammatory stimuli and will further our understanding of disease pathogenesis in horses.

MATERIALS AND METHODS

Materials

Equine serum was purchased from Hyclone (Logan, UT), RPMI-1640, PBS and penicillin/streptomycin were from Mediatech, Inc. (Herndon, VA). *Escherichia coli* 0111:B4 LPS was purchased from List Biological Laboratories, Inc. (Campbell, CA), Poly I:C (Polyinosine-polycytidylic acid) was from InvivoGen (San Diego, CA). RNeasy Mini Kits and the RNase-Free DNase Set were purchased from Qiagen Inc. (Valencia, CA). High Capacity cDNA archive kits, SYBR Green PCR master mix, and Universal eukaryotic 18S rRNA Taqman kits were purchased from Applied Biosystems (Foster City, CA). PMA, Histopaque-1077 and all other high grade chemicals were from Sigma–Aldrich (St. Louis, MO).

Horses

Three adult horses determined to be healthy on the basis of clinical examination were used for the study. A venipuncture site over a jugular vein on each horse was aseptically prepared, and blood samples were collected into syringes containing EDTA as an anticoagulant. The experimental protocol was reviewed and approved by the Animal Care and Use Committee at The University of Georgia.

Isolation of equine peripheral blood monocytes

Mononuclear cells were isolated by density-gradient centrifugation over Histopaque 1077 as previously described.[23] Viability was greater than 98% by trypan blue exclusion. Approximately 1 x 10^7 mononuclear cells were added to sterile 60 x 15-mm Petri dishes and

incubated for 2 h at 37°C in 5% CO2 atmosphere. After incubation, non-adherent cells were removed by washing with RPMI-1640. Adherent monocytes were overlaid with RPMI-1640 supplemented with 10% equine serum containing 100 U of penicillin/ml and 100 μg of streptomycin/ml. Monocytes were incubated for 20 h in the presence of either 100 pg/ml *Escherichia coli* 0111:B4 LPS, 250 μg/ml Poly I:C or 10^{-6} M PMA.

RNA extraction and cDNA synthesis for mRNA expression analysis

After incubation, cells were washed with cold phosphate buffered saline, scraped from the plates in RNA cell lysis solution and stored at –80°C. Total RNA was extracted from the cell lysates using the RNeasy mini RNA extraction kit according to manufacturer's protocol and treated by incubation with DNase I at 24°C for 30 min. Only samples having 260:280 nm absorbance ratios between 2.0 and 2.2 as measured on a NanoDrop spectrophotometer (Thermo Fisher Scientific;Wilmington, DE) were processed for cDNA synthesis using the High Capacity cDNA Archive Kit with 100 ng of RNA as template.

Primer design

Oligonucleotide primers were designed with Primer Express software (Applied Biosystems, Foster City, CA), with equine sequences available from the GenBank database. The primer and the predicted melting temperature (Tm) were calculated using the nearest-neighbor algorithm[24] using the same software. The primer pairs were selected using the following criteria: primer Tm (58 – 60°C), primer length (18 – 24 mer), limit repeats of guanosine and cytosine, and amplicon size (80 – 160 bp). Table 4.1 lists the twenty-nine genes, with their respective

accession number, forward and reverse sequence, amplicon size, predicted melting temperature and biological function.

Optimizing primer concentrations and PCR efficiency

The objective of this process was to identify the lowest primer concentration that yielded the lowest C_T (cycle number at which the fluorescence generated within a reaction crosses the threshold line), the maximum ΔRn (the magnitude of the signal generated by the given set of PCR conditions) and the generation of a single amplicon of correct size and with a melting temperature close to that predicted. In these experiments, all combinations of three concentrations (50, 300, 900 nM) of forward and reverse primers for eight genes (CCL5, IFN-ß, IL-1, IL-6, IL-10, IP-10, TNF-α, and TRAF1) were used to generate optimal amplification plots. PCR specificity for each gene was determined by detection of fluorescence generated with a C_T value less than 30, dissociation curve analysis and gel electrophoresis. For gel electrophoresis samples were resolved at 80V in a 2% agarose gel with 0.5X Tris-borate/EDTA buffer and stained with ethidium bromide to visualize products. PCR efficiency was determined using standard curves generated with ½ log dilutions (0.1 pg – 100 pg/ml) of cDNA template for each target gene.

Real-time RT-PCR (qRT-PCR)

Real-time qPCR assays using SYBR Green as a detector were performed in an Applied Biosystems 7900HT (Foster City, CA) sequence detection system. Conditions for amplification were 2 minutes at 50°C, 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C, and 60 seconds at 60°C for primer annealing and elongation. This was followed by a dissociation

stage for 15 s at 95°C to ensure the presence of a single amplicon. The PCR reactions contained 300 nM of each primer, 2.5 µl of a 2X SYBR Green master mix, and 1 µl of the diluted cDNA sample in a final volume of 5 µl. Inter-assay variation was evaluated by measuring either 18S rRNA or TNF-α from a pooled cDNA sample prepared from RNA of cells stimulated with *E. coli* 0111:B4 LPS (100 pg/ml) for 4 hours. A no-template control was included for every PCR run. All reactions were performed in triplicate wells of a 384-well microtiter plate.

RESULTS

Optimization of primer concentrations

Of the combinations of primer concentrations evaluated, 300 nM of the forward and reverse primers used to target the eight representative genes revealed good amplification (Fig. 4.1). The product generated by each primer pair yielded single products on inspection of the dissociation curves (Fig. 4.2) and a single band on agarose gel electrophoresis (Fig. 4.3). A concentration of 300 nM was used for the other 21 sets of primers. In all of those assays, each primer pair yielded a single specific PCR product as determined by dissociation curve analysis and gel electrophoresis.

Size and Tm of amplicons

All amplicons were of the correct predicted size, ranging between 81 and 153 bp (examples in Figure 4.3). Because all the amplicons utilized were of a consistent small size, we were able to combine annealing and elongation into a single step. The observed Tm for each amplicon, which is dictated by the sequence and length of the amplicon, was similar to the

predicted Tm value. In fact, the average difference between predicted and observed Tm for all amplicons was 0.04 ± 0.25 (mean ± SEM). For example, the observed Tm for CD44 was 82.8°C (Fig 4.2), which confirmed the predicted Tm of 83°C (Table 4.1).

PCR efficiency

The PCR efficiency was determined from the slope of the standard curve generated with serial dilutions of cDNA template for each gene. It was calculated using the equation $Ex = (10^{-1/slope}) - 1$, where Ex is PCR efficiency. A slope of -3.32 is indicative of a PCR efficiency of 1 (100%). For the majority of the primer pairs, the PCR standard curve efficiencies derived from LPS-stimulated monocytes generated a PCR efficiency of approximately 100% (Table 4.2). In contrast, for genes with low expression in response to LPS, such as ß-defensin, IFN-ß, IFN-γ, and TLR3 the apparent efficiencies ranged from 175 to 472%. As a result, monocytes were incubated with PMA to yield increased expression of ß-defensin and IFN-γ, and with Poly I:C for IFN-ß (Fig. 4.4 A-B) and TLR-3. Table 4.2 lists the range of C_T values, PCR efficiency mean slope, percent efficiency, and the mean slope for the validation experiment for each gene transcript. The PCR efficiency variation of -3.45 to -3.13 reported in this study was within the range reported by others.[25,26]

Selection of a housekeeping gene

For a valid quantitative assay using the comparative C_T method, the efficiency of the target amplification and the efficiency of amplification of the housekeeping gene must be equivalent. The housekeeping gene should serve as an endogenous reference, in which its expression remains constant within cells during the course of the experiments and between

different experimental samples. In this study, the eukaryotic 18S rRNA was selected as the housekeeping gene, primarily because it shows limited change in expression under different experimental conditions.[27-29] Because 18S rRNA sequences are highly conserved among eukaryotes, a single assay can be used as housekeeping gene measurement for studies involving cells from many species. In addition, our laboratory has used this housekeeping gene for gene expression studies in cells from mice, humans, dogs, cows, and horses. To determine if the two amplification reactions had the same PCR efficiency, ΔC_T (C_T target – C_T 18S mRNA) was calculated, and ΔC_T values were plotted vs. the log cDNA input. For the results of these experiments to be considered valid, the absolute value of the slope for each target gene had to be <0.1 (Fig. 4.4 C-D). The slopes for all primer pairs except HYAL1, HYAL2 and IL-8 were ≤ 0.1. As indicated in Table 4.2, the slopes for primer pairs for 10 genes were determined using values obtained from a single horse. The amplification efficiency of 18S rRNA was 96% using equine RNA as template (Fig. 4.5).

Inter-assay variability

Inter-assay variability was measured using a pooled cDNA samples prepared from RNA from cells incubated with *E. coli* 0111:B4 LPS (100 pg/ml) for 4 hours. For every RT-qPCR assay either 18S rRNA or TNF-α were measured from this pooled sample. The mean $C_T \pm$ SEM and coefficient of variance C_T values for 18s rRNA and TNF-α were 12.9 ± 0.2, 8.1% and 23.6 ± 0.1, 2.2%, respectively.

DISCUSSION

This study describes the steps used to optimize and validate a reliable set of twenty-nine equine primer pairs for RT-qPCR assays using the fluorogenic DNA binding dye SYBR Green as the detection method. Although the use of SYBR Green does not permit development of multiplex reactions, if properly validated it does provide highly reliable and accurate quantification of changes in gene expression. The accuracy of the data obtained using this dye depends on satisfying a specific set of criteria, as described below. Furthermore, SYBR Green can be used to detect accumulation of PCR products without requiring the synthesis of a fluorescent probe specific for each individual template. Finally, the costs associated with making these assessments with SYBR Green are considerably less than those typically associated with use of the fluorescently-labeled oligonucleotide probes such as the hydrolysis probe (e.g. TaqMan chemistry), hybridization probes, and hairpin probes (e.g. molecular beacons).

The accuracy of gene expression measurements relies on the linearity and efficiency of product formation during the PCR amplification steps. In this study, the PCR efficiency for each gene was assessed using a standard curve generated with increasing amounts of cDNA from equine monocytes stimulated with LPS for 20 h. Because the PCR efficiency for expression of genes for ß-defensin, IFN-ß, IFN-γ, and TLR3 using this stimulant was poor, additional experiments were performed in which monocytes were stimulated with other pro-inflammatory agents. The agonists selected were PMA, a direct activator of PKC that has numerous effects on cellular gene expression, and Poly I:C, a synthetic analog of double-stranded RNA. The latter agonist contains molecular patterns recognized by TLR3 and known to induce type 1 interferon expression.[30] Stimulation of the monocytes with PMA decreased the range of C_T values and

improved the PCR efficiency for ß-defensin and IFN-γ. Incubation of cells with Poly I:C improved the accuracy and efficiency of the PCR reactions for IFN-ß and TLR3, thereby allowing identification of increased expression of these two genes. As a consequence of these findings, when validating a set of primer pairs, it is important to consider the degree of expression induced by a specific stimulant and the time at which maximum expression of the gene will occur.

The most commonly used methods to quantify PCR data are the absolute method using a standard curve and the comparative C_T method. The latter method is a mathematical model that calculates a relative fold change in gene expression between experimental and calibrator samples. This method is useful when assaying a large number of samples, as it avoids the necessity for generating a standard curve for every run. However, the comparative C_T method can only be used if the amplification kinetics of the target and housekeeping genes are approximately equal,[31] as determined in a validation experiment. While it is desirable that the slope generated in the validation experiment be < 0.10, as occurred for the majority of the primer pairs tested in this study (Table 4.2), this may not always be achievable. A small group of primer pairs (HYAL1, HYAL2 and IL-8) generated slopes between 0.15 and 0.19. Thus, if the efficiencies of the two amplicons are not approximately equal, then the analysis may need to be performed via the absolute quantification method using standard curves.

Normalization of gene expression relative to a reliable housekeeping gene is essential for accurate data interpretation. The most commonly used reference genes include ß-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18S ribosomal RNA. Both ß-actin and GAPDH previously have demonstrated variability in expression during cell activation, differentiation or proliferation, precluding their use as a housekeeping gene in certain cell types

or studies in which cells are stimulated with a pleiotropic agent such as LPS.[32-34] In contrast, 18S rRNA has demonstrated a more consistent level of expression under different conditions,[27-29] and has been advocated as the housekeeping gene of choice for studies on gene expression in equine macrophages.[10] Based on these data and concerns about other housekeeping genes, 18S rRNA was used as the housekeeping gene in this study.

To calculate the inter-assay variation in the measurements made in this study, 18S rRNA (as housekeeping gene) or TNF-α (as an inducible gene) mRNA expression were evaluated using a pooled cDNA sample prepared from monocytes stimulated with LPS. The inter-assay mean C_T ± SEM for 18S rRNA and TNF-α expression were 12.9 ± 0.2 and 23.6 ± 0.1, respectively, allowing these measurements to serve as a quality control index to identify factors that might account for spurious results in an experiment (e.g., faulty reagents, equipment malfunction). In addition, a "no template" control was included on each plate to prove that amplicon contamination was absent, and in initial experiments, control RT-qPCR reactions were performed in which reverse-transcriptase was omitted. The failure to generate fluorescent signal in the latter reactions provided convincing evidence regarding the efficacy of the DNAse I treatment during RNA extraction.

As depicted in Figure 4.6, it is important that every primer pair validation assay be performed in an organized and methodical fashion. First, primers should be designed according to a set of predefined criteria. Secondly, an RT-qPCR reaction for primer optimization is required. Once an optimal concentration for the forward and reverse primer combination has been determined, the next step is to show an amplification plot with a C_T range within 15-35 cycles. Thereafter, the presence of a single specific PCR product as demonstrated by dissociation curve analysis and a single band on gel electrophoresis must be established.

Subsequently, the PCR efficiency and validation experiments (linearity of amplification), as described in this study, should be performed. If the efficiency and validation trial fails to provide evidence of sufficient amplification, an alternative agonist should be used to establish sufficient mRNA levels to allow for assessment. Once all of these steps have been completed successfully, the RT-qPCR assay is ready for routine use. In one instance in our laboratory, quantitative detection of MMP-2 transcripts using primers designed for SYBR detection failed to pass the validation process, and redesigned primers did not improve the results. In that case, we were forced to move to a probe using TaqMan chemistry to generate high quality validated data (unpublished data). This is an example of the value of utilizing a fully pre-established validation protocol for new RT-qPCR primers before incorporating them into an experimental system.

This paper reports the validation of a reliable set of twenty-nine equine primer pairs using RT-qPCR with SYBR Green as a detection method. The genes represent a variety of biological functions relevant to the activation of equine leukocytes. Using the information provided herein, investigators will be able to implement RT-qPCR assays for these genes without having to repeat the laborious validation process, as long as they adhere to the conditions that have been validated by our trials. Furthermore, this paper outlines the procedures and techniques required to develop a straight forward validation system for the establishment of primers to be used in RT-qPCR assays for other genes of interest in the horse or any other species.

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Table 4.1. Equine primer sets for real-time quantitative PCR

Gene	GenBank Accession	Primer sequences forward/reverse (5′ to 3′)	Amplicon Size (bp)	Predicted Tm (^{o}C)	Function
ß-defensin	AY170305	GCAGGCATAGAAACTTCGTTCA TGACCCAGGCAAGATACAAGTG	101	81	Antimicromial peptide
Cathelicidin 2	AJ224928	CTCAACCAGCGGTCCTCAGA CCTTCACCGTGAAGCTCACA	112	84	
L-selectin	CD535275	AGATCGTCGGGATTGAAGAAAC TCCCCAAATCAGGTGCTGTT	122	81	Cell adhesion molecule
MD-2 (myeloid differentiation factor-2)	NM_001081898	GTGATAACATGAAGTCCCCGATTT GGAACGAAGAGCATGTGCAA	91	78	Cell surface receptor
CD44	X66862	TCCACCCCAACTCCATCTGT CGGAGCTGAGGCATTGAAG	101	83	
TLR2 (Toll-like receptor 2)	AY429602	GGCACTGGACCAGATCCTGAT TGGCATTCAGAGACCGAGAGA	111	84	
TLR3	DQ266434	CAAACCCTGGTGGTCCTGTT GAAGGCCTCTGCTGGGATCT	101	79	
TLR4	AY005808	ATGCCCGTGCTGGGTTTTA ACTTTTTGCAGCCAGCAAGAA	151	78	
CCL5 (RANTES)	AF506970	CACTGCCACCTTCTGCACTC CGGGAGATGTAGGCAAAGCA	81	85	Chemokine
IL-8 (interleukin-8)	AF062377	TTGGCCGTCTTCCTGCTTT GGTTTGGAGTGCGTCTTGATG	101	80	
IP-10 (interferon-inducible protein 10)	CD470151	CCTCCAGTTGCAGCACCAT TTCCTTGAGTTCCACTCAGAGTCA	81	78	
MCP-3 (macrophage chemoattractant protein-3)	MONO1_14_F10	GAAGATCCCCATCCAGAAGCT AGACCTGTTTGGCCAGTTTGG	101	81	

Table 4.1. Equine primer sets for real-time quantitative PCR (continuated)

Gene	GenBank Accession	Primer sequences forward/reverse (5′ to 3′)	Amplicon size (bp)	Predicted Tm (^{o}C)	Function
IFN-ß (interferon-ß)	AF134227	CCCCGAGGACACAATGAACT ACCAATGCAGCATCCTCCTT	81	82	Cytokine
IFN-γ	EU000433	CCAGCGCAAAGCAATAAGTG GGCCTCGAAACGGATTCTG	101	80	
IL-1ß	ECU92481	ATGACTTACTGCAGCGGCAAT GTCTTGGAAGCTGCCCTTCA	81	80	
IL-6	ECU64794	TGCTGGCTAAGCTGCATTCA GGAAATCCTCAAGGCTTCGAA	81	79	
IL-10	U38200	GCCTTGTCGGAGATGATCCA TTTTCCCCCAGGGAGTTCAC	81	83	
IL-12	AY686642	CCAGACGCTGTGCCTTAGC TCTGCCTCTGAGGATCTATCAACA	101	80	
IL-1RA (interleukin-1 receptor antagonist)	D83714	TGTCTCCAGCCTCCTCAGCTA GGCCCGGATTTTATCCTGAA	131	81	
TNF-α (tumor necrosis factor-alpha)	AB035735	AAAGGACATCATGAGCACTGAAAG GGGCCCCCTGCCTTCT	81	83	
COX-2 (cyclooxygenase-2)	DQ480158	CAGCATAAACTGCGCCTTTTC AGGCGGGTAGATCATTTCCA	111	78	Enzyme
HYAL 1 (hyaluronidase)	XM_001493665	ACATCCCAGGACATCCAGGAT CGCTGCCGGTAAATGTCTTT	125	83	
HYAL 2	XM_001493640	CGTGTACCGCAGGTCATCAC AGCGCAGTGTCTCCAGCAT	132	82	
MMP9 (matrix metalloproteinase 9),	EU025852	CGGTAAGGTGCTGCTGTTCA AGCTTTCTCTCGGTACTGGAAGAC	151	85	
IFIT1 (interferon-induced protein with tetratricopeptide repeats 1)	CD465351	CTGCAATGCCTGATTTGGAA GGCCTTTCAGGTGTTTCACATAG	111	78	Mediator of signal transduction
TRAF1 (TNF receptor-associated factor 1)	BI961513	GTCCCGTCCCCATTCAGATA CAGCCTCTTCTCCCCAGCTT	81	80	
PAI1 (plasminogen activator inhibitor-1)	AF508034	TCTGCCCTCACCAACATTCTG TGTCGGTCATCCCCAAGTTC	151	83	Serine protease inhibitor
SOD2 (superoxide dismutase 2)	AB001693	TGGTGGAGGCCATATCAATCA ACCAGCCGATACAGCAGTCAA	151	80	Superoxide dismutase
TGF-ß (transforming growth factor beta)	AF175709	AGAGCTGCGCCTCCTAAGG ACGACTCCGGTGACATCAAAG	153	82	Growth factor

Table 4.2. Standard curve data from real-time quantitative PCR

Gene	Range of C_T values	PCR efficiency Mean slope	%	Validation Mean slope
ß-defensin*	21 – 34	-3.28 ± 0.34	98	0.02 ± 0.35
Cathelicidin2	27 – 33	-3.14 ± 0.32	108	0.07 ± 0.22†
CD44	17 – 26	-3.34 ± 0.20	99	0.08 ± 0.17
COX2	21 – 30	-3.35 ± 0.29	99	0.06 ± 0.27
HYAL 1	27 – 37	-3.37 ± 0.34	98	0.15 ± 0.41
HYAL 2	27 – 38	-3.13 ± 0.45	109	0.16 ± 0.52
IFIT1	22 – 30	-3.40 ± 0.15	97	0.01 ± 0.09
IFN-ß#	24 – 32	-3.32 ± 0.17	100	0.09 ± 0.14
IFN-γ*	24 – 32	-3.19 ± 1.30	106	0.01 ± 0.18†
IL-1ß	15 – 23	-3.27 ± 0.16	102	0.04 ± 0.08†
IL-6	22 – 29	-3.28 ± 0.15	102	0.06 ± 0.09†
IL-8	16 – 24	-3.22 ± 0.29	104	0.19 ± 0.28
IL-10	23 – 32	-3.22 ± 0.24	104	0.09 ± 0.25
IL-12	25 – 34	-3.20 ± 0.34	105	0.10 ± 0.08†
IL-1RA	15 – 23	-3.29 ± 0.19	101	0.02 ± 0.10†
IP-10	23 – 32	-3.33 ± 0.19	106	0.08 ± 0.15
L-selectin	24 – 32	-3.45 ± 0.20	95	-0.03 ± 0.20
MCP-3	21 – 29	-3.32 ± 0.10	100	0.09 ± 0.09
MD-2	24 – 32	-3.24 ± 0.19	104	0.01 ± 0.18†
MMP9	22 – 30	-3.41 ± 0.20	96	-0.03 ± 0.20
CCL5	19 – 27	-3.30 ± 0.09	101	0.05 ± 0.06†
SOD2	18 – 26	-3.36 ± 0.11	98	0.06 ± 0.07
TGF-ß	21 – 29	-3.34 ± 0.18	99	0.07 ± 0.17
TLR2	22 – 30	-3.43 ± 0.15	96	-0.01 ± 0.10
TLR3#	24 – 30	-3.28 ± 0.14	102	0.02 ± 0.08†
TLR4	21 – 30	-3.26 ± 0.21	103	0.10 ± 0.23
TNF-α	20 – 27	-3.33 ± 0.19	100	0.09 ± 0.15
TRAF1	21 – 28	-3.23 ± 0.12	103	0.08 ± 0.09
PAI1	26 – 31	-3.35 ± 0.21	99	0.09 ± 0.09†

* Standard curve generated from samples stimulated with 10^{-6} M PMA.
# Standard curve generated from samples stimulated with 250 µg/ml Poly I:C.
† Mean slope generated with data from a single horse.

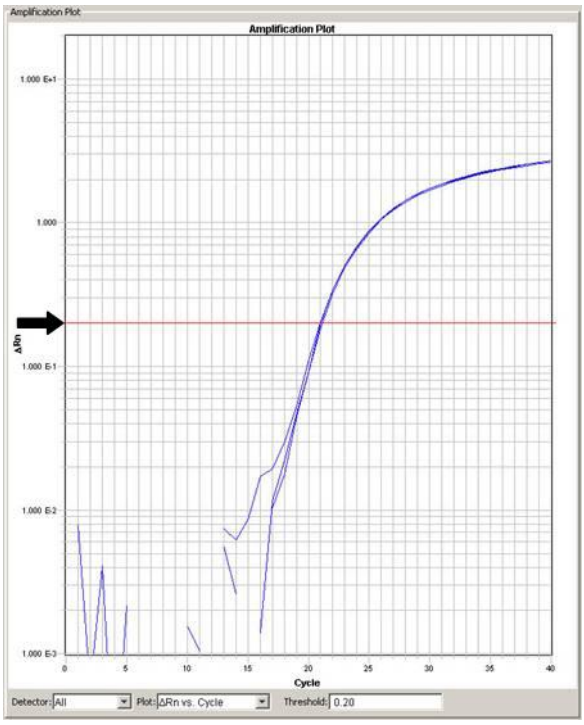

Figure 4.1 Representative amplification plot for equine CD44. The amplification graph is a plot of cycle vs. ΔRn (the magnitude of the signal generated by the given set of PCR conditions), with the horizontal line (→) representing the threshold. The intersection between the amplification plot and the threshold line defines the C_T (cycle number at which the fluorescence generated within a reaction crosses the threshold line).

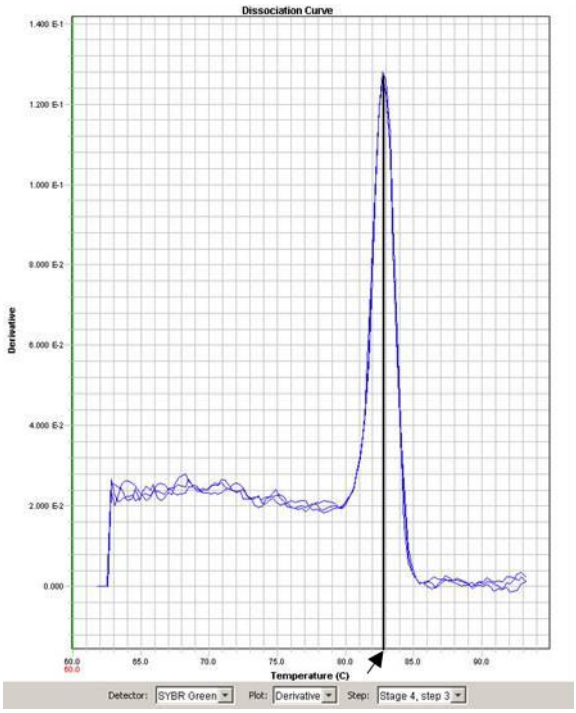

Figure 4.2 Representative dissociation curve for equine CD44. The dissociation curve is a plot of temperature (x-axis) vs. change in fluorescence (y-axis) using SYBR Green detection. Observed Tm (temperature when 50% of the double-stranded DNA is dissociated) is 82.8 ^{o}C (↑).

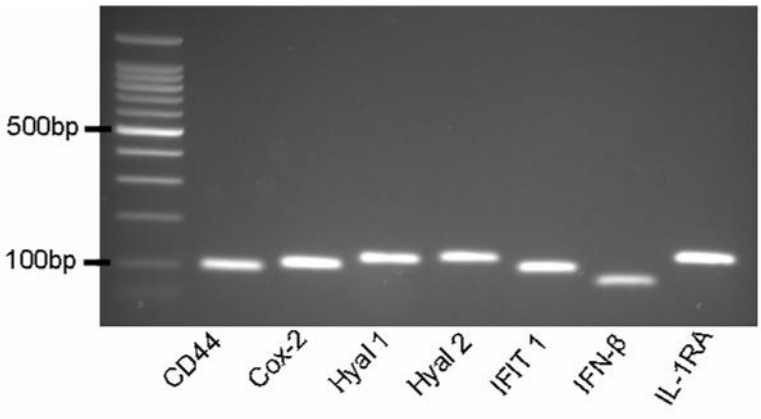

Figure 4.3 Electrophoresis of selected PCR products (lanes 2 to 7) in a 2% agarose gel. The molecular marker (100bp ladder) is in lane 1. A single band is evident for each amplicon.

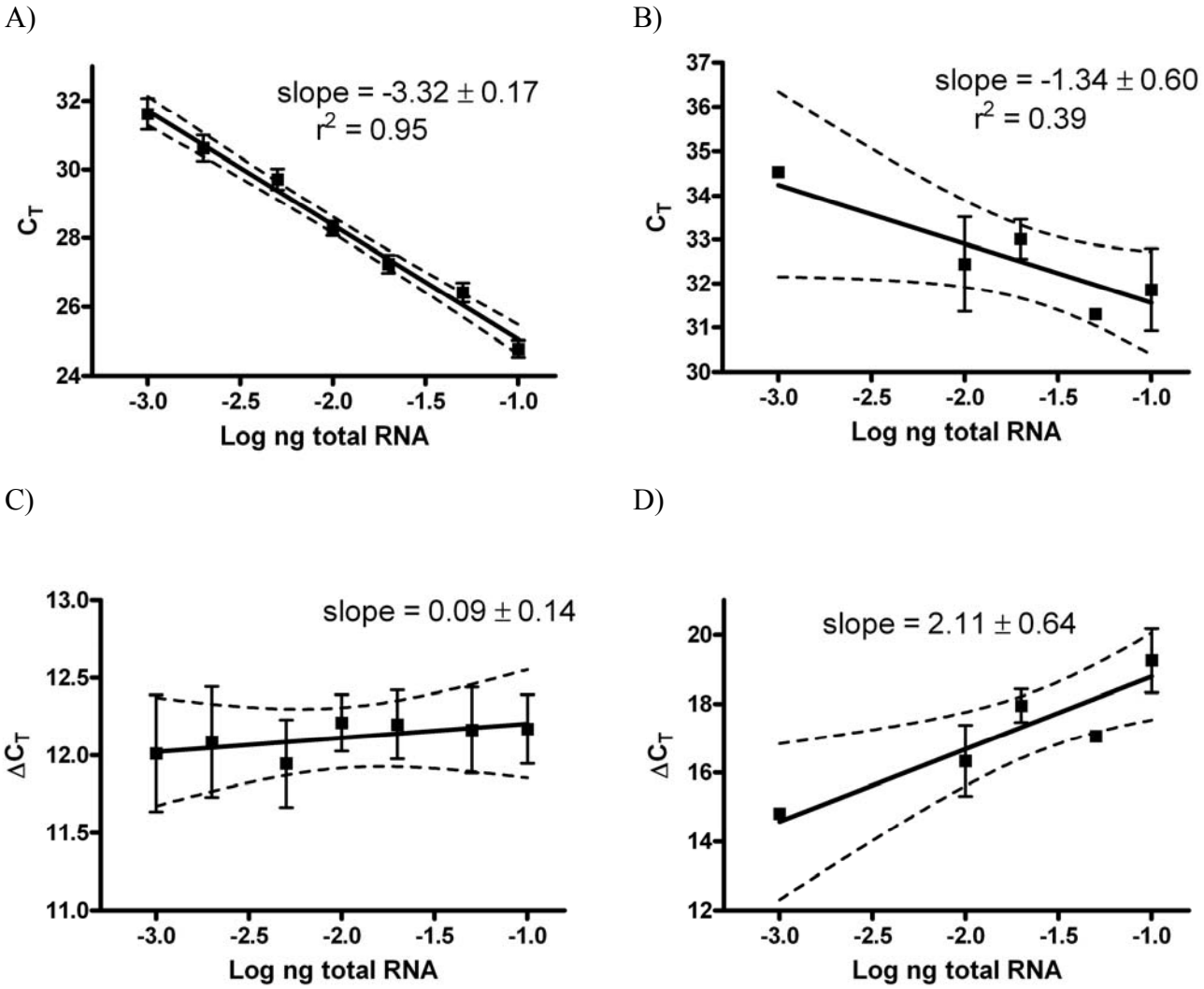

Figure 4.4 Representative standard curves for equine IFN-ß. (A) Good PCR efficiency generated from samples stimulated with 250 ug/ml Poly I:C. As defined for optimal PCR efficiency, the slope is -3.32. (B) Poor PCR efficiency generated from samples stimulated with 100 pg/ml LPS. (C) Equivalent amplification efficiencies of the target gene and the housekeeping gene, from samples stimulated with 250 ug/ml Poly I:C. As defined for optimal amplification, the slope should be < 0.1. (D) Poor amplification efficiencies of the target gene and the housekeeping gene, from samples stimulated with 100 pg/ml LPS. Data are presented as mean ± SEM, n= 3.

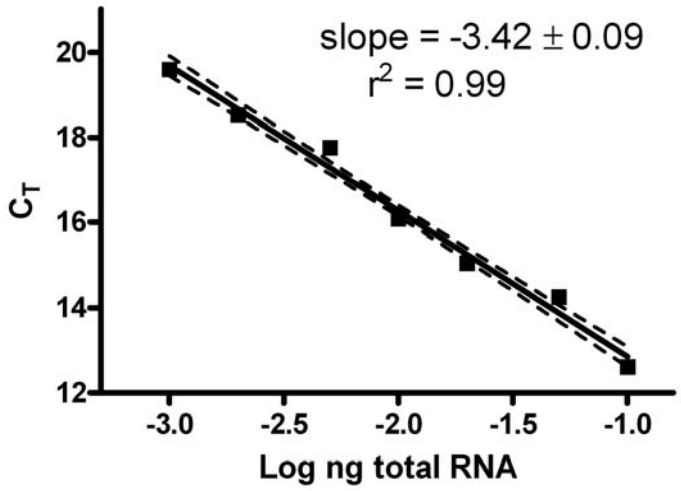

Figure 4.5 Representative standard curve for 18S rRNA. As defined for optimal PCR efficiency, the slope is -3.32; data are presented as mean ± SEM, n= 3.

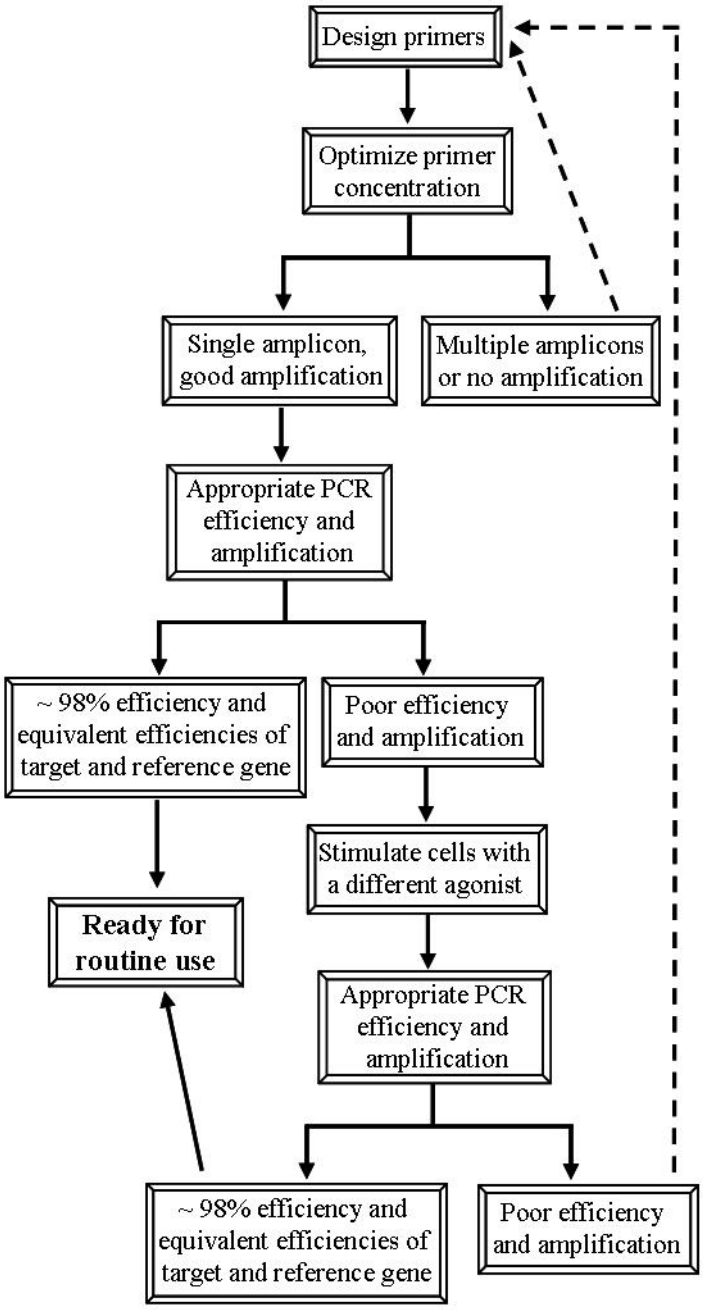

Figure 4.6 A step-wise approach to validating primers for use in SybrGreen-based RT-qPCR assessment of gene expression. The dashed lines represents the approach that should be taken for two of the most commonly encountered problems during the validation process (i.e., presence of multiple amplicons or poor efficiency due to an inappropriate agonist).

CHAPTER 5

DIFFERENTIAL INDUCTION OF MYD88 AND TRIF DEPENDENT PATHWAYS IN EQUINE MONOCYTES BY TOLL-LIKE RECEPTOR AGONISTS[1]

[1]M.D. Figueiredo, M.L. Vandenplas, D.J. Hurley, J.N. Moore. Accepted by *Veterinary Immunology and Immunopathology,* 09/30/2008
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ABSTRACT

Our understanding of the innate immune response in the horse has been limited by a lack of definitive data concerning cell signaling in response to microbial products. Toll–like receptors (TLR) recognize conserved molecular motifs of microbes and elicit immune responses through their coupling with intracellular adaptor molecules, particularly MyD88 and TRIF. To provide a more definitive characterization of TLR signaling in the horse, the objectives of this study were to: (1) Characterize the responses of equine monocytes to TLR ligands that signal through MyD88, TRIF or both, and (2) Determine the profiles of gene expression initiated utilizing these adaptor molecules. Monocytes were used to establish concentration response curves for *E. coli* lipopolysaccharide (LPS; TLR4 ligand) and Pam_3CSK_4 (TLR2 ligand) based on expression of procoagulant activity (PCA) and production of tumor necrosis factor–alpha (TNF–α); effects of Poly I:C (TLR3 ligand) were determined by quantifying expression of mRNA for interferon-beta (IFN–ß). Expression of genes associated with the MyD88- (TNF-α, IL-1ß, IL-6 and IL-10) and TRIF-dependent pathways (IFN-ß, IP-10, RANTES and TRAF1) were measured at intervals spanning 20 h. LPS and Pam_3CSK_4 induced significantly higher expression of TNF–α, IL–1ß, and IL–10 than did Poly I:C. Poly I:C induced significantly higher expression of IFN–ß, IP–10 and RANTES than did either the TLR2 or TLR4 ligands. High concentrations of *E. coli* LPS did not significantly increase expression of genes associated with the TRIF-dependent pathway. The results of this study suggest that equine monocytes utilize a common intracellular pathway in response to TLR2 and TLR4 ligands, but a distinct pathway in response to TLR3 ligands.

INTRODUCTION

Inflammation is a significant component of the host response to infection, trauma and vaccine adjuvants. This is especially true for the horse, as inflammation plays a prominent role in the pathogenesis of many equine diseases. Therefore, it is important that we have a clear understanding of the factors that initiate inflammatory responses and lead to innate and adaptive immune responses in the horse.

The most well characterized family of pathogen-associated molecular pattern receptors that initiate inflammatory responses are the Toll-like receptors (TLR). TLRs are vital components of the host's system for sensing dangerous pathogens, and for initiating inflammatory and immune responses directed against these pathogens. These responses are elicited through specific intracellular adaptor molecules, most notably MyD88 and TRIF.[1-4] The importance of MyD88 and TRIF lies in the finding that each leads to a distinct profile of immune mediators that in turn determine the phenotype of the cells that primarily are responsible for the development of adaptive immune responses.[5-8] Consequently, activation of TLRs is critical not only as part of an animal's response to microbial infections, but also in their response to vaccines. Therefore, it should be possible to characterize cellular responses to ligands that selectively activate specific TLRs, and then utilize this information to improve our understanding of disease pathogenesis and to design more effective vaccine adjuvants.

In recent years, knowledge regarding the expression of various TLRs in the horse has increased. For example, there is evidence that TLR9 is expressed in dendritic cells, macrophages,[9] peripheral blood leukocytes, spleen and lymph nodes in horses.[10] We have also used RT-PCR assays to demonstrate expression of TLR2, 3, 4, 6, and 9 in isolated equine

monocytes (unpublished data). Furthermore, there is evidence that expression of TLR2 and TLR4 increases in the lungs of horses administered LPS and in the liver after induction of laminitis.[11,12] Similarly, expression of TLR4 is increased in adult horses with airway inflammation[13] and in foals with sepsis.[14] To date, little is known regarding MyD88 and TRIF in horses, although mRNA sequences for these equine proteins have been published[15] or made accessible through GenBank (GenBank XM_001493990).

In species studied to date, LPS signaling through TLR4 utilizes both MyD88 and TRIF pathways.[16,17] TLR2 (in combination with TLR1) is activated by lipoproteins that are present in the walls of Gram-positive bacteria;[18] the effects of lipoproteins are mimicked by Pam_3CSK_4, a synthetic compound. The TLR2/1 receptor complex signals only through MyD88. In contrast, TLR3 signals only via the TRIF pathway and it is activated by double-stranded RNA, an effect that is mimicked by its synthetic analog Poly I:C.[19] Furthermore, activation of the intracellular TRIF and MyD88 pathways distal to TLR4 also leads to time-dependent differences in activation of the transcription factor NFκB; activation of the MyD88-dependent pathway results in rapid activation of NFκB, whereas this response is delayed when the TRIF-dependent pathway is used. The TRIF dependent pathway also leads to activation of another transcription factor, IRF-3, which results in expression of IFN-ß, RANTES and IP-10.[20-22] Activation of the TRIF-dependent pathway has a profound impact on dendritic cell maturation, an important link between innate and adaptive immune responses and to the function of vaccines.[5-8]

In this study, we characterized the responses of equine monocytes to ligands that initiate cellular activation via the TLR 2, 3 or 4 receptors. These ligands have been reported to rely specifically on MyD88, TRIF or a combination of these two molecules to induce receptor-mediated signaling in other species.[23] We chose to study the responses of equine monocytes to

microbial ligands primarily because horses are exquisitely sensitive to the deleterious effects of the quintessential TLR4 ligand, LPS, and they suffer from diseases that have inflammatory responses to LPS in their pathogenesis.[24,25] Furthermore, little is known about the response of equine cells to TLR2 and TLR3 ligands that may be important in bacterial and viral diseases.

The hypothesis underlying this study was that selective activation of TLRs 2, 3 or 4 on equine monocytes, using well-defined ligands, induces distinct profiles of gene expression. Our objectives were to: (1) Characterize the responses of equine monocytes to TLR ligands shown in other species to signal through MyD88, TRIF or both adaptor proteins and, (2) Determine the profiles of TLR ligand–induced gene expression initiated through either MyD88, TRIF, or a combination of both pathways.

MATERIALS AND METHODS

Materials

Equine serum was purchased from Hyclone (Logan, UT), RPMI-1640, PBS and penicillin/streptomycin were from Mediatech, Inc. (Herndon, VA). *Escherichia coli* 0111:B4 LPS was purchased from List Biological Laboratories, Inc. (Campbell, CA), Polymyxin B was purchased from Bedford Laboratories (Beford, OH), Pam_3CSK_4 (N- Palmitoyl- S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-[S]-seryl-[S]-lysyl-[S]-lysyl-[S]- lysyl-[S]-lysine x 3 HCl) and Poly I:C (Polyinosine-polycytidylic acid) were from InvivoGen (San Diego, CA) and reconstituted as indicated by the manufacturer. RNeasy Mini Kits and the RNase-Free DNase Set were purchased from Qiagen Inc. (Valencia, CA). The High Capacity cDNA archive kit, SYBR Green PCR master mix, and Eukaryotic 18S rRNA were purchased from Applied

Biosystems (Foster City, CA). Histopaque 1077 and all other high grade chemicals were from Sigma–Aldrich (St. Louis, MO).

Horses

A group of 15 adult horses determined to be healthy on the basis of clinical examination were used for the study. A venipuncture site over a jugular vein on each horse was aseptically prepared, and blood samples were collected into syringes containing EDTA as an anticoagulant. The experimental protocol was reviewed and approved by the Animal Care and Use Committee at The University of Georgia.

Isolation of equine peripheral blood monocytes

Mononuclear cells were isolated by density-gradient centrifugation over Histopaque 1077 as previously described.[26] Viability was greater than 98% by trypan blue exclusion. Mononuclear cells were suspended in RPMI-1640 at a final concentration 4 x 10^6/ml; 2 x 10^6 mononuclear cells were added to each well of a sterile 24-well polystyrene plate and incubated for 2 h at 37°C, in a 5% CO_2 atmosphere. After incubation, non-adherent cells were removed by washing with warm RPMI-1640. Adherent monocytes were then incubated in RPMI-1640 containing 10% equine serum supplemented with 100 units/ml penicillin and 100 μg/mL streptomycin.

Determination of concentration response curves for E. coli LPS, Pam_3CSK_4 and Poly I:C

Control samples used in the study contained 10% equine serum in RPMI 1640 (negative control sample) and PMA 10^{-6} M (positive control sample). Monocytes were incubated either

with *E. coli* LPS 0111:B4 (3 fg/mL – 30 ng/ml), Pam_3CSK_4 (1 pg/ml – 10 μg/ml) or Poly I:C (300 ng/ml – 300 μg/ml) using ½ log increments in concentration. Cells were incubated for 6 h after which cell lysates and supernatants from the LPS, Pam_3CSK_4 and Poly I:C stimulated samples were assayed for PCA and TNF-α. As there are currently no reliable quantitative assays for determining TRIF dependent mediators (eg. IFN-ß, IP-10 and RANTES) in equine cell supernatants, we used RT-qPCR as described below to establish concentration responses for Poly I:C using IFN-ß mRNA expression as a readout. To ensure that any effects ascribed to Pam_3CSK_4 were not due to LPS in the solution, monocytes from three horses were incubated either with *E. coli* LPS 0111:B4 (1 pg/mL – 100 ng/ml) or Pam_3CSK_4 (10 pg/ml – 1 μg/ml) in the presence and absence of polymixin B (PMB; 13 units/ml). Cells were incubated for 6 h, after which the cell supernatants were assayed for TNF-α. All experiments were done in triplicate for each horse.

TNF-α assay

The TNF-α protein concentration in monocyte cell supernatants was measured by use of an equine TNF-α ELISA using a recombinant equine TNF-α standard as previously described.[27]

PCA assay

An automated 1-stage clotting assay was used to determine the effects of cell lysates on calcium-induced clotting of pooled equine plasma. The PCA activity of samples was determined by comparing the results with those obtained for a standard curve generated using equine brain thromboplastin.[28]

RNA extraction and cDNA synthesis for mRNA expression analysis

Approximately 1 x 10^7 isolated mononuclear cells were added to sterile 60 x 15-mm Petri dishes and incubated for 2 h at 37°C in 5% CO_2 atmosphere. After incubation, non-adherent cells were removed by washing with warm RPMI-1640. Adherent monocytes were overlaid with RPMI-1640 supplemented with 10% equine serum containing 100 U of penicillin/ml and 100 μg of streptomycin/ml. Monocytes were incubated for 1, 4, 8, 12 and 20 h in the presence or absence of either 100 pg/ml LPS, 30 ng/ml Pam_3CSK_4 or 250 μg/ml Poly I:C. High concentrations of *E. coli* LPS (incremental concentrations up to 10 μg/ml) were used to determine if cellular activation via TLR4 could induce TRIF-dependent gene expression.

After incubation, 1 ml of supernatant was collected for TNF-α assay and cells were washed with cold phosphate buffered saline, scraped from the plates with RNA cell lysis solution and stored at –80°C. Cell lysates were thawed, total RNA was extracted using the RNeasy Kit according to the manufacturer's protocol and treated by incubation with DNase I at 24°C for 30 min. Only samples having 260:280 nm absorbance ratios between 2.0 and 2.2 as measured on a NanoDrop spectrophotometer (Thermo Fisher Scientific;Wilmington, DE) were processed for cDNA synthesis using the High Capacity cDNA Archive Kit (Foster City, CA) with 100 ng of RNA as template.

Real-time quantitative RT-PCR

Real-time qPCR assays using SYBR Green as a detector were performed in an Applied Biosystems 7900HT (Foster City, CA) sequence detection system, with eukaryotic 18S ribosomal RNA TaqMan serving as endogenous controls.[29-31] Conditions for amplification were 2 minutes at 50°C, 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C, and 60

seconds at 60°C. This was followed by a dissociation stage for 15 s at 95°C to ensure the presence of a single amplicon. The eight target genes of interest in this study were equine TNF-α, IL-1ß, IL-6, IL-10, IFN-ß, IP-10, RANTES and TRAF1 (Table 1). Oligonucleotide primers used for the detection of cDNA specific for equine cytokines were derived from the GenBank database and designed with Primer Express software (Applied Biosystems, Foster City, CA). Dissociation curve analysis and agarose gel electrophoresis revealed single products for all primers, the PCR amplification efficiency was ~ 100%, and amplification efficiencies of the target and reference (18S rRNA) were equivalent. The PCR assays contained 300 nM of each primer as optimized concentration, 5 µl 2X master mix, and 2 µl of the diluted cDNA sample in a final volume of 10 µl. Intra-assay variation was evaluated using a pooled cDNA sample prepared from RNA of cells stimulated with *E coli* 0111:B4 LPS (100 pg/ml) for 4 hours. Changes in cytokine expression were calculated by relative quantification against 18S rRNA using the $\Delta\Delta C_T$ method; where $\Delta\Delta C_T = [(\text{gene of interest } C_T - \text{18S rRNA } C_T)_{\text{sample}} - (\text{gene of interest } C_T - \text{18S rRNA } C_T)_{\text{calibrator}}]$, C_T is defined as the amplification cycle at which amplification reached a comparable threshold level of template concentration. Fold changes in gene expression were calculated as $2^{-\Delta\Delta CT}$. Results were expressed as the mean fold change in gene expression at each time point. The C_T for the non-stimulated control sample was used as the calibrator and assigned a fold change in expression of 1. All reactions were performed in triplicate wells.

Statistical analyses

Data were analyzed by fitting a logistic expression to concentration-response data using GraphPad Prism Software (San Diego, CA). This analysis allowed determination of EC_{50} and

maximum response values with their associated 95% confidence intervals. To identify significant differences between concentration response curves generated in the presence and absence of PMB, the best fit values for three parameters (log EC_{50}, top and bottom) were compared pairwise using F tests. The distribution of quantitative variables was tested for normality and data without a normal distribution were log transformed before analysis. Differences between treatments and time points were detected by a repeated measures analysis using PROC MIXED in SAS. Multiple comparisons were adjusted for using Tukey's test. All data are reported as mean ± SEM. Significance was set at $P < 0.01$.

RESULTS

Concentration response curves

Optimal concentrations of *E. coli* LPS and Pam_3CSK_4 were determined from the concentration response curves for PCA and TNF-α production (Fig. 5.1 and 5.2), whereas responses to Poly I:C were monitored using an RT-qPCR assay for IFN-ß mRNA expression (Fig. 5.3). The EC_{50} values for LPS were 13 pg/ml [95% confidence interval (CI), 3-57 pg/ml] for PCA expression and 30 pg/ml (95% CI, 8-100 pg/ml) for TNF-α production. The EC_{50} value for Pam_3CSK_4 was 4.7 ng/ml (95% CI, 0.6-30 ng/ml) for PCA expression and 1 ng/mL (95% CI, 0.08-12 ng/ml) for TNF-α production. Poly I:C induced IFN-ß mRNA expression in a concentration-dependent manner (Fig. 5.3). Because the highest concentrations of Poly I:C achievable did not result in a plateau in IFN-ß mRNA expression, it was not possible to fit a sigmoidal concentration response curve to these data. The concentrations used for LPS and Pam_3CSK_4 for the real-time RT-qPCR studies were based on the minimum concentration

yielding maximal expression of PCA; these concentrations were approximately the EC_{90} for each ligand. The concentration selected for Poly I:C was based on a concentration that induced strong induction of IFN-ß mRNA; this concentration has been used *in vitro* studies with cells from other species.[32,33]

Co-incubation of Pam_3CSK_4 with polymyxin B did not inhibit TNF-α production

To evaluate cellular responses to microbial ligands, highly purified preparations free of contaminating LPS must be used.[34] PMB is a cationic antibiotic that neutralizes the endotoxicity of LPS by binding directly with the lipid A moiety.[35] Co-incubation of LPS and PMB caused a significant ($P < 0.0001$) rightward shift of the concentration response curve for TNF-α production (Fig 5.4A). Conversely, co-incubation of Pam_3CSK_4 and PMB did not significantly alter the concentration response curve for TNF-α production (EC_{50} for Pam_3CSK_4 alone = 3.1 ng/ml and in the presence of PMB = 5.8 ng/ml) (Fig 5.4B).

Differential induction of MyD88 and TRIF dependent pathway

In mammalian species studied to date, TLR2 only signals through MyD88, TLR3 utilizes only TRIF, and TLR4 recruits both MyD88 and TRIF. The recruitment of MyD88 is essential for the production of pro-inflammatory cytokines such as TNF-α, IL-1ß and IL-6 and the anti-inflammatory cytokine IL-10. Genes that are dependent on TRIF activation include IFN-ß, RANTES, IP-10, and TRAF1. There were no significant differences between the expression of genes induced by the TLR4 (LPS) and TLR2 (Pam_3CSK_4) ligands that signal via MyD88 (Fig. 5.5A-D), except that expression of TRAF1 was significantly increased at 20 h in cells incubated with LPS (Fig. 5.5D). LPS and Pam_3CSK_4 induced significantly higher expression of TNF–α at

1 h (Fig. 5.5A), IL-10 at 4 h (Fig. 5.5B) and IL–1ß at all time points (Fig. 5.5C) in comparison to Poly I:C. The mRNA expression of IL-10 at 8 h was significantly increased by Pam_3CSK_4 in comparison to Poly I:C. Although IL-6 did not reach statistical significance, expression was increased at 4, 8 and 12 h by LPS and Pam_3CSK_4. In contrast, the TLR3 (Poly I:C) ligand that signals through TRIF, induced significantly higher expression of IFN–ß at all time points in comparison to the other two ligands, except at 1 h at which IFN-ß was only significantly increased in comparison to Pam_3CSK_4 (Fig. 5.6A). IP–10 was significantly increased by Poly I:C at 8, 12 and 20 h (Fig. 5.6B) and RANTES at 20 h in comparison to both LPS and Pam_3CSK_4 and at 12 h was only significantly different from Pam_3CSK_4 (Fig. 5.6C). Poly I:C also induced significantly higher expression of IL-10 at 20 h in comparison to the other two ligands (Fig. 5.5B).

The individual microbial ligands also induced gene expression profiles that differed from each other in a temporal manner. For example, expression of mRNA for TNF-α was significantly increased by Poly I:C at 20 h when compared to expression after 1 h. The expression of IL-1ß was significantly increased by all ligands at 8, 12 and 20 h when compared to values obtained after 1 h. Interferon-ß, IP-10 and RANTES were significantly increased by Poly I:C at 20 h when compared to values at 1, 4 and 8 h.

TNF-α production by monocytes

Supernatant concentrations of TNF-α were significantly increased by LPS and Pam_3CSK_4 at 4, 8, 12 and 20 h in comparison to the negative control (media in 10% equine serum) (Fig. 5.7). LPS-induced TNF-α production was also significantly greater than Pam_3CSK_4-induced TNF-α at 4, 8 and 12 h. Poly I:C did not significantly increase TNF-α

production at any time point when compared to the negative control. Collectively, these data indicate that only LPS and Pam_3CSK_4 were potent inducers of genes utilizing the MyD88-dependent pathway.

LPS stimulation did not induce mRNA expression of IFN-ß, IP-10 or RANTES

Although MyD88 is a prominent adaptor molecule in TLR4 signaling, MyD88-independent pathways, in particular those that utilize TRIF, also have been described for TLR4.[16] However, in this study incubation of equine monocytes with concentrations of LPS as high as 10 µg/ml for 20 h resulted in a 2.3 fold change in IL-1ß (Fig. 5.8A) and in only a 0.5, 1.0 and -0.1 fold change in IFN-ß, IP-10 and RANTES mRNA expression, respectively (Fig 5.8B and Fig 5.9A-B). In contrast, incubation of monocytes from the same horses with 250 µg/ml Poly I:C resulted in a 2.8, 2.5 and 0.7 fold increase in IFN-ß, IP-10 and RANTES mRNA expression, respectively. Collectively, these data suggest that LPS is at best weakly coupled to TRIF mediated gene expression events in equine monocytes.

DISCUSSION

The role of TLRs in the link between innate and adaptive immune responses has received considerable attention in recent years, primarily because recognition of ligands by TLRs results in activation of monocyte-derived dendritic cells, and other antigen-presenting cells, to secrete pro-inflammatory cytokines that promote dendritic cell maturation, antigen uptake and presentation. TLRs may signal through either the MyD88 or TRIF adaptor molecules, resulting in the expression of genes having quite different biological effects. Consequently, it may be

possible to develop immunomodulating compounds that can be used to minimize the deleterious effects of TLR activation induced by microbial ligands and to serve as better vaccine adjuvants.[36-39]

The interplay between the signaling pathways utilizing MyD88 and TRIF has been illustrated by microarray studies that examined LPS-induced changes in gene expression profiles of macrophages isolated from wild-type, MyD88$^{-/-}$, TRIF$^{-/-}$ and MyD88$^{-/-}$ TRIF$^{-/-}$ mice.[20,21] Results from those studies demonstrated that LPS-inducible genes were completely abrogated in the MyD88$^{-/-}$ TRIF$^{-/-}$ mice; these genes can be classified into clusters based on their dependence on MyD88 and/or TRIF for expression. The cluster of genes regulated primarily by MyD88 included genes commonly associated with pro-inflammatory responses (e.g. TNF-α, IL-1ß, IL-6). The cluster that was dependent on both MyD88 and TRIF included IL-1α and IL-12a, and the cluster that was dependent primarily on TRIF included the IFN-inducible genes, IFN-ß, IP-10, and RANTES.

Based on the results of the above studies, we monitored expression of four genes from the MyD88-dependent pathway (TNF-α, IL-1ß, IL-6 and IL-10) and four genes from the TRIF-dependent pathway (IFN-ß, IP-10, RANTES and TRAF1). The availability of equine gene sequences for these eight mediators allowed us to perform the RT-qPCR assays needed to monitor cellular responses induced via MyD88 or TRIF. Results of this study showed that expression of TNF-α at 1 h, IL-1ß at all time points and IL-10 at 4 h, correlated most strongly with utilization of the MyD88 pathway. Conversely, expression of IFN-ß and IP-10 at 8, 12 and 20 and RANTES at 20 h were discriminatory for utilization of the TRIF pathway. Therefore, equine monocytes appear to be an excellent model for dissecting the effects of ligands that function via the MyD88 or TRIF dependent pathways. Further, we report that concentrations of

E. coli LPS as high as 10 μg/mL do not induce significant expression of TRIF-dependent genes, indicating that LPS activation of equine monocytes occurs almost exclusively through the MyD88 pathway. These findings are in contrast to results reported for two principle species studied, human and mice that utilize both MyD88 and TRIF pathways in their response to LPS.[20,40,41]

In the current study, we evaluated three ligands that have been reported to rely specifically on MyD88, TRIF or a combination of these two pathways to induce receptor-mediated signaling. To identify the concentrations of LPS, Pam_3CSK_4 and Poly I:C to be used to compare gene expression profiles induced by these ligands, we first selected three readouts of cellular activation, namely expression of PCA, production of TNF-α, and expression of mRNA for IFN-ß. We elected to measure changes in expression of PCA and production of TNF-α, not because we expected either to discriminate MyD88-dependent events from those mediated via TRIF, but because both readouts are sensitive indicators of monocyte activation. PCA, also called tissue factor, reflects the ability of stimulated cells to produce a protein that shortens the coagulation time of plasma.[42] Expression of the gene encoding tissue factor is regulated by NFκB,[43] and thus is a reliable indicator of LPS-induced activation of equine monocytes.[42,44] In the present study, both LPS and Pam_3CSK_4 induced a concentration-dependent expression of PCA with EC_{50} values of 13 pg/ml and 4.7 ng/ml, respectively. The other cellular readout used was TNF-α, a cytokine that is often used as a reliable, representative indicator of inflammatory cell activation.[45] Again, LPS and Pam_3CSK_4 both induced concentration-dependent increases in TNF-α production with EC_{50} values of 30 pg/ml and 1 ng/ml, respectively. In contrast, Poly I:C only weakly induced expression of PCA and induced moderate TNF-α production at the highest concentration used, 300 μg/ml (*data not shown)*.

While the results of these experiments provided clear EC_{50} values for LPS and Pam_3CSK_4, incubation of the monocytes with the highest achievable concentration of Poly I:C never resulted in a clear maximal value, and thus did not yield an EC_{50} value for use in our subsequent studies. As a result, we then monitored changes in expression of IFN-ß mRNA to assess the effects of Poly I:C, and decided to use 250 μg/ml of Poly I:C in the subsequent studies in which changes in expression of the other genes were determined. This concentration of Poly I:C has also been used in *in vitro* studies performed in other species.[32,33]

When cellular responses to microbial ligands are evaluated, the purity of the preparation is of utmost importance.[34] A number of strategies can be used to determine whether a component of the cellular responses detected are due to LPS contamination. These include, assaying the preparation for endotoxic activity using the *Limulus* amebocyte lysate assay, heating the preparation to detoxify LPS, co-incubating the cells with specific TLR4 antagonists, or, as was done in this study, co-incubating the cells with PMB to inhibit the biological activities of LPS. Because of the similarities in the responses of monocytes incubated with either LPS or Pam_3CSK_4, we performed additional experiments to compare responses to Pam_3CSK_4 in the presence or absence of PMB. While PMB significantly inhibited LPS induced production of TNF-α, it did not alter production of TNF-α by Pam_3CSK_4. Thus, we concluded that the preparation of Pam_3CSK_4 used in this study was not contaminated by LPS.

In the present study, LPS and Pam_3CSK_4 significantly induced the expression of the following MyD88-dependent genes: TNF-α at 1 h, IL-10 at 4 h and IL-1ß at all time points. TNF-α induces the production of inflammatory factors, such as IL-1ß and IL-6, activates inflammatory cells, increases expression of adhesion molecules, and increases production of nitric oxide and reactive oxygen species. The biological activities of IL-1ß are similar to those

of TNF-α, as both induce the systemic inflammatory response syndrome. IL-6 is responsible for inducing fever, B cell maturation and the synthesis of acute phase proteins. Interleukin-10 is an anti-inflammatory cytokine that inhibits production of TNF-α, IL-1ß and IL-6. In the present study, we determined that Poly I:C significantly induced mRNA expression of IL-10 at 20 h. This finding is in agreement with increases in plasma concentrations of IL-10 detected after administration of Poly I:C in mice.[46] It has also been shown that MyD88 dependent activation of signal transduction events (e.g. NFκB nuclear translocation and MAPK activation) are more rapid than those induced through TRIF. In agreement, our data indicate that LPS and Pam_3CSK_4-induced activation of TNF-α gene expression is maximal at 1 h and decreases thereafter. In contrast, Poly I:C-induced expression of TNF-α peaked at 20 h (Fig. 5.5A). Interestingly, incubation with Poly I:C did not result in increased concentrations of TNF-α in the monocyte cell supernatant at any time point. The discrepancy between increased TNF-α mRNA at 20 h and the lack of TNF-α protein could be due to several factors; TNF-α is produced primarily as a membrane bound protein and must be released from cells by protease cleavage.[47] Cell stimulation also induces both release of TNF-α and the upregulation of its receptors that bind and remove TNF-α from the medium.[48] Thus, secretion of TNF-α is a highly regulated process that, in addition to the aforementioned factors, may require utilization of components of the MyD88 pathway.

Classically, genes induced through the TRIF pathway include: IFN-ß, RANTES, IP-10, and TRAF1. TRIF dependent IFN-ß expression occurs through the activation of IRF-3. Interferon-ß is a type I interferon with potent antiviral activities and it induces the expression of RANTES and IP-10. The latter are members of the CCL5 and CXC chemokine subfamilies, respectively, and are involved in leukocyte trafficking. In the present study, the Poly I:C–induced

expression of mRNA for IFN- ß preceded the expression of the other two TRIF dependent genes, RANTES and IP-10 (Fig. 5.6A-C). As the expression of the latter two genes is dependent on the synthesis and secretion of IFN- ß, these differences in the timing of gene expression events are in agreement with data obtained from other species.[49] The TRAF family is a group of adapter proteins that links a wide variety of cell surface receptors. For example, TRAF1 is a TRIF-associated protein that is a key regulator of the cellular stress response and a negative regulator of TNF-α activity.[50,51] Therefore, it is possible that the increased expression of TRAF1 at 20 h induced by LPS represents a negative feedback response to the LPS-induced production of TNF-α that occurred at 1 h. In equine monocytes, however, there appears to be little induction of TRAF1 gene expression in response to the TLR3/TRIF ligand, Poly I:C, suggesting that changes in expression of TRAF1 are not indicative of TRIF recruitment.

TLR4 activation by LPS in other species leads to recruitment of TRIF and up-regulation of IFN-regulated genes.[16] However, in the present study with equine monocytes, incubation with LPS resulted in minimal expression of IFN-regulated genes, suggesting that TLR4 appears to be strongly linked to MyD88, and at best weakly coupled to TRIF in the horse. To examine the possible role that LPS serotype-specificity might play in the lack of induction of TRIF-dependent genes in our studies, we have recently compared the responses of equine monocytes to five structurally distinct *E. coli* lipid A compounds. In that study, all five lipid A compounds induced genes classically associated with recruitment of MyD88, but not those associated with TRIF (unpublished data).

In summary, the results of this study indicate that ligand-induced expression of TNF-α, IL-1ß, IL-6, IFN-ß, IP-10 and RANTES can be used to discriminate between MyD88 and TRIF-dependent cellular activation of equine monocytes. Specifically, the increased expression of

TNF-α, IL-1ß and IL-10 at the time points reported in this paper are linked to the early activation of the MyD88 pathway, whereas up-regulation of IFN-ß, IP-10 and RANTES are linked to the TRIF-dependent pathway. Upregulation of IL-10 expression does not appear to discriminate between the two pathways, as it was linked with activation of MyD88-dependent genes at 4 h but with TRIF activation at 20 h. Further, changes in expression of these genes can be utilized to identify specific intracellular signaling pathways involving either MyD88 or TRIF that may be induced by adjuvant candidates, particularly those based on TLR features. Collectively, these results provide a basis for the rational design of vaccines and application of these methods can be used to provide a better understanding of innate immune responses in horses.

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Table 5.1. Real-time quantitative RT-PCR primer pairs used for the RT-qPCR assays

Gene	GenBank accession No.	Forward and reverse sequence (5′ to 3′)
TNF–α	AB035735	AAGGACATCATGAGCACTGAAAG GGGCCCCCTGCCTTCT
IL–1ß	ECU92481	ATGACTTACTGCAGCGGCAA GTCTTGGAAGCTGCCCTTCA
IL–6	ECU64794	TGCTGGCTAAGCTGCATTCA GGAAATCCTCAAGGCTTCGAA
IL–10	U38200	GCCTTGTCGGAGATGATCCA TTTTCCCCCAGGGAGTTCAC
IFN–ß	AF134227	CCCCGAGGACACAATGAACT ACCAATGCAGCATCCTCCTT
IP–10	CD470151	CCTCCAGTTGCAGCACCAT TTCCTTGAGTTCCACTCAGAGTCA
RANTES	AF506970	CACTGCCACCTTCTGCACTC CGGGAGATGTAGGCAAAGCA
TRAF1	BI961513	GTCCCGTCCCCATTCAGATA CAGCCTCTTCTCCCCAGCTT

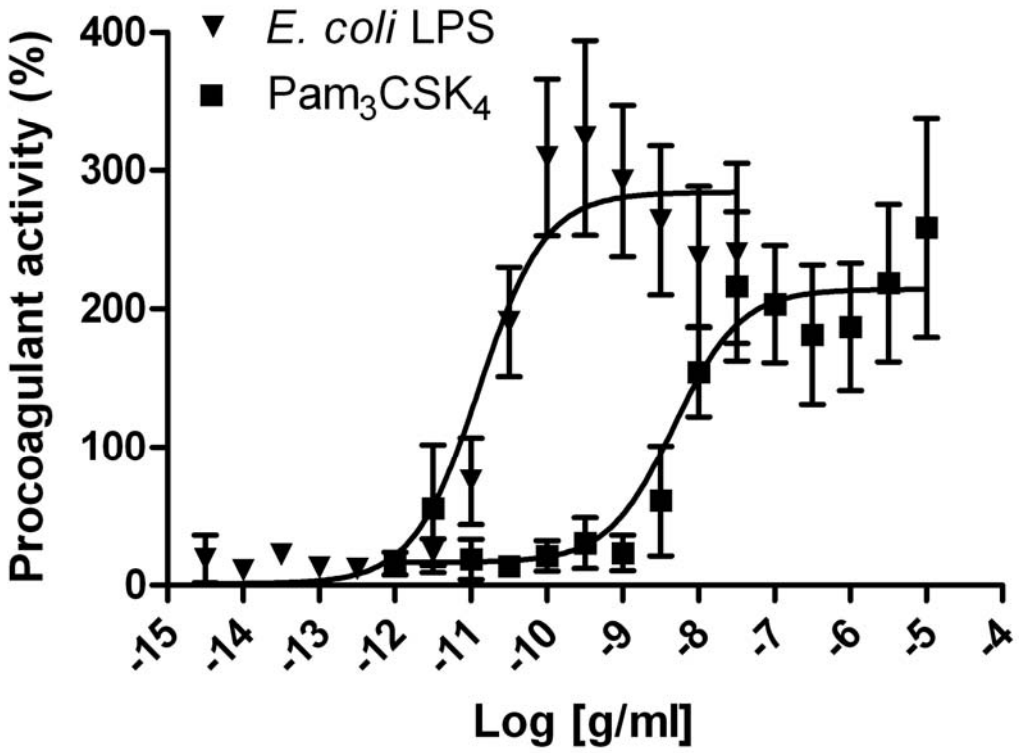


Figure 5.1 Expression of PCA (mean ± SEM) by monocytes incubated with *E. coli* LPS (▼) (3 fg/ml to 30 ng/ml) and Pam_3CSK_4 (■) (1 pg/ml to 10 μg/ml) for 6 hours; EC_{50} values are 13 pg/ml (95% CI, 3–57 pg/ ml) and 4.7 ng/ml (95% CI, 0.6 –30 ng/ml) respectively; n= 15 horses.

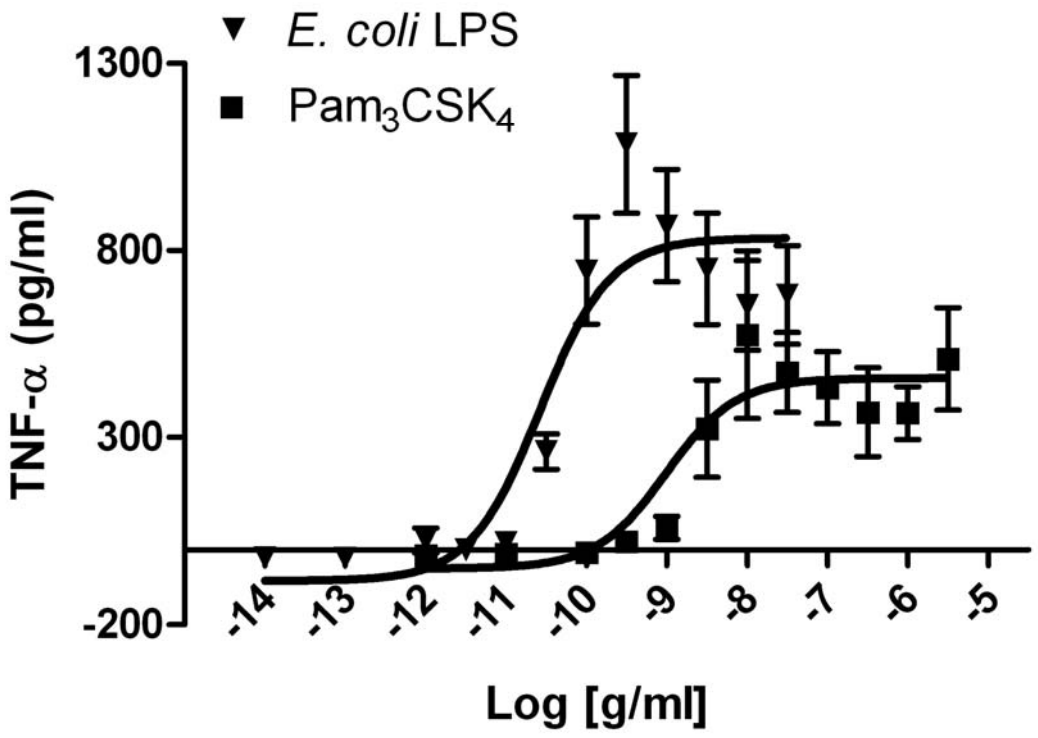


Figure 5.2 TNF-α production (mean ± SEM) by monocytes incubated with *E. coli* 0111:B4 LPS (▼) (10 fg/ml to 30 ng/ml) and Pam_3CSK_4 (■) (1 pg/ml to 3 μg/ml) for 6 hours; EC_{50} values are 30 pg/ml (95% CI, 8 – 100 pg/ml) and 1 ng/ml (95% CI, 81 pg – 12 ng/ml) respectively. All values were normalized by subtracting the mean value for medium with serum from each horse from all other results within each trial; n= 13 horses.

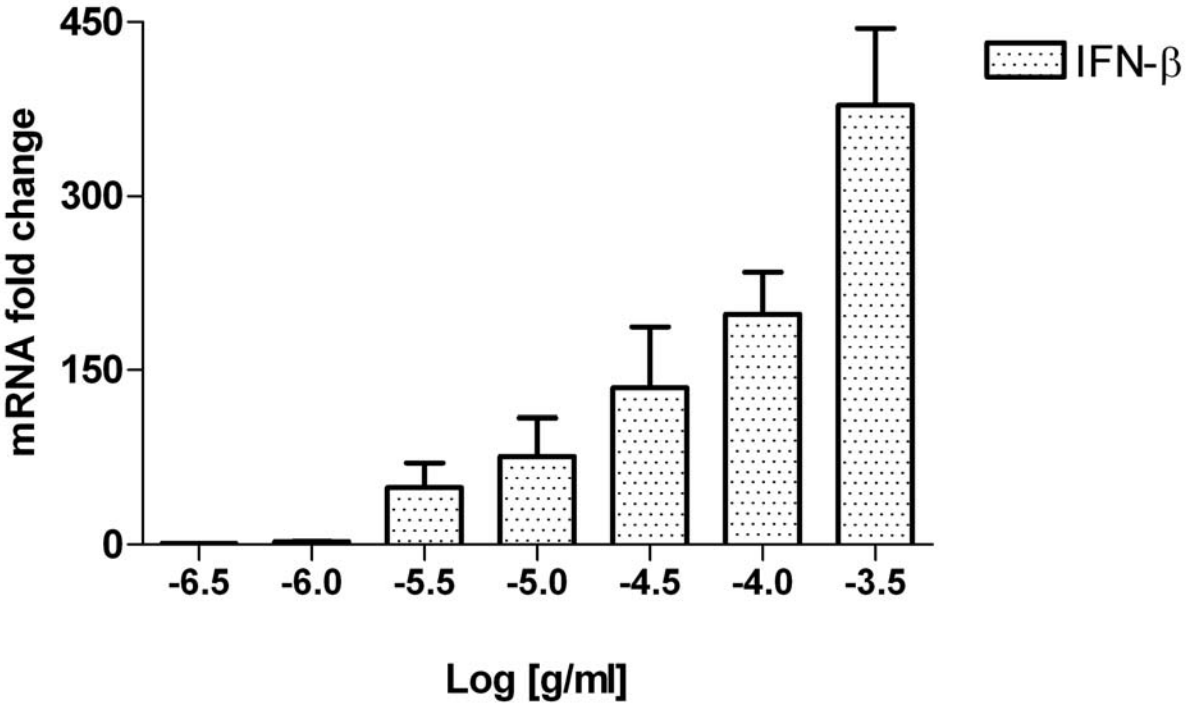
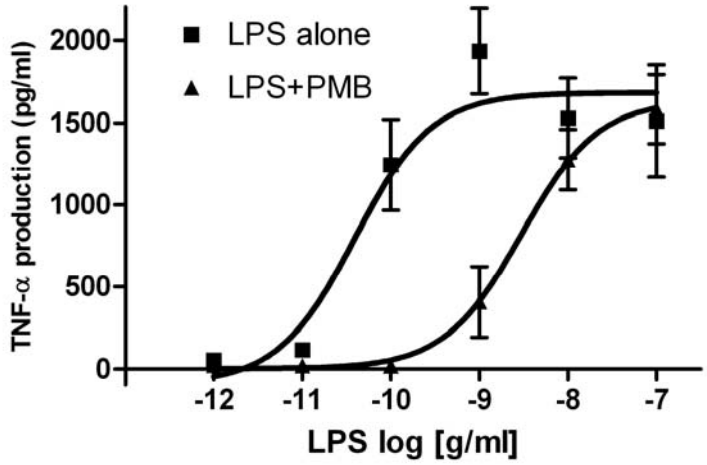


Figure 5.3 The mean (±SEM) relative expression of IFN–ß in monocytes incubated with Poly I:C (300 ng/ml to 300 μg/ml) for 20 hours; n= 3 horses.

A)



B)

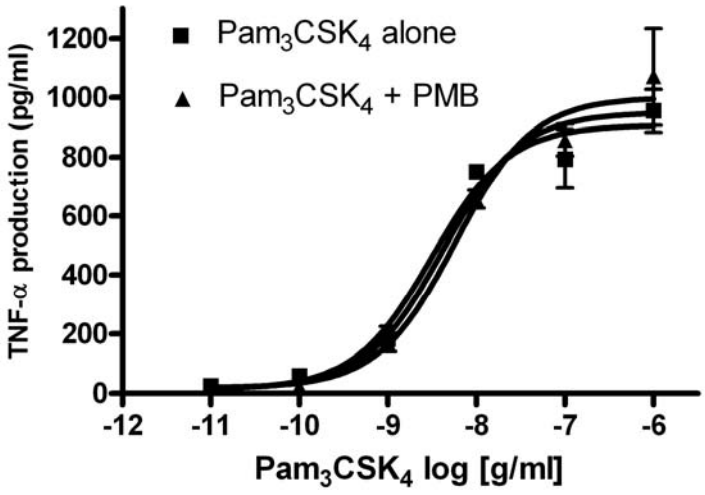


Figure 5.4 TNF–α protein production (mean ± SEM) in supernatants of monocytes (A) incubated with *E. coli* 0111:B4 LPS alone (■) (1 pg/ml to 100 ng/ml) and in the presence of polymyxin B (▲) (13 units/ml). (B) Cells incubated with Pam_3CSK_4 alone (■) (10 pg/ml to 1 g/ml) and in the presence of polymyxin B (▲) (13 units/ml) for 6 hours; n= 3 horses.

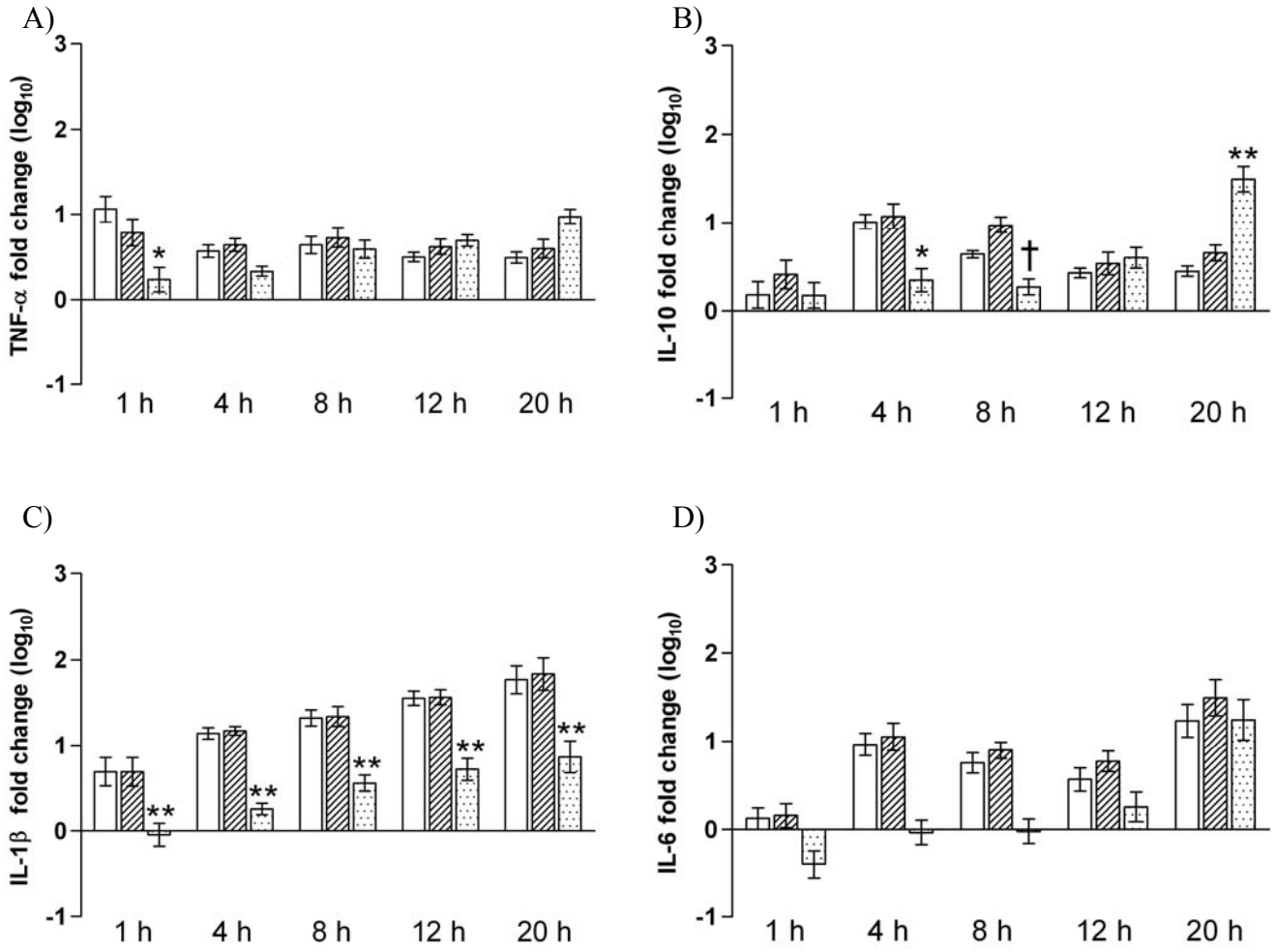


Figure 5.5 The mean (±SEM) fold difference mRNA expression of TNF–α (A), IL–10 (B), IL-1ß (C) and IL-6 (D) in monocytes incubated with *E. coli* LPS (clear bars) (100 pg/ml), Pam_3CSK_4 (dashed bars) (30 ng/ml) and Poly I:C (dotted bars) (250 µg/ml) for 1, 4, 8, 12 and 20 hours. * Significantly different from other ligands at corresponding times ($P < 0.01$). ** Significantly different from other ligands at corresponding times ($P < 0.001$). † Significantly different only from Pam_3CSK_4 ($P < 0.01$); n= 10 horses.

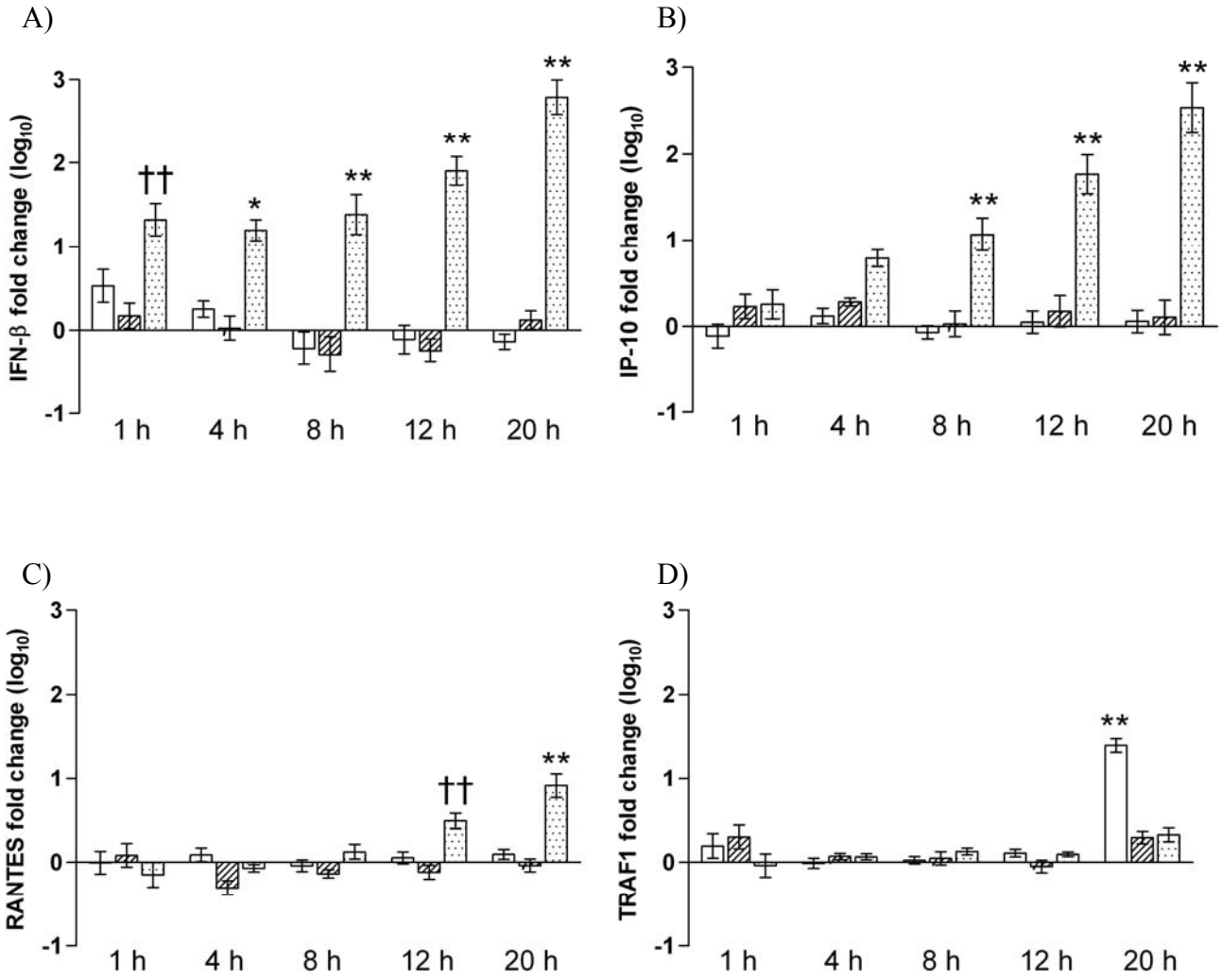


Figure 5.6 The mean (±SEM) fold difference mRNA expression of IFN–ß (A), IP–10 (B), RANTES (C) and TRAF1 (D) in monocytes incubated with *E. coli* LPS (clear bars) (100 pg/ml), Pam_3CSK_4 (dashed bars) (30 ng/ml) and Poly I:C (dotted bars) (250 µg/ml) for 1, 4, 8, 12 and 20 hours. * Significantly different from other ligands at corresponding times ($P < 0.01$). ** Significantly different from other ligands at corresponding times ($P < 0.001$). †† significantly different only from Pam_3CSK_4 ($P < 0.001$); n= 10 horses.

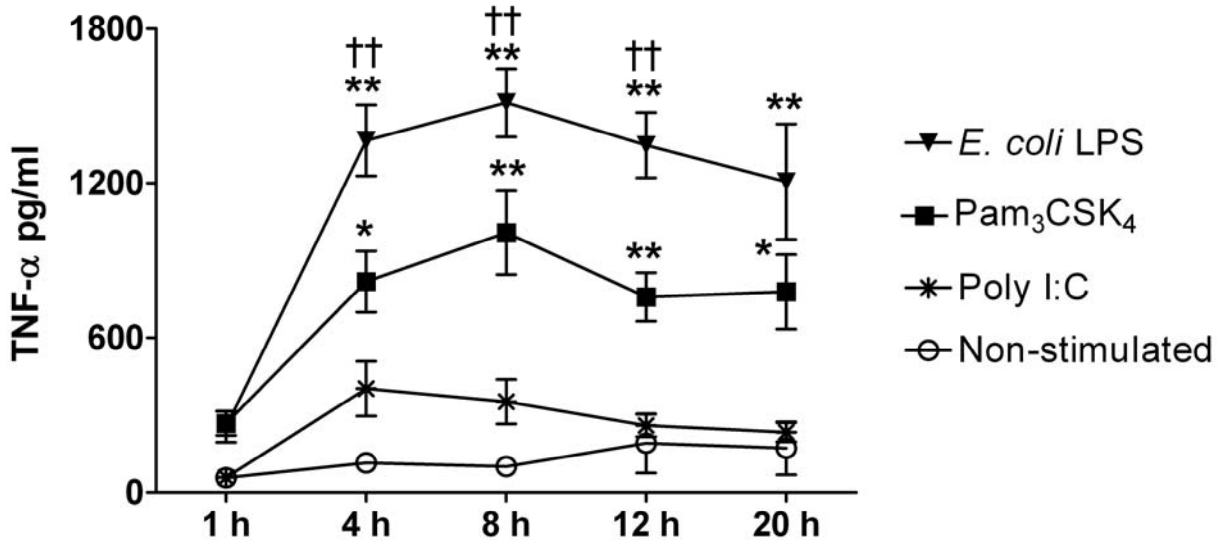
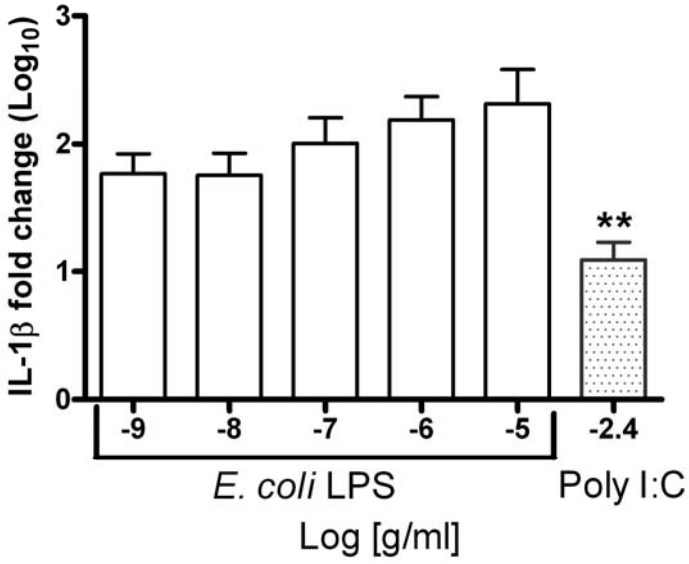


Figure 5.7 TNF–α protein production (mean ± SE) in supernatants of monocytes incubated with *E. coli* LPS (▼) (100 pg/ml), Pam_3CSK_4 (■) (30 ng/ml), Poly I:C (*) (250 μg/ml) and non-stimulated sample (10% equine serum in media) (o) for 1,4, 8, 12 and 20 hours. * Significantly different from Poly I:C and non-stimulated samples at corresponding times ($P < 0.01$). ** Significantly different from Poly I:C and non-stimulated samples at corresponding times ($P < 0.001$). †† Significantly different only from Pam_3CSK_4 at corresponding times ($P < 0.001$); n= 10 horses.

A)



B)

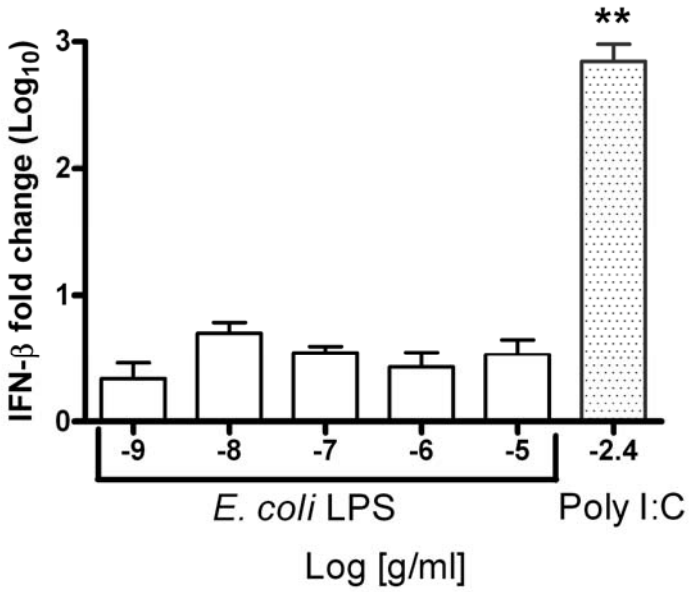
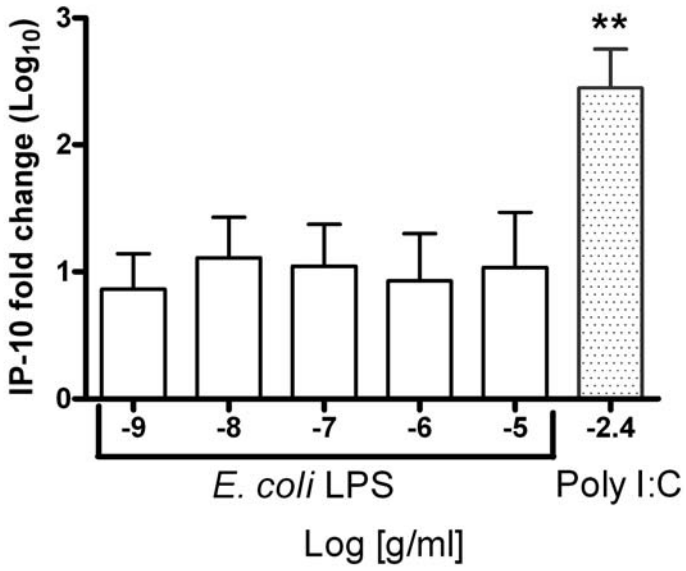


Figure 5.8 The mean (±SEM) fold difference mRNA expression of IL-1ß (A) and IFN-ß (B) in monocytes incubated with *E. coli* LPS (clear bars) (1 ng/ml to 10 µg/ml) and Poly I:C (dotted bars) (250 µg/ml) for 20 hours. ** Significantly different from all concentrations of *E. coli* used ($P < 0.001$); n= 4 horses.

A)



B)

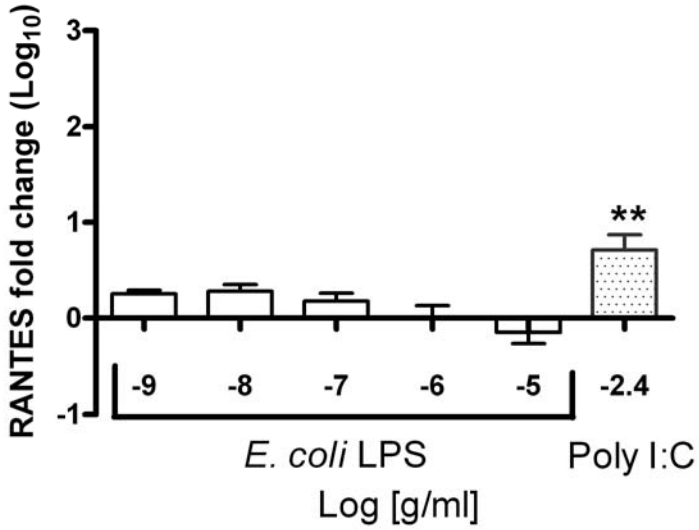


Figure 5.9. The mean (±SEM) fold difference mRNA expression of IP-10 (A) and RANTES (B), in monocytes incubated with *E. coli* LPS (clear bars) (1 ng/ml to 10 μg/ml) and Poly I:C (dotted bars) (250 μg/ml) for 20 hours. ** Significantly different from all concentrations of *E. coli* used ($P < 0.001$); n= 4 horses.

CHAPTER 6

RESPONSE OF EQUINE MONOCYTES TO FOUR STRUCTURALLY DISTINCT *E. COLI* LIPID *A* COMPOUNDS[1]

[1] M.D. Figueiredo, C.E. Salter, WC. Sun, Y Zhang, M.A. Wolfert, M.L. Vandenplas, D.J. Hurley, J.N. Moore, G.J. Boons. To be submitted to *Veterinary Immunology and Immunopathology.*

ABSTRACT

Due to the inherent heterogeneity of the lipid A region that initiates the deleterious effects of lipopolysaccharide (LPS), it has been difficult to identify structural features of LPS responsible for these effects. In this study, the biological activities of four structurally distinct *E. coli* lipid A compounds, natural lipid A and three synthetic compounds lacking the KDO moiety of natural lipid A, were investigated. The three synthetic compounds were lipid A with long fatty acids, lipid A with short chain fatty acids and lipid A with only one phosphate group (SMPLA). The biological activities of the compounds were investigated by determining their ability to induce production of tumor necrosis factor α (TNF-α) and expression of procoagulant activity (PCA) by equine monocytes. Furthermore, the effects of polymyxin B (PMB), a compound known to bind lipid A of LPS, and E5564, a synthetic TLR4 antagonist, on induction of TNF-α production by the four lipid A compounds were evaluated, as were the profiles of gene expression induced by the four lipid A compounds.

The synthetic lipid A compound with short fatty acids was a more potent inducer of PCA and TNF-α in comparison to other synthetic compounds. Inhibition of TNF-α production by cells co-incubated with PMB and E5564 exceeded 90%, except for SMPLA co-incubated with PMB. There were no significant differences between the expression of genes induced by the synthetic lipid A compounds, however, the natural lipid A induced significantly greater expression of IL-6 and IL-10 than the three synthetic lipid A's. These findings indicate that both natural and synthetic lipid As initiate inflammatory responses in equine monocytes via activation of TLR4 and primarily induce expression of genes (TNF-α, IL-1β, IL-6 and IL-10) associated with recruitment of the MyD88 adaptor protein. The results of this study underscore the

importance of the KDO moiety in the natural lipid A in initiation of gene expression, and that alterations in the length of fatty acids in lipid A alter the biological responses.

INTRODUCTION

Lypopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, induces profound effects on the host immune system.[1] A molecule of *E. coli* LPS consists of three regions: the O-chain, the core and the lipid A portion. The O-chain and core form the polysaccharide component of the molecule. The polysaccharide component is bound to the lipid A portion through a unique sugar called 2-keto-3-deoxyoctulosonic acid (KDO). The outermost layer, O-chain, is highly variable among bacteria and determines the antigenic (serological) specificity of LPS. The inner most layer, lipid A, which is responsible for the toxicity associated with LPS, typically consists of six fatty acyl chains linked to two phosphorylated glucosamine residues.[2] LPS is recognized by Toll-like receptor 4 (TLR4), a member of the TLR family of receptors that recognize conserved microbial structures.[3,4] After binding of LPS to TLR4, two main intracellular pathways may be activated, which leads to the production of inflammatory mediators. One of these pathways recruits the adaptor molecule MyD88 and the other recruits the TRIF adaptor protein.[5,6] The MyD88 dependent pathway leads to the production cytokines, such as tumor necrosis factor α (TNF-α), interleukin 1ß (IL-1ß), IL-6 and IL-10, whereas the TRIF-dependent pathway leads to the production of interferon (IFN) and IFN-inducible genes, such as, IFN-ß, IP-10 and CCL5.[7,8]

Until recently, it was presumed that the structure of lipid A was highly conserved among Gram-negative bacteria. However, the results of recent studies have demonstrated that the fatty

acid acylation pattern, the carbohydrate backbone and the degree of phosphorylation can vary considerably among bacterial species.[9,10] Furthermore, altering the number or length of the attached fatty acids or altering the charge of lipid A may alter the magnitude of the cell response and induce different patterns of pro-inflammatory responses.[11,12] Differential patterns of cytokine induction are produced by LPS molecules isolated from different Gram-negative bacteria. For example, LPS from *E. coli* primarily induces the production of pro-inflammatory mediators (TNF-α, IL-1ß and IL-6), monophosphoryl lipid A (MPLA) from *Salmonella* induces mainly IFN-ß inducible genes, and LPS from *N. meningitides* induces both sets of genes.[13] Collectively, these results indicate that LPS derived from different bacteria can differentially induce genes associated with the MyD88-dependent and the TRIF-dependent pathways.

Due to the heterogeneity in lipid A, it has been difficult to identify the specific structure-function relationships controlling activation of differential inflammatory pathways using preparations isolated from bacterial cell walls. Furthermore, the lipid A preparations purified from bacterial cultures may suffer by being heterogenous in composition and inconsistent in performance. In order to determine how the structure of the lipid affects cell activation, more recent studies have utilized chemically synthesized and synthetically modified lipid As.[14-16] The majority of the synthetic lipid A structures synthesized contain the typical ß-(1-6)-linked D-glucosamine dissacharide, but differ in patterns of acylation and phosphorylation. The generation of synthetic lipid A analogues is a route to excellent consistency and purity in the products being tested.

Therefore, the hypothesis underlying this study was that lipid A compounds, based on the structure of *E. coli* lipid A, having defined differences in their chemical structures induce different cellular responses in equine monocytes, and that these responses are structurally

specific. The objectives of this study were to: (1) Characterize responses of equine monocytes to four structurally distinct *E. coli* based lipid A compounds: natural diphosphoryl lipid A with KDO and long chain fatty acids (NLA), synthetic diphosphoryl lipid A with long chain fatty acids (SLA), synthetic diphosphoryl lipid A with short chain fatty acids (SLAsFA) and synthetic monophosphoryl lipid A with long chain fatty acids (SMPLA) (Figure 6.1), using TNF-α production and expression of procoagulant activity (PCA) as readouts, (2) Determine whether these compounds are differentially inhibited by polymyxin B (PMB) and E5564, and (3) Determine the gene expression profiles induced by the four lipid A compounds.

MATERIALS AND METHODS

Materials

Equine serum was purchased from Hyclone (Logan, UT), RPMI-1640, PBS and penicillin/streptomycin were from Mediatech, Inc. (Herndon, VA). *Escherichia coli* 0111:B4 LPS and *E. coli* K12 lipid A were purchased from List Biological Laboratories, Inc. (Campbell, CA). Polymyxin B was purchased from Bedford Laboratories (Beford, OH). Synthetic MPLA and Poly I:C (Polyinosine-polycytidylic acid) were from InvivoGen (San Diego, CA) and reconstituted as indicated by the manufacturer. RNeasy Mini Kits and the RNase-Free DNase Set were purchased from Qiagen Inc. (Valencia, CA). The High Capacity cDNA archive kit, SYBR Green PCR master mix, and Eukaryotic 18S rRNA were purchased from Applied Biosystems (Foster City, CA). E5564 was generously donated by EISAI Inc. (Woodcliff Lake, NJ) and the other two synthetic *E. coli* lipid A compounds were synthesized by Dr. G-J. Boons

laboratory at the Carbohydrate Research Center at the University of Georgia. Histopaque 1077 and all other high-grade chemicals were from Sigma–Aldrich (St. Louis, MO).

Horses

Six adult horses determined to be healthy on the basis of clinical examination were used for the study. A venipuncture site over a jugular vein on each horse was aseptically prepared, and blood samples were collected into syringes containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. The experimental protocol was reviewed and approved by the Animal Care and Use Committee at the University of Georgia.

Isolation of equine peripheral blood monocyte

Mononuclear cells were isolated by density-gradient centrifugation over Histopaque 1077 as previously described.[17] Viability was greater than 98% by trypan blue exclusion. Mononuclear cells were suspended in RPMI-1640 at a final concentration 4 x 10^6/mL; 2 x 10^6 mononuclear cells were added to each well of a sterile 24-well polystyrene plate and incubated for 2 h at 37°C, in a 5% CO_2 atmosphere. After incubation, non-adherent cells were removed by washing with warm RPMI-1640. Adherent monocytes were then incubated in RPMI-1640 containing 10% equine serum supplemented with 100 units/ml penicillin and 100 µg/mL streptomycin and collected according to the individual assays as described below. All experiments were done in triplicate for each horse.

Concentration response curves for NLA, SLA, SLAsFA and SMPLA

Negative and positive control samples used in the study contained 10% equine serum in RPMI 1640 and 100 pg/ml of *E. coli* 0111:B4 LPS, respectively. Monocytes were incubated with ranges of either NLA (1 pg/ml – 10 μg/ml), SLA (1 fg/ml – 10 μg/ml), SLAsFA (1 fg/ml – 10 μg/ml) and SMPLA (1 fg/ml – 10 μg/ml) using whole log increments in concentration. Cells were incubated for 6 h after which cell lysates and supernatants were assayed for PCA and TNF-α production, respectively. Analysis of the concentration response curves for each compound generated an EC_{50} (concentration that produces 50% of the maximum plateau effect) value for expression of PCA and TNF-α production. The concentrations used in the assays described below were based on the EC_{50} generated from supernatant concentrations of TNF-α induced by a range of concentrations of each compound.

Effects of polymyxin B and E5564 on induced TNF-α production

Negative control samples included cells incubated with 10% equine serum in RPMI 1640, 13 units/ml of PMB, or 100 nM of E5564. The positive control samples included cells incubated with 100 pg/ml of *E. coli* 0111:B4 LPS in RPMI 1640. Monocytes were incubated with the calculated EC_{50} for each compound, 0.30 ng/ml of LPS, 4.2 ng/ml of NLA, 0.90 ng/ml of SLA, 0.014 ng/ml of SLAsFA and 25 ng/ml of SMPLA. All cells were incubated for 4 h after which supernatants were assayed for TNF-α concentration.

TNF-α assay

The TNF-α protein concentration in monocyte supernatants was measured by use of an equine TNF-α ELISA using a recombinant equine TNF-α standard as previously described.[18]

PCA assay

An automated 1-stage clotting assay was used to determine the effects of cell lysates on calcium-induced clotting of pooled equine plasma. The PCA activity of the samples was determined by comparing the results with those obtained for a standard curve generated using equine brain thromboplastin.[19]

RNA extraction and cDNA synthesis for mRNA expression analysis

Approximately 1 x 10^7 isolated mononuclear cells were added to sterile 60 x 15-mm Petri dishes and incubated for 2 h at 37°C in 5% CO_2 atmosphere. After incubation, non-adherent cells were removed by washing with warm RPMI 1640. Adherent monocytes were overlaid with RPMI 1640 supplemented with 10% equine serum containing 100 U of penicillin/mL and 100 μg of streptomycin/mL. Monocytes were incubated for 4 and 20 h with the approximate EC_{90} for each compound, namely 42 ng/ml of NLA, 9 ng/ml of SLA, 0.140 ng/ml of SLAsFA and 250 ng/ml of SMPLA. Ligands for TLR3 (Poly I:C, 250 μg/mL) and TLR4 (*E. coli* LPS, 100 pg/mL) were used as control compounds that primarily activate the TRIF-dependent and MyD88-dependent pathways, respectively.

After incubation, 1 ml of supernatant was collected for TNF-α assay and cells were washed with cold phosphate buffered saline, scraped from the plates with RNA cell lysis solution and stored at –80°C. After the cells were thawed, total RNA was extracted according to the manufacturer's protocol and treated by incubation with DNase at 24°C for 30 min. Only samples having 260:280 nm absorbance ratios between 2.0 and 2.2 as measured on a NanoDrop spectrophotometer (Thermo Fisher Scientific;Wilmington, DE) were processed for cDNA

synthesis using the High Capacity cDNA Archive Kit (Foster City, CA) with 100 ng of RNA as template.

Real-time quantitative RT-PCR

Real-time qPCR assays using SYBR Green as a detector were performed in an Applied Biosystems 7900HT (Foster City, CA) sequence detection system, with eukaryotic 18S ribosomal RNA assays serving as endogenous controls.[20-22] Conditions for amplification were 2 minutes at 50°C, 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C, and 60 seconds at 60°C. This was followed by a dissociation stage for 15 s at 95°C to ensure the presence of a single amplicon. The eight target genes of interest in this study were equine TNF-α, IL-1ß, IL-6, IL-10, IFN-ß, IP-10 and CCL5 (Table 6.1). Oligonucleotide primers used for the detection of cDNA specific for equine cytokines were derived from the GenBank database and designed with Primer Express software (Applied Biosystems, Foster City, CA). Dissociation curve analysis and agarose gel electrophoresis revealed single products for all primers, the PCR amplification efficiency was ~100%, and amplification efficiencies of the target and reference (18S rRNA) were equivalent. The PCR assays contained 300 nM of each primer as optimized concentration, 2.5 μL 2X master mix, and 1 μL of the diluted cDNA sample in a final volume of 5 μL. Intra-assay variation was evaluated using a pooled cDNA sample prepared from RNA of cells stimulated with *E. coli* 0111:B4 LPS (100 pg/mL) for 4 hours. Changes in cytokine expression were calculated by relative quantification against 18S rRNA with the $\Delta\Delta C_T$ method; where $\Delta\Delta C_T = [(\text{gene of interest } C_T - \text{18S rRNA } C_T)_{sample} - (\text{gene of interest } C_T - \text{18S rRNA } C_T)_{calibrator}]$, C_T is defined as the amplification cycle at which amplification reached a comparable threshold level of template concentration. Fold changes in

gene expression were calculated as $2^{-\Delta\Delta CT}$. Results were expressed as the mean fold change in gene expression at each time point. The C_T for the non-stimulated control sample was used as the calibrator and assigned a fold change in expression of 1.

Statistical analyses

Data were analyzed by fitting a logistic expression to concentration-response data using GraphPad Prism Software (San Diego, CA). This analysis allowed determination of EC_{50} and maximum response values with their associated 95% confidence intervals (CI). Data were analyzed using a one-way ANOVA and Bonferroni post-hoc test. All data are reported as mean ± SEM. Significance was set at $P < 0.05$.

RESULTS

Concentration response curves

The EC_{50} values were derived from the concentration response curves generated by each compound (Figures 6.2A-B and 6.3A-B). The EC_{50}, 95% CI and maximal response values for all compounds tested are presented in Table 6.2. The SLAsFA compound was a more potent inducer of PCA and TNF-α in comparison to the other synthetic compounds. The maximal responses generated by these compounds in descending order were the following: NLA > SLA > SMPLA > SLAsFA, for both expression of PCA and TNF-α production.

Polymyxin B and E5564 inhibited TNF-α production by all synthetic compounds

Co-incubation of monocytes with either PMB or E5564 inhibited TNF-α production induced by the lipid A compounds more than 90%, except for SMPLA co-incubated with PMB. PMB reduced the production of TNF-α induced by SLA and SLAsFA by approximately 99%, whereas it reduced that induced by SMPLA by only 40% (Table 6.3).

Gene expression profiles induced by NLA and the synthetic compounds

NLA and the synthetic lipid A compounds primarily induced expression of inflammatory genes (TNF-α, IL-1β, IL-6 and IL-10) (Figures 6.4A-D and 6.5 A-C). While there were no significant differences in the gene expression profiles induced by the synthetic lipid A compounds, NLA induced significantly greater expression of IL-6 and IL-10 at 4 h of incubation, when compared the synthetic compounds. NLA also induced significantly greater expression of TNF-α at 4 h and IL-1β at 20 h when compared to SLAsFA, and significantly greater expression of IL-6 at 20 h when compared to SLA and SLAsFA.

TNF-α production by monocytes

Supernatant concentrations of TNF-α were significantly increased by NLA and SMPLA at 4 h when compared to the SLAsFA (Figure 6.6A). At 20 h, NLA induced significantly higher production of TNF-α when compared to SLAsFA (Figure 6.6B).

DISCUSSION

To identify relationships between combinations of lipid A structures and cellular activation, several recent studies have compared cellular responses to chemically synthesized and modified lipid A compounds containing different patterns of acylation and degrees of phosphorylation. For example, lipid A analogs with only one phosphate at either end have been shown to induce weak production of TNF-α and IL-1 ß in comparison to *E. coli* lipid A. Furthermore, lipid A compounds containing both phosphates, but with a reduced number of fatty acids did not induce TNF-α and IL-1ß production in murine cells, and synthetic lipid A molecules based on the lipid A structures of *Rhodobacter sphaeroides* and *R. capsulatus*, and natural *R. sin*-1 function as antagonists, rather than agonists, of enteric LPS in human and murine cells.[23-29]

However, there are also species-specific differences in cellular responses to different lipid A compounds. For example, *R. spahaeroides* lipid A stimulates production of inflammatory mediators by equine and hamster leukocytes, whereas the same lipid A compound is an antagonist in human cells.[30,31] Furthermore, a synthetic lipid A, E5531, based on the structure of *R. capsulatus* is a potent antagonist of LPS in human cells, but functions as an agonist in equine leukocytes.[32,33] In contrast, E5564, the synthetic lipid A based on the structure of *R. sphaeroides*, does not induce inflammatory responses in equine cells and, in fact, functions as a potent LPS antagonist in equine whole blood and monocytes.[18] Therefore, it is important to establish the biological activities of compounds acting via TLR4 in the species of interest rather than trying to extrapolate results across species. This is the first study to investigate the

responses of equine monocytes to synthetic lipid A compounds based on *E. coli* LPS that differ in their acylation pattern or the number of phosphate groups they contain.

To assess the responses of equine monocytes and to identify concentrations of NLA, SLA, SLAsFA and SMPLA for later assays in the study, we first selected two readouts of cellular activation, namely expression of PCA and production of TNF-α. Procoagulant activity, which is also called tissue factor or thromboplastin, reflects the ability of stimulated cells to decrease the coagulation time of plasma.[34] In previous studies, LPS has been shown to increase expression of PCA by monocytes and endothelial cells.[35] The importance of PCA in horses was exemplified by the fact that an increase in monocyte PCA was significantly associated with coagulopathy and poor prognosis in horses with colic.[36] Tumor necrosis factor-α is a central mediator synthesized in response to LPS.[37] As such, it induces production of other pro-inflammatory mediator, including IL-1ß and IL-6, activates inflammatory cells and increases expression of adhesion molecules. Increases in serum concentrations of TNF-α have been associated with a poor prognosis in septic equine neonates and in horses with colic.[38,39] Therefore, TNF-α is often used as a reliable, representative indicator of activation of inflammatory cells in horses and other species.

The natural lipid A containing the KDO moiety showed the maximal level of production of TNF-α and PCA in comparison to the synthetic compounds, which lack the KDO moiety. These results demonstrate the importance of the KDO moiety for biological activity. Of the synthetic lipid A compounds tested, the compound with shorter fatty acids (SLAsFA) lacking two carbons at three of the acylation sites was the most potent inducer of PCA and TNF-α. Although the maximal biological responses to this compound were not significantly different from the other synthetic compounds, the magnitude of response was consistently greater. Thus,

the data suggest that substitution of shorter fatty acid chains to the *E. coli* lipid A backbone in the absence of KDO enhances its ability to activate signaling/induce cellular activation in equine monocytes. Aside from SMPLA, which yielded markedly different EC_{50} values from the two cellular readouts (PCA and TNF-α), the other synthetic compounds had similar potencies and efficacies (Table 6.2). Since TNF-α is often used as a reliable indicator of activation of inflammatory cells; the concentrations used for the later assays were based on the results from the lipid A induced TNF-α concentration response curves.

Polymixin B is a cationic antibiotic that neutralizes the endotoxic effects of LPS by binding directly with lipid A.[40] E5564, a synthetic lipid analogue A, based on the structure of the nontoxic LPS from the bacterium *Rhodobacter sphaeroids*, antagonizes the proinflammatory effects of LPS by preventing the interaction between LPS and MD2. E5564 binds to MD2 and competes for the LPS binding site.[41] Both PMB and E5564 inhibited TNF-α production induced by all the lipid A compounds tested in this study. The inhibition by E5564 further substantiates the specificity of the sythetic lipid A structures for binding and activation of TLR4. The reason why PMB partially inhibited SMPLA induced TNF-a production in comparison to other compounds is not yet clear, and warrants further investigation.

In mammalian species studied to date, TLR4 recruits both MyD88 and TRIF, whereas TLR3 recruits only TRIF. The recruitment of MyD88 is essential for the production of pro-inflammatory cytokines, such as TNF-α, IL-1ß and IL-6 and the anti-inflammatory cytokine IL-10.[7] Genes that depend on TRIF activation include IFN-ß, IP-10 and CCL5.[19] The importance of the differential induction of the MyD88- and TRIF-dependent pathway lies in the finding that each leads to a distinct profile of immune mediators that in turn determine the phenotype of the cells that primarily are responsible for the development of adaptive immune responses. In the

present study, all synthetic compounds induced similar patterns of gene expression, with induction of genes typically associated with the MyD88-dependent pathway. The one compound (NLA) that containined the KDO moiety induced significantly greater expression of IL-6 and IL-10 than the other compounds at 4 h, suggesting that the KDO moiety is important for cellular activation and induction of inflammatory genes.

In summary, the results of this study indicate that natural lipid A, and the three synthetic lipid A compounds, based on the structure of *E. coli* lipid A without KDO, all signal through the TLR4 receptor complex. Further, each of these lipid A compounds induced expression of genes that are typically associated with utilization of the MyD88-dependent pathway. The natural lipid A, containing KDO, induced significantly greater expression of inflammatory cytokines than the synthetic compounds that lacked KDO. Furthermore, differences in the length of the fatty acid chains attached to the lipid A backbone induce different levels of biological responses from equine monocytes. Collectively, these findings indicated that components of the lipid A core and the acylation pattern are important factors in initiation of biological responses by monocytes.

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Table 6.1. Real-time quantitative RT-PCR primer pairs used for the qRT-PCR assays.

Gene	GenBank accession No.	Forward and reverse sequence (5′ to 3′)
TNF–α	AB035735	AAGGACATCATGAGCACTGAAAG GGGCCCCCTGCCTTCT
IL–1ß	ECU92481	ATGACTTACTGCAGCGGCAA GTCTTGGAAGCTGCCCTTCA
IL–6	ECU64794	TGCTGGCTAAGCTGCATTCA GGAAATCCTCAAGGCTTCGAA
IL–10	U38200	GCCTTGTCGGAGATGATCCA TTTTCCCCCAGGGAGTTCAC
IFN–ß	AF134227	CCCCGAGGACACAATGAACT ACCAATGCAGCATCCTCCTT
IP–10	CD470151	CCTCCAGTTGCAGCACCAT TTCCTTGAGTTCCACTCAGAGTCA
CCL5	AF506970	CACTGCCACCTTCTGCACTC CGGGAGATGTAGGCAAAGCA

Table 6.2. EC_{50} (ng/ml), 95% CI (ng/ml) and maximal response values of natural *E. coli* LPS, natural lipid A and synthetic compounds derived from *E. coli* lipid A.

Compounds	**PCA (%)**		**TNF-α (pg/ml)**	
	EC_{50}	Max response	E_{50}	Max response
Natural lipid A	2.7 (0.5 to 9.6)	1211	4.2 (1.4 to 13)	2950
Synthetic compounds				
Lipid A	0.36 (0.07 to 1.7)	294	0.90 (0.3 to 2.4)	1238
Lipid A with shorter lipids	0.024 (0.003 to 0.2)	183	0.014 (0.002 to 0.08)	1095
Monophosphoryl lipid A	0.47 (0.05 to 4.6)	249	25 (6.6 to 97)	1234

Table 6.3. Percent inhibition of TNF-α production (mean ± SEM) by monocytes incubated with 10% equine serum in RPMI 1640 (media), plain LPS (0.03 ng/ml), NLA (4.2 ng/ml), SLA (0.9 ng/ml), SLAsFA (0.014 ng/ml), SMPLA (25 ng/ml) and in the presence of either or PMB (13 units/ml) and E5564 (100 nM) for 4 hours; n= 4 horses. Cells incubated with the compound alone were assigned a value of 100%.

	% inhibition	
Natural compounds	**+ PMB**	**+ E5564**
LPS	95.1 ± 4.9	90.5 ± 8.1
Natural lipid A	96.4 ± 1.1	99.3 ± 0.5
Synthetic compounds		
Lipid A	99.4 ± 0.6	99.0 ± 0.8
Lipid A with shorter lipids	98.9 ± 1.1	98.5 ± 1.5
Monophosphoryl lipid A	40.6 ± 8.5	99.2 ± 0.8

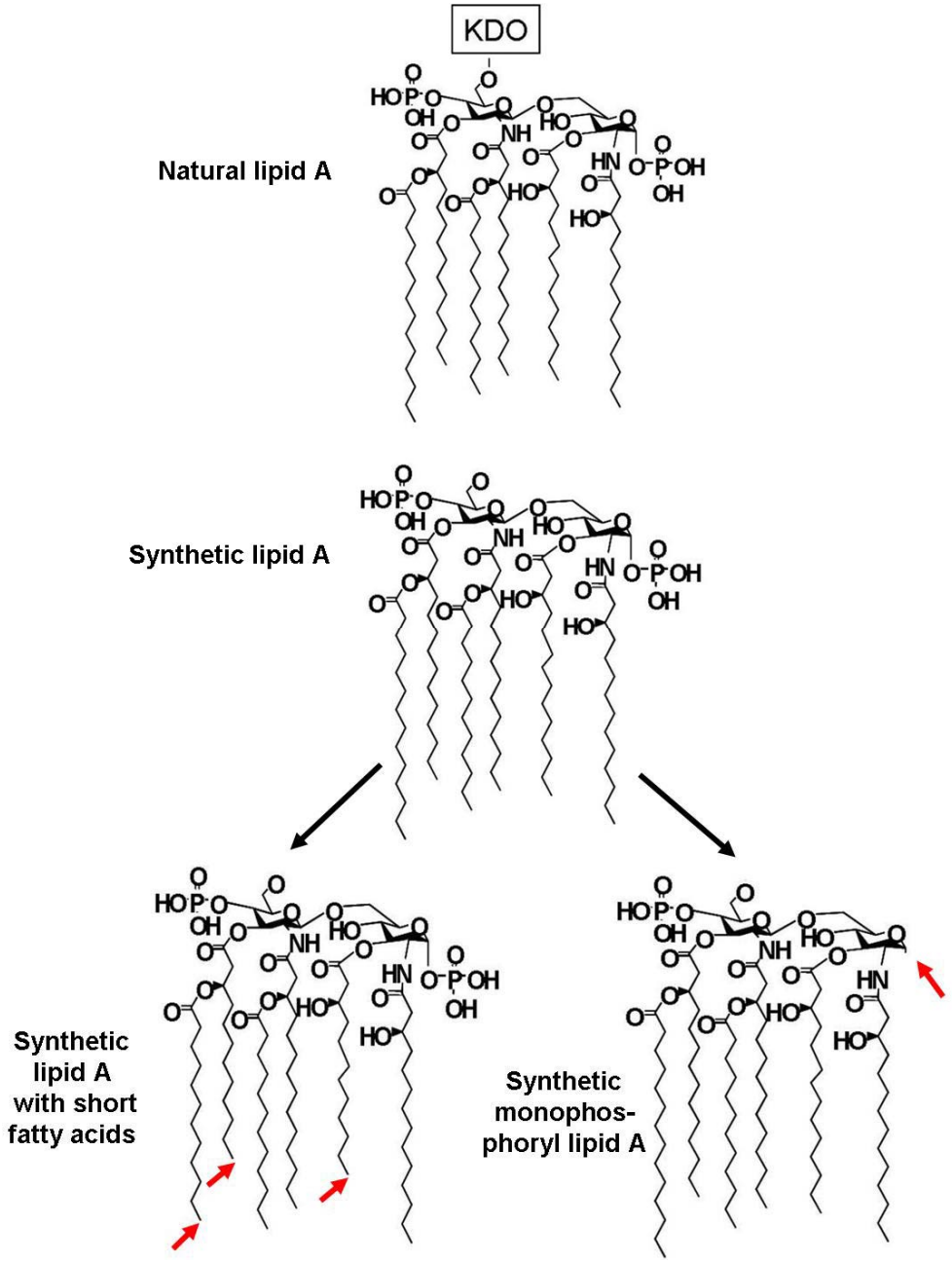
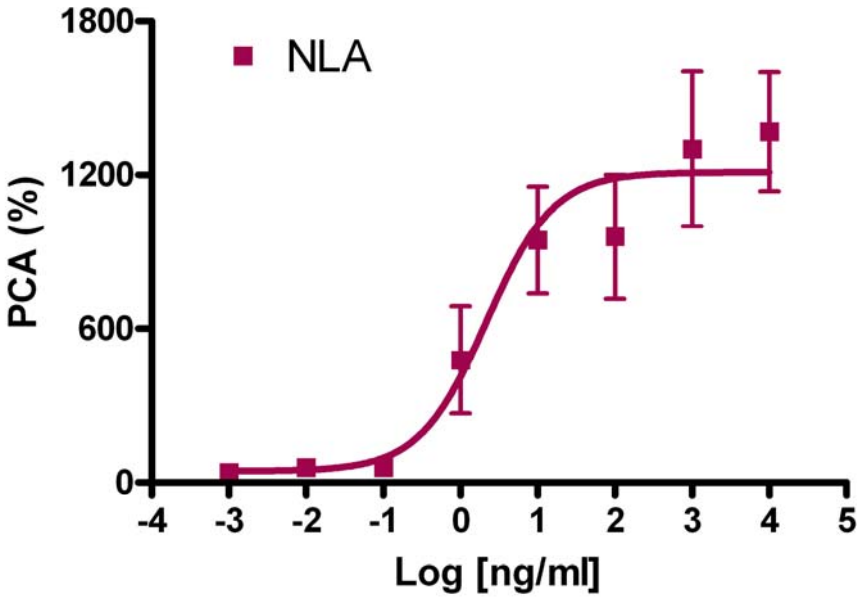


Fig 6.1 Chemical structure of *E. coli* Lipid A and the three synthetic compounds used in the study. Natural lipid A (NLA) contains long chain fatty acids and KDO, Synthetic lipid A (SLA) is the same as NLA, except that it does not contain KDO, Synthetic lipid A with short fatty acids (SLAsFA) features fatty acid chains that are 2 carbons shorter at three of the acylation sites and no KDO, Synthetic monophosphoryl lipid A (SMPLA) has both one phosphate and the KDO removed, but typical fatty acid chain length.

A)



B)

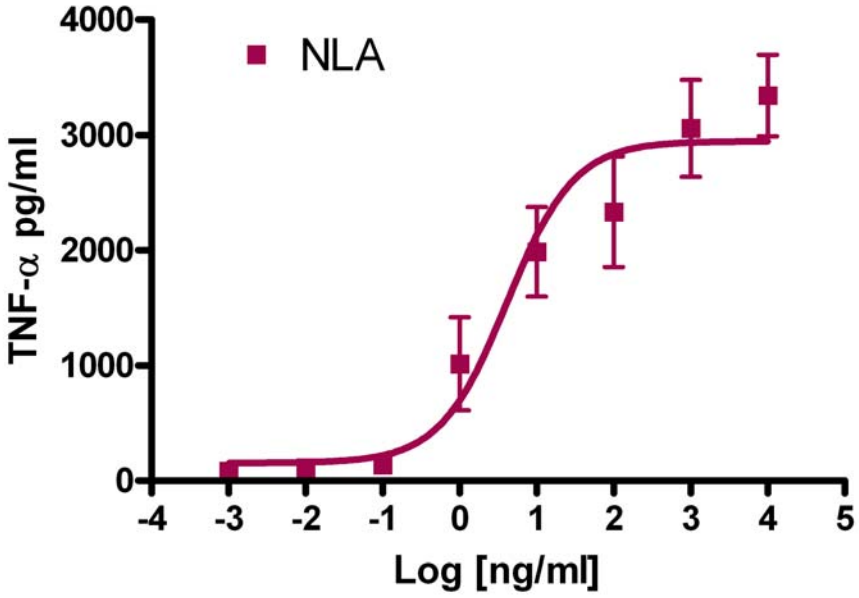
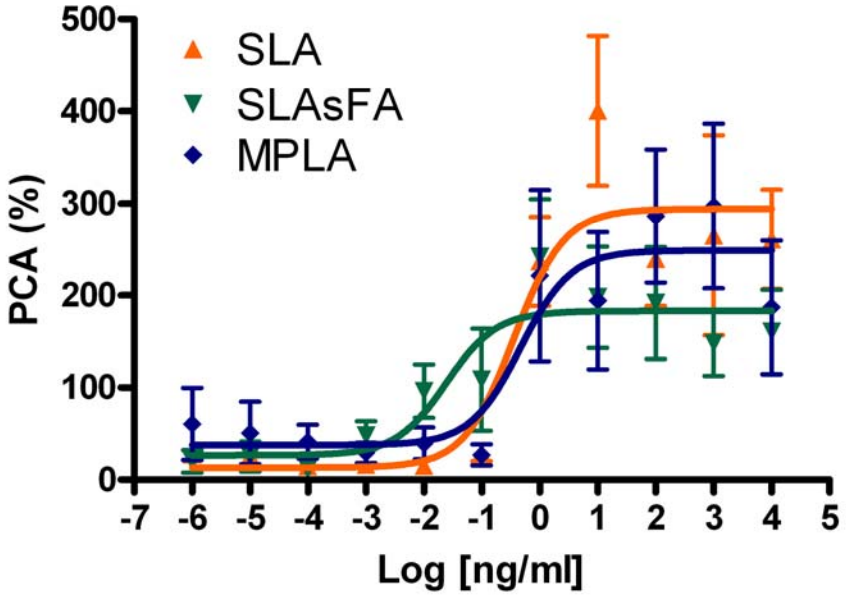


Fig 6.2 PCA and TNF-α production (mean ± SEM) by monocytes incubated with NLA (■) (1 pg/ml – 10 μg/ml) for 6 hours; n= 6 horses.

A)



B)

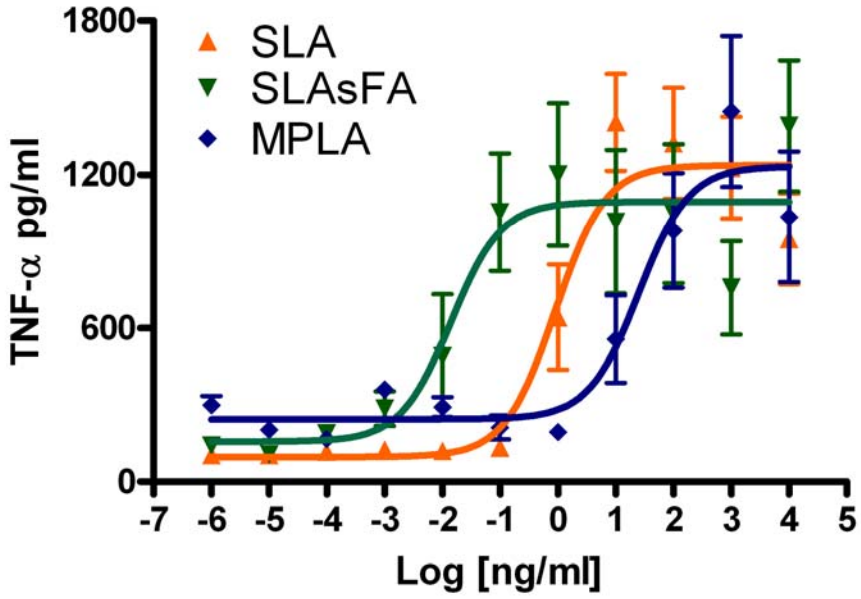


Fig 6.3 PCA and TNF-α production (mean ± SEM) by monocytes incubated with SLA (▲) (1 fg/ml – 10 μg/ml), SLAsFA (▼) (1 fg/ml – 10 μg/ml) and SMPLA (♦) (1 fg/ml – 10 μg/ml) for 6 hours; n= 6 horses.

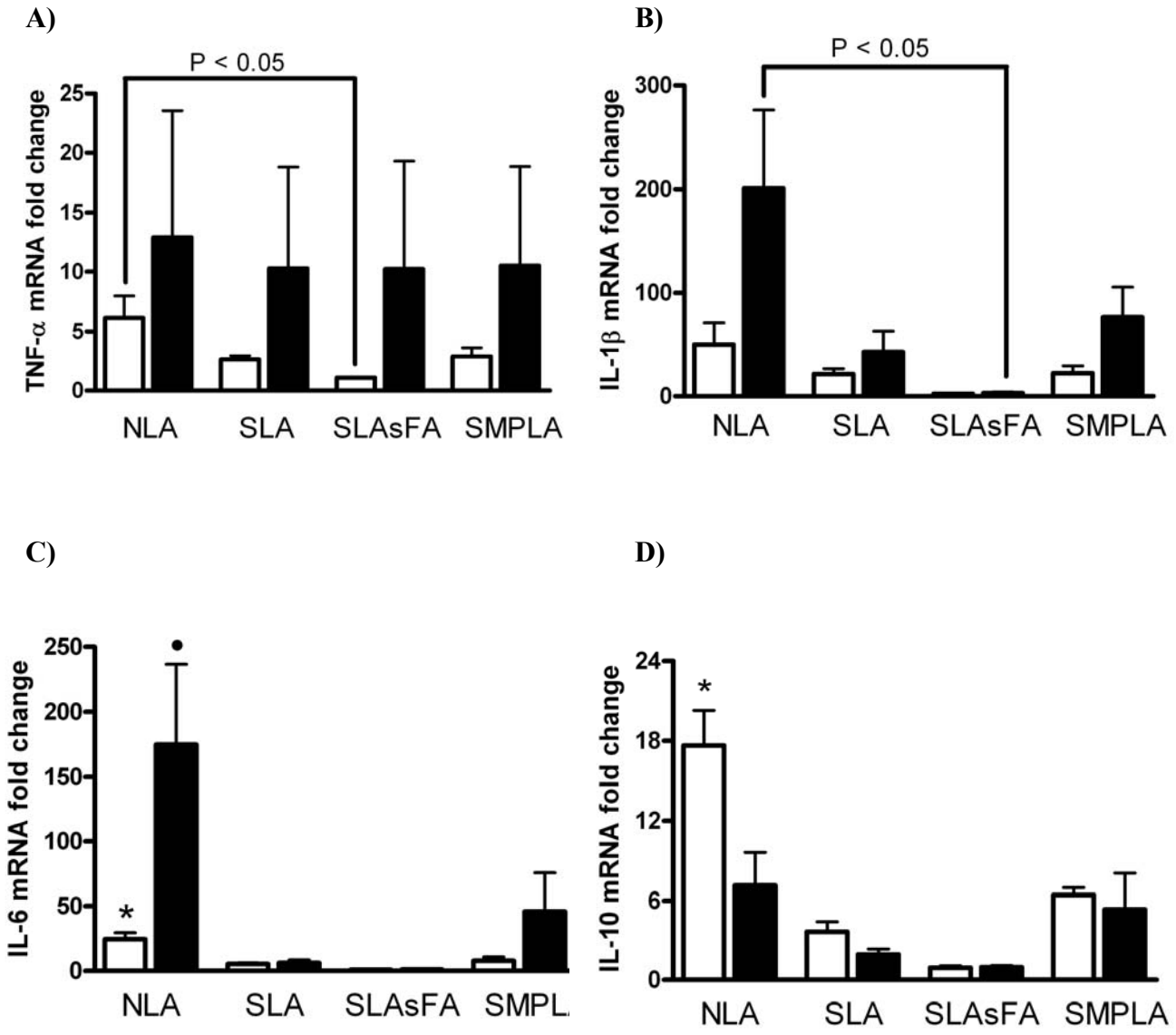


Figure 6.4 The mean (± SEM) relative mRNA expression of TNF–α (A), IL–1ß (B), IL-6 (C) and IL-10 (D) in monocytes incubated with NLA (42 ng/ml), SLA (9 ng/ml), SLAsFA (140 pg/ml) and SMPLA (250 ng/ml) for 4 (clear bars) and 20 (dark bars) hours. * Significantly different from other compounds at corresponding times. • Significantly different from other compounds at corresponding times except for SMPLA; n= 4 horses.

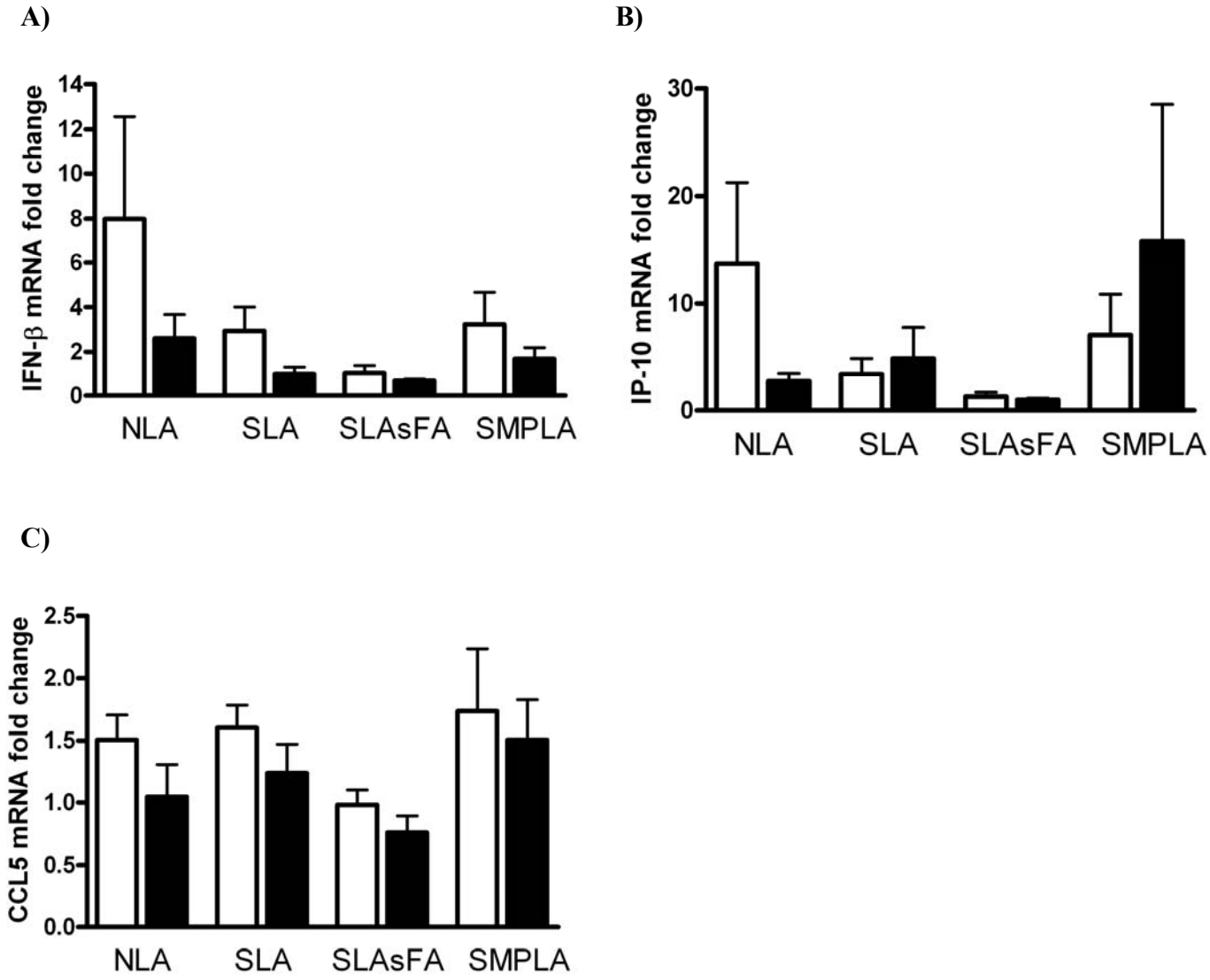
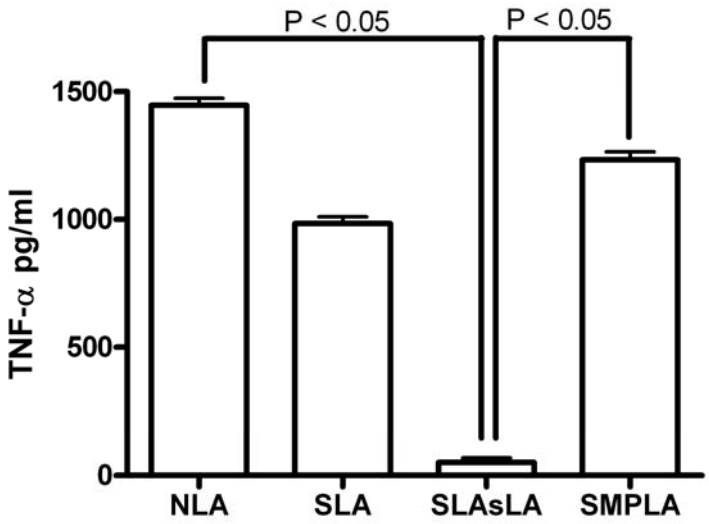


Figure 6.5 The mean (± SEM) relative mRNA expression of IFN-β (A), IP-10 (B) and CCL5 (C) in monocytes incubated with NLA (42 ng/ml), SLA (9 ng/ml), SLAsFA (140 pg/ml) and SMPLA (250 ng/ml) for 4 (clear bars) and 20 (dark bars) hours; n= 4 horses.

A)



B)

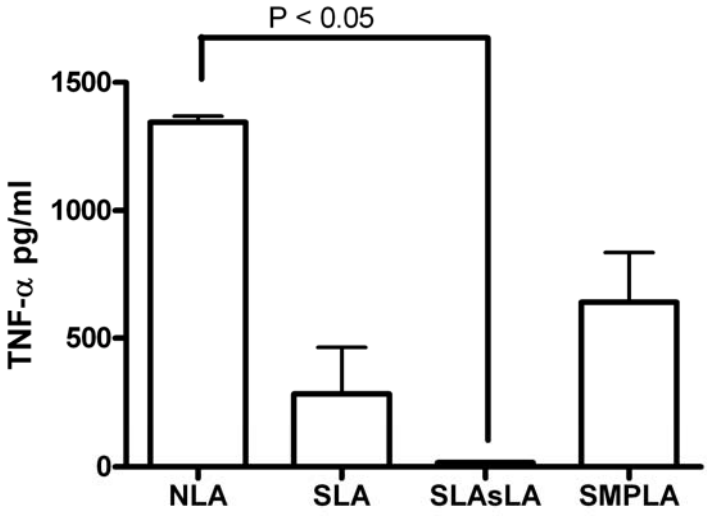


Figure 6.6 TNF–α protein production (mean ± SEM) in supernatants of monocytes incubated with NLA (42 ng/ml), SLA (9 ng/ml), SLAsFA (140 pg/ml) and SMPLA (250 ng/ml) for 4 (A- clear bars) and 20 (B- dark bars) hours; n= 3 horses.

CHAPTER 7

EFFECTS OF THE SECOND-GENERATION SYNTHETIC LIPID *A* ANALOGUE E5564 ON RESPONSES TO ENDOTOXIN IN EQUINE WHOLE BLOOD AND MONOCYTES[1]

[1]M.D. Figueiredo, J.N. Moore, M.L. Vandenplas, W.C. Sun, T.F. Murray. 2008 *American Journal of Veterinary Research* 69 (6) 796-803
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ABSTRACT

Endotoxin, a structural component of Gram-negative bacteria, gains access to the circulation in acute gastrointestinal diseases in horses and in septicemia in neonatal foals. E5564 is a synthetic second generation lipid A analog that blocks the effects of LPS in humans and laboratory animals. We hypothesized that E5564 would inhibit the endotoxin response in equine whole blood and monocytes.

Whole blood and monocytes were incubated with *Escherichia coli* 0111:B4 LPS, E5564, or E5564 plus *E. coli* 0111:B4 LPS. Whole blood and cell supernatants were assayed for TNF-α, and cell lysates were assayed to determine PCA. Expression of mRNA for TNF-α, IL-1ß, IL-6, and IL-10 by monocytes was determined by use of real-time quantitative polymerase chain reaction assay.

Minimal proinflammatory effects were detected in whole blood or monocytes. In addition, E5564 inhibited LPS-induced PCA and TNF-α production in a concentration-dependent manner. Furthermore, E5564 significantly inhibited LPS-induced mRNA expression of TNF-α, IL-1ß and IL-10 and decreased LPS-induced expression of IL-6.

The second-generation synthetic lipid A analogue E5564 lacked agonist activity in equine whole blood and monocytes and was a potent antagonist of enteric LPS. Therefore, E5564 appeared to be the first lipid A analogue that has potential as an effective therapeutic agent in horses with endotoxemia.

INTRODUCTION

Endotoxemia is a leading cause of morbidity and fatalities in adult horses and foals,[1] and it has been associated with acute abdominal disease, laminitis, strenuous exercise, and neonatal sepsis.[2,3] Current methods of treatment often are ineffective, hence the need for new therapeutic approaches.

The hydrophobic lipid A region of LPS (ie, endotoxin) is responsible for initiating various innate immune responses that result in development of the systemic inflammatory response syndrome. Because the lipid A region is relatively conserved among a variety of gram-negative bacteria, this molecule is an attractive target for the development of LPS antagonists.[4] Lipid A and LPS isolated from *Rhodobacter sphaeroides*, as well as E5531 (a synthetic analogue of the lipid A region from *Rhodobacter capsulatus*) are potent inhibitors of enteric LPS in several species. For example, diphosphoryl lipid A from *R sphaeroides* inhibits cytokine release in response to cell stimulation by enteric LPS in murine[5,6] and human[7] cells, expression of LPS-inducible genes,[8] and binding of enteric LPS to a murine cell line.[9] In vivo experiments have identified an inhibitory effect of lipid A on LPS-induced lethality in mice.[10] Compound E5531 acts as an LPS antagonist in murine and human cells as well as in whole blood,[11,12] protects mice after injection with a lethal dose of viable *E. coli* bacteria,[13] and completely blocks signs and symptoms attributable to sepsis in human volunteers with experimentally induced endotoxemia.[14] However, results of studies indicate that *R sphaeroides* LPS and E5531 both elicit strong proinflammatory responses in equine cells and equine whole blood, which makes it impossible to use these compounds in the treatment of endotoxemic horses.[15,16]

Compound E5564 (eritoran tetrasodium) is a second-generation synthetic lipid A analogue derived from the structure of *R sphaeroides*. It is approximately 10 times as potent, has a longer duration of action, and is more stable than E5531.[4] In addition, E5564 blocks the action of LPS at its cell-surface receptor (Toll-like receptor 4), thereby preventing in vitro and in vivo induction of cellular mediators in rodents and humans[17] and improving survival in LPS-challenged mice.[18] Furthermore, E5564 completely blocks the effects of experimentally induced endotoxemia in humans but does not have agonistic LPS-like activity.[19] Currently, E5564 is in phase III clinical trials in human patients with severe sepsis.

To determine the therapeutic potential of this compound in horses, there were 2 objectives for the study reported here. The first was to evaluate E5564 for proinflammatory effects in equine peripheral whole blood and isolated monocytes. The second was to evaluate the ability of E5564 to prevent LPS-induced expression of proinflammatory and anti-inflammatory cytokine genes by equine monocytes. We hypothesized that E5564 would not induce agonist responses but would antagonize the effects of enteric LPS in equine whole blood and monocytes and inhibit LPS-induced expression of mRNA for TNF-α, IL-1ß, IL-6, and IL-10 by monocytes.

MATERIALS AND METHODS

Horses

A group of 19 adult horses determined to be healthy on the basis of clinical examination were used for the study. A venipuncture site over a jugular vein on each horse was aseptically prepared, and blood samples were collected. The Animal Care and Use Committee at the University of Georgia approved the experimental protocol.

Preparation of LPS and E5564

Lipopolysaccharide was reconstituted in DPBSS without calcium and magnesium (LPS concentration, 1 mg/mL). Prior to use, LPS was sonicated for 60 seconds and further diluted in RPMI 1640 containing 1% HIFBS.[20] Compound E5564 was reconstituted in RPMI 1640 at a concentration of 100μM and further diluted in the same medium. All experiments were conducted in triplicate for each horse.

Stimulation of whole blood

Heparinized whole blood (1 unit of heparin/mL) was divided into aliquots and placed in 1.5-mL microcentrifuge tubes. In preliminary experiments that involved use of a limited range of concentrations (10, 100, or 1,000nM), E5564 was tested for agonist activity in whole blood samples obtained from 3 healthy horses. In addition, *E. coli* 0111:B4 LPS (1 ng/mL) was included as a positive control sample in these experiments.

In an additional set of experiments that involved use of a wider range of concentrations of LPS, it was determined that 300 pg/mL was the lowest concentration to yield the maximal increase in TNF-α production. In subsequent experiments, E5564 was evaluated for antagonist effects in whole blood samples obtained from 7 healthy horses. In those experiments, LPS was added to achieve a final concentration of 300 pg/mL, and E5564 was added to achieve final concentrations of 0.01, 0.1, 1, 10, 100, 1,000, and 10,000nM. Additional DPBSS was added to bring the total volume of cells and reagents to 1 mL. Samples were incubated on an orbital mixer at 37°C for 6 hours. At the end of the incubation period, plasma was collected by centrifugation (6,000g at 4°C for 10 minutes) and stored frozen (–80°C) until assayed for TNF-α.

Stimulation of monocytes

Mononuclear cells were isolated by density-gradient centrifugation with a solution of polysucrose and sodium diatrizoate.[20] One-milliliter aliquots containing 4 X 10^6 mononuclear cells were added to sterile 12 X 75-mm polysterene tubes and incubated for 2 hours (37°C and 5% carbon dioxide). After incubation, nonadherent cells were removed with 1 wash of warm RPMI 1640. Adherent monocytes were overlaid with RPMI 1640 containing 100 U of penicillin/mL and 100 µg of streptomycin/mL. Control samples used in the study contained 1% HIFBS in RPMI 1640 (negative control sample) and *E. coli* 0111:B4 LPS (100 pg/mL) in RPMI 1640 with 1% HIFBS (positive control sample). To test for agonist activity of E5564, cells from 6 healthy horses were incubated in culture medium containing E5564 or its vehicle at final concentrations of 0.1, 1, 10, 100, and 1000nM. To test for antagonist activity of E5564, cells from 19 horses were incubated with E5564 at final concentrations of 0.1,1, 10, 100, and 1,000nM for 15 minutes and then stimulated with *E. coli* 0111:B4 LPS (100 pg/mL). To measure the potency of E5564, monocytes were incubated with RPMI 1640 containing 1 of 4 concentrations of E5564 (0.1, 1, 10, and 100nM) and *E. coli* 0111:B4 LPS at 0.0001, 0.001, 0.01, 0.1, 1, 10, and 100 ng/mL. Cells were incubated for 6 hours (37°C and 5% carbon dioxide), after which cell lysates and supernatants were assayed for PCA and TNF-α, respectively.

TNF-α assays

The TNF-α activity in whole blood samples was determined by modification of an in vitro cytotoxicity bioassay that used the murine fibrosarcoma cell line WEHI-164 clone 13, as described elsewhere.[21] The TNF-α protein concentration in whole blood and monocyte cell supernatants was measured by use of an equine TNF-α ELISA (developed with commercially

available reagents) and a recombinant equine TNF-α standard. Standard ELISA plates (96-well flat-bottom plates) were coated with equine TNF-α polyclonal antibody that recognized equine TNF-α. The antibody was diluted 1:333 in a 0.05M carbonate-bicarbonate buffer at pH 9.6, and 100 μL of antibody was added to each well. Microtiter plates were incubated overnight at 4°C, after which antibody was removed and the wells were filled with 100 μL of blocking buffer (1% bovine serum albumin in 1X DPBSS). Plates were incubated for 1 hour at 24°C. Then, plates were washed 3 times with DPBST. Next, 100 μL of each sample or standard was added to duplicate wells, and plates were incubated at 37°C for 2 hours. Plates were washed 3 times with DPBST. Equine TNF-α biotin-labeled polyclonal antibody against TNF-α was diluted 1:277 in DPBST, and 100 μL was added to each well. Plates were incubated at 37°C for 90 minutes, after which they were washed 3 times with DPBST. Next, 100 μL of avidin-horseradish peroxidase, diluted 1:5,000 in DPBST, was added to each well and incubated for 1 hour at 37°C. Plates were then washed 5 times with DPBST. Finally, 100 μL of substrate was added to each well. Plates were incubated for 30 minutes at 24°C and measurements made at 405 nm on an automated microplate reader.

PCA assay

An automated 1-stage clotting assay was used to determine the effects of cell lysates on calcium-induced clotting of pooled equine plasma. The PCA of samples was determined by comparing the results with those obtained for a standard curve generated by use of equine brain thromboplastin.[20]

RNA extraction and cDNA synthesis

Mononuclear cells were isolated by density-gradient centrifugation with a solution of polysucrose and sodium diatrizoate.[20] Approximately 8 X 10^7 mononuclear cells were added to sterile 150 X 15-mm Petri dishes and incubated for 2 hours (37°C and 5% carbon dioxide). After incubation, nonadherent cells were removed with 1 wash of warm RPMI 1640. Adherent monocytes were overlaid with RPMI 1640 containing 100 U of penicillin/mL and 100 μg of streptomycin/mL. Monocytes were incubated for 1 hour and 4 hours in media alone (nonstimulated control sample), media containing E5564 (100nM), media containing *E. coli* 0111:B4 LPS (100 pg/mL), or media containing E5564 and *E. coli* 0111:B4 LPS. After incubation, cells were washed with cold DPBSS and scraped from the plates, isolated by centrifugation, suspended and homogenized in lysis solution, and stored at –80°C. Cell pellets were thawed and total RNA was extracted by use of a commercially available kit (performed in accordance with the manufacturer's protocol) and treated by incubation with DNase I at 24°C for 30 minutes. Only samples with 260:280 nm absorbance ratios between 2.0 and 2.2 (as measured on the spectrophotometer) were processed for cDNA synthesis with a commercially available kit.

Real-time quantification of mRNA expression

A real-time qPCR assay that used a nucleic acid dye was performed in a sequence detection system, with 18S ribosomal RNA used as an endogenous control sample. Conditions for the detection system were 2 minutes at 50°C, 10 minutes at 95°C, 40 cycles of 15 s/cycle at 95°C, and 60 seconds at 60°C. Oligonucleotide primers used for the detection of cDNA specific for equine cytokines were derived from the GenBank database and designed by use of commercially available software (Table 7.1). The PCR assays contained 300nM of each primer,

master mix, and 2 μL of the diluted cDNA sample in a final volume of 10 μL. Intra-assay variation was evaluated with a pooled cDNA sample prepared from RNA of cells stimulated with *E. coli* 0111:B4 LPS (100 pg/mL). Changes in cytokine expression were calculated by relative quantification against 18S rRNA by use of the ΔΔCT method and plain media used as the calibrator. Fold changes in gene expression among treatments for each sample were calculated as $2^{-\Delta\Delta CT}$.

Data analysis

Data were analyzed by fitting a logistic expression to concentration-response data by use of commercial software. This analysis allowed determination of IC_{50} and maximum response values with associated 95% CIs. The affinity constant for E5564 as an antagonist of the *E. coli* 0111:B4 LPS concentration–response curve was derived from Schild analysis.[22] Plots of the logarithm (dose ratio – 1) as a function of the negative logarithm of the concentration of the antagonist were analyzed by linear regression. The pA_2 values were determined from the intercept of the regression line with the x-axis on the Schild plots.[23] Gene expression of proinflammatory cytokines was analyzed by use of an unpaired *t* test with the Welch correction. Significance was set at $P < 0.05$. All data were reported as mean ± SE. Because of the horse-to-horse variation in LPS-induced production of TNF-α and expression of PCA, all data were reported as a percentage of the maximal response to LPS.

RESULTS

Effects of *E. coli* LPS and E5564 in equine whole blood

Activity and protein production of TNF-α in whole blood incubated with *E. coli* 0111:B4 LPS increased significantly in all horses (data not shown), whereas E5564 at final concentrations of 10, 100, and 1,000nM did not stimulate TNF-α activity. Mean ± SE TNF-α activity in whole blood incubated with E5564 was not significantly different from values for DPBSS (DPBSS, 4.6 ± 0.8 units/mL; 10nM E5564, 3.8 ± 0.6 units/mL; 100nM E5564, 4.5 ± 0.8 units/mL; and 1,000nM E5564, 3.5 ± 0.7 units/mL); however, it did differ significantly ($P = 0.04$) from values obtained with *E. coli* 0111:B4 LPS at 1 ng/mL (19.2 ± 0.8 units/mL). To assess the ability of E5564 to antagonize LPS-induced TNF-α production, whole blood was coincubated with these compounds. Analysis of the results of these experiments indicated that E5564 inhibited LPS-induced TNF-α protein production in a concentration-dependent manner, with a calculated IC_{50} value of 0.9nM (95% CI, 0.4 to 1.9nM) (Figure 7.1).

Effects of E5564 on isolated monocytes

Monocyte production of TNF-α protein and expression of PCA were increased by incubation with *E. coli* 0111:B4 LPS (Figures 7.2 and 7.3). Use of E5564 inhibited LPS-induced TNF-α protein production and PCA expression in isolated equine monocytes in a concentration-dependent manner, with calculated IC_{50} values of 4.6nM (95% CI, 1.7 to 12nM) and 4.4nM (95% CI, 1.8 to 11nM), respectively. Analysis of Schild plots of pooled LPS concentration–PCA response curves for 3 horses performed by use of 4 concentrations of E5564 yielded a pA_2 value

of 10.17 and a slope of 1.04 (Figure 7.4). Monocytes incubated with medium alone, E5564 alone, or vehicle did not express PCA (data not shown).

Effects of E5564 on expression of proinflammatory and anti-inflammatory cytokine mRNA

The concentrations of RNA isolated and the CT values for 18s rRNA did not vary significantly among samples (data not shown). Analysis of real-time qPCR dissociation curves revealed single products for TNF-α, IL-1ß, IL-6, and IL-10. The PCR amplification efficiency was approximately 100%, and all primers passed the validation experiment, which indicated that the efficiencies of the target amplification and reference (18S rRNA endogenous control sample) amplification were approximately equal. Expression of mRNA for TNF-α, IL-1ß, IL-6, and IL-10 were increased by incubation with *E. coli* 0111:B4 LPS (Figure 7.5). Use of E5564 significantly inhibited LPS-induced TNF-α mRNA expression at 1 hour, but the inhibition was not significant ($P = 0.06$) at 4 hours. In addition, E5564 significantly inhibited LPS-induced IL-1β mRNA expression at 1 and 4 hours. There was no significant effect of E5564 on LPS-induced IL-6 gene expression at 1 or 4 hours, although the pattern of modulation was similar to that for the other cytokines. The LPS-induced expression of mRNA for the anti-inflammatory cytokine IL-10 was significantly inhibited by E5564 at 1 hour, but the inhibition was not significant ($P = 0.07$) at 4 hours. There was no significant difference in cytokine mRNA expression between values obtained from monocytes incubated with E5564 alone and E5564 plus LPS at 1 or 4 hours.

DISCUSSION

To assess the therapeutic potential of E5564 in horses, we investigated the pharmacologic actions of this compound by monitoring the synthesis of TNF-α in equine peripheral whole blood and isolated monocytes, expression of PCA by monocytes, and alteration of mRNA expression for 3 proinflammatory and 1 anti-inflammatory mediator that play important roles in the response of horses to LPS.[24]

In contrast to results reported for LPS from *R sphaeroides* and E5531, a first-generation lipid A analogue, E5564 did not have agonist activity in equine whole blood or in isolated equine monocytes. Although E5531 and E5564 have similar structures, E5564 lacks a secondary side chain at position 3′ and there is an extended side chain with a *cis*-double bond at position 2′.[18] The mechanisms responsible for the agonist activity of E5531 in equine whole blood are unknown, but it is possible that the activity of the drug may be modified in whole blood as result of deacylation.[15]

In one study, it was reported that neither E5664 nor its vehicle stimulated production of TNF-α after IV injection in mice, guinea pigs, and rats.[25] In another study, it was reported that E5564 was safe for use in healthy male humans who received a 30-minute IV infusion of 0.35 to 3.5 mg.[26] In the study reported here, we determined that E5564 at all concentrations evaluated (10 to 1,000nM) did not stimulate TNF-α production in equine whole blood, nor did it induce expression of PCA in monocytes for a similar concentration range (0.1 to 1,000nM). The IC_{50} values identified in this study for inhibition of LPS-induced TNF-α protein production in equine whole blood (0.9nM) and supernatants of monocytes (4.6nM) and for expression of PCA by monocytes (4.4nM) were similar to those reported for inhibition of TNF-α production by human

(1.6nM) and murine (20nM) cells.[25] Collectively, these findings suggest that E5564, in contrast to other LPS antagonists, should be safe for administration to horses, although in vivo studies will need to be performed before this compound can be used in the treatment of horses with endotoxemia.

To test the ability of E5564 to prevent expression of a membrane-bound proinflammatory mediator, we evaluated the effect of the compound on LPS-induced PCA expression in monocytes. Procoagulant activity, which is also called tissue factor or thromboplastin, reflects the ability of stimulated cells to decrease the coagulation time of plasma.[20,27] When LPS is included, PCA expression is increased by circulating cells (particularly monocytes) and endothelial cells.[28] The importance of PCA in horses was exemplified by the fact that an increase in monocyte PCA has been significantly associated with coagulopathy and poor prognosis in horses with colic.[29] Furthermore, activation of coagulation by increased PCA of cells initiates pathologic responses, such as disseminated intravascular coagulation, which is important in the pathogenesis of sepsis-associated organ injury.[30,31] Our finding that E5564 significantly reduced expression of PCA by equine monocytes incubated with LPS suggested that this compound may be of use in endotoxemic horses at increased risk for development of coagulopathy.

In the study reported here, TNF-α activity and TNF-α protein production were measured with an in vitro cytotoxicity bioassay and ELISA, respectively. In the first experiment performed, the bioassay was used to evaluate E5564 at concentrations ≤ 1,000nM for potential agonist activity in whole blood. Once it was determined that the compound lacked significant agonist activity in that concentration range, experiments were then designed to assess the ability of E5564 to antagonize the effects of LPS. Because of the labor-intensive nature of the bioassay,

those experiments were performed with a newly established ELISA for equine TNF-α. To ensure a sufficient number of data points from which to calculate an IC_{50} value for E5564, the concentration range was extended to include 10,000nM. Because E5564 at 10,000nM (when LPS was included) did not result in production of TNF-α as determined by the ELISA, it is highly unlikely that this concentration would have agonist effects on its own.

In the subsequent experiments, isolated equine monocytes were incubated with E5564 before LPS was added. On the basis of the encouraging results obtained with this study design, additional experiments were then performed in which LPS and E5564 were added simultaneously to whole blood samples in an effort to more closely simulate events in some clinical settings. Although it would have been optimal to measure other cytokine proteins, there are no validated equine cytokine protein assays other than the one for TNF-α. Although the horse-to-horse variability in response to LPS was large (5- to 30-fold, depending on the mediator), the inhibitory effects of E5564 were consistent. This variation in response to LPS is in keeping with the results of other studies[32,33] on the effects of LPS in horses.

Tumor necrosis factor-α is a central mediator synthesized in response to LPS.[34] As such, it induces production of inflammatory factors (such as IL-1ßand IL-6), activates inflammatory cells, increases expression of adhesion molecules, and increases production of nitric oxide and reactive oxygen species.[35] Increases in serum concentrations of TNF-α are detected in septic neonates and in horses with colic.[36,37] Therefore, TNF-α is often used as a reliable, representative indicator of activation of inflammatory cells.[24] The biological activity of IL-1ß is similar to that of TNF-α because both induce the systemic inflammatory response syndrome. Interleukin-6 is responsible for inducing the synthesis of acute-phase proteins[2,35] and can have anti-inflammatory properties.[38] Interleukin-10 is an anti-inflammatory cytokine that inhibits production of TNF-α,

IL-1ß, and IL-6.[39] Increases in concentrations of IL-10 have been reported in patients with sepsis and humans with experimentally induced endotoxemia.[40-42]

In the study reported here, E5564 clearly prevented *E. coli* LPS-induced expression of mRNA for TNF-α, IL-1ß, and IL-10. These findings are consistent with the fact that E5564 inhibits intracellular production of TNF-α and IL-6 in LPS-stimulated human monocytes, as assessed by flow cytometry.[43] Of the 4 genes monitored in our study, expression of IL-6 and IL-10 in response to LPS varied most among the horses. Three of 5 horses had modest increases in IL-6 and IL-10 mRNA in response to LPS, whereas the other 2 horses expressed considerably more IL-6 and IL-10 mRNA. Despite this inherent horse-to-horse variability in gene expression in response to LPS, E5564 reduced expression of mRNA for all 4 cytokines in all horses to values that were indistinguishable from those identified for monocytes incubated in E5564 alone. The 2 time points (1 hour and 4 hours) were chosen to evaluate early gene response (TNF-α) and a later gene response (IL-1ß, IL-6, and IL-10), similar to studies conducted by others.[44-46] Additional studies would be needed to determine whether E5564 prevents *E. coli* LPS-induced expression of mRNA for TNF-α, IL-1ß, IL-6, and IL-10 at later time points.

Treatment of patients with endotoxemia involves IV administration of fluids and use of nonsteroidal anti-inflammatory drugs and antimicrobials.[47] The use of nonsteroidal anti-inflammatory drugs in horses has potential adverse effects, such as development of gastrointestinal ulcers and renal papillary necrosis.[48] The use of antimicrobials in patients with endotoxemia is controversial. Synthetic lipid A analogues inhibit LPS interaction with cells and prevent initiation of intracellular signaling pathways. Therefore, concurrent treatment with a lipid A antagonist could halt or reduce undesired inflammatory responses during endotoxemia. Other treatments with similar activity used in horses with endotoxemia are polymyxin B and

anti-LPS hyperimmune plasma. Polymyxin B is a polycationic antimicrobial that has the ability to bind and neutralize LPS. Studies have revealed beneficial effects of polymyxin B in horses with endotoxemia;[49,50] however, the use of hyperimmune plasma has yielded conflicting results.[51.52]

Analysis of results of the study reported here indicated that E5564 lacked agonist activity in equine whole blood and monocytes and was a potent antagonist of enteric LPS. Furthermore, E5564 inhibited LPS-induced expression of PCA; TNF-α protein production; and mRNA expression of TNF-α, IL-1ß, and IL-10. Therefore, E5564 appears to have potential as an effective therapeutic agent in horses with endotoxemia.

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Table 7.1. Forward and reverse primers used for the detection of mRNA for equine TNF-α, IL-1ß, IL-6, and IL-10 by use of a real-time qPCR assay.

Cytokine	Genbank accession No.	Primer	Sequence (5′ to 3′)	Predicted size (bp)
Equine TNF-α	AB035735	Forward	AAAGGACATCATGAGCACTGAAAG	82
		Reverse	GGGCCCCCTGCCTTCT	
Equine IL-1ß	ECU92481	Forward	ATGACTTACTGCAGCGGCAAT	84
		Reverse	GTCTTGGAAGCTGCCCTTCA	
Equine IL-6	ECU64794	Forward	TGCTGGCTAAGCTGCATTCA	81
		Reverse	GGAAATCCTCAAGGCTTCGAA	
Equine IL-10	U38200	Forward	GCCTTGTCGGAGATGATCCA	101
		Reverse	TTTTCCCCCAGGGAGTTCAC	

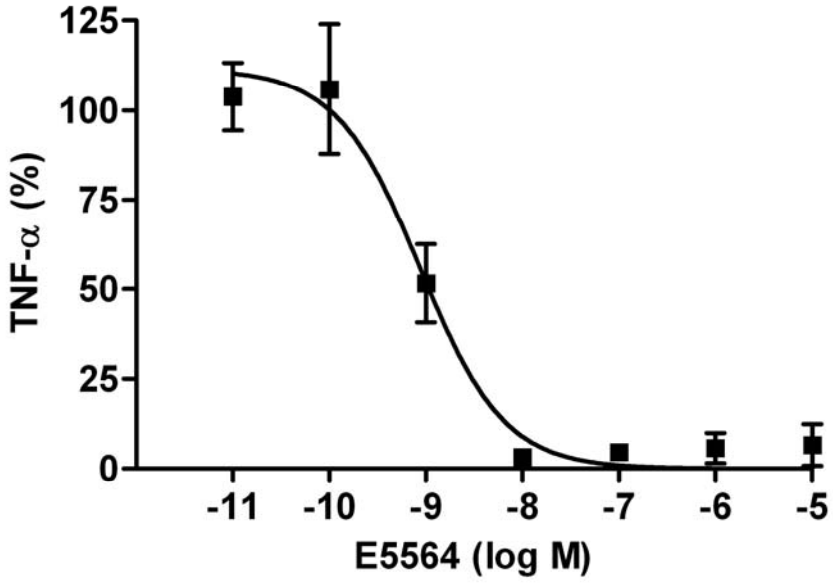

Figure 7.1 Production of TNF-α protein in equine whole blood incubated with *Escherichia coli* 0111:B4 LPS (300 pg/mL) and the second-generation synthetic lipid A analogue E5564 (10pM to 10 μM). Results are expressed as the mean ± SE percentage of the maximal response to *E. coli* LPS (assigned a value of 100%) for samples obtained from 7 horses. The IC_{50} is 0.9nM (95% CI, 0.4 to 1.9nM).

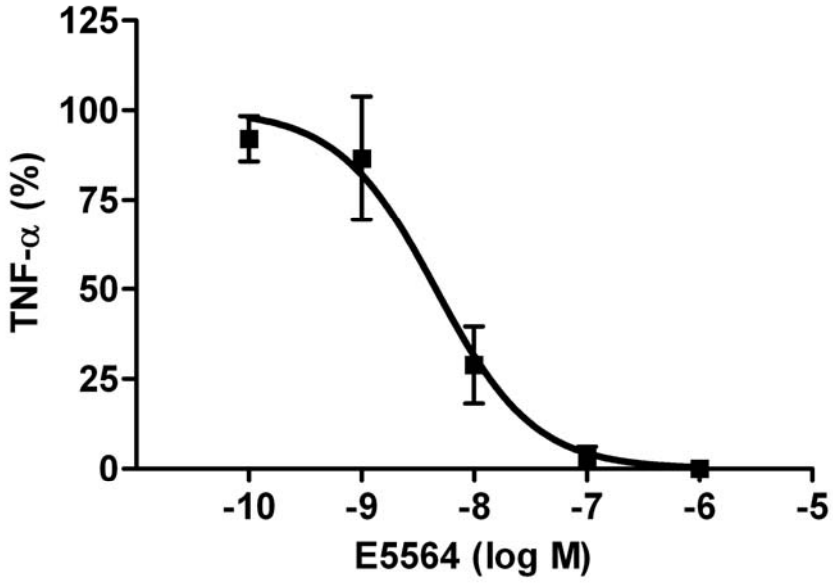

Figure 7.2 Production of TNF-α protein in supernatants of monocytes incubated with *E. coli* 0111:B4 LPS (100 pg/mL) and E5564 (0.1nM to 1μM). Results are expressed as the mean ± SE percentage of the maximal response (ie, 100%) to *E. coli* LPS for samples obtained from 6 horses. The IC_{50} is 4.6nM (95% CI, 1.7 to 12nM).

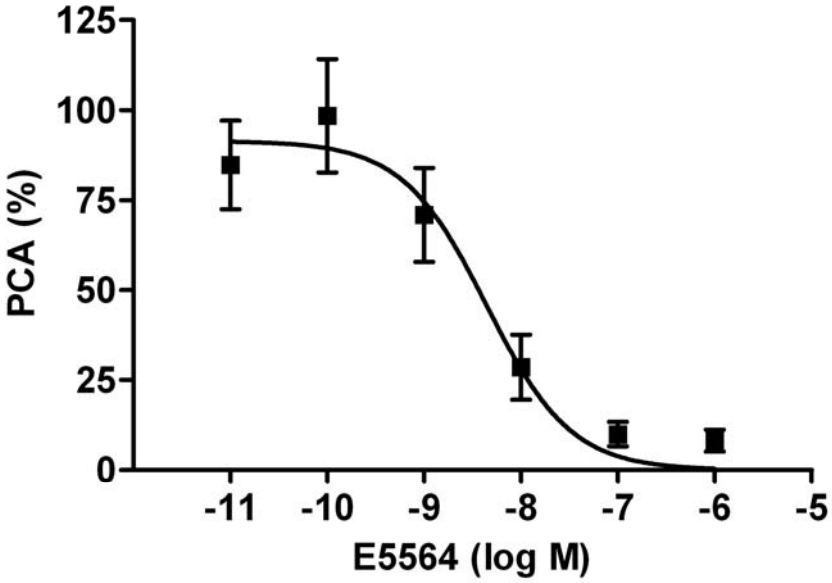

Figure 7.3 Expression of PCA by monocytes coincubated with *E. coli* 0111:B4 LPS (100 pg/mL) and E5564 (10pM to 1μM). Results are expressed as the mean ± SE percentage of the maximal response (ie, 100%) to *E. coli* LPS for samples obtained from 19 horses. The IC_{50} is 4.4nM (95% CI, 1.8 to 11nM).

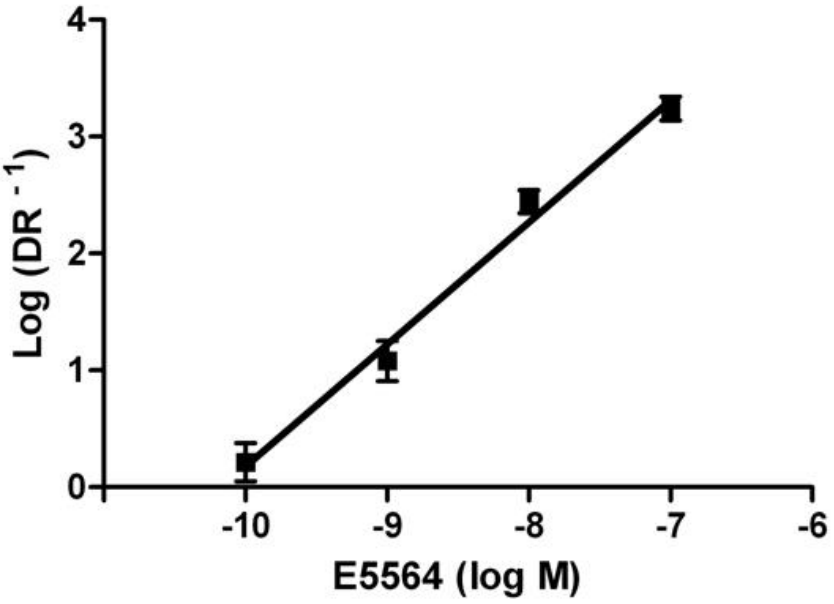

Figure 7.4 Schild plot of the effect of various concentrations of E5564 (0.1 to 100nM) on inhibition of LPS-induced PCA on monocytes obtained from 3 horses. In this plot, dose ratio (DR) is calculated from EC_{50} values determined for LPS in monocytes incubated with and without E5564. The value for pA_2 derived from these analyses was 10.17 (95% CI, 11.0 to 9.67) with a slope of 1.04.

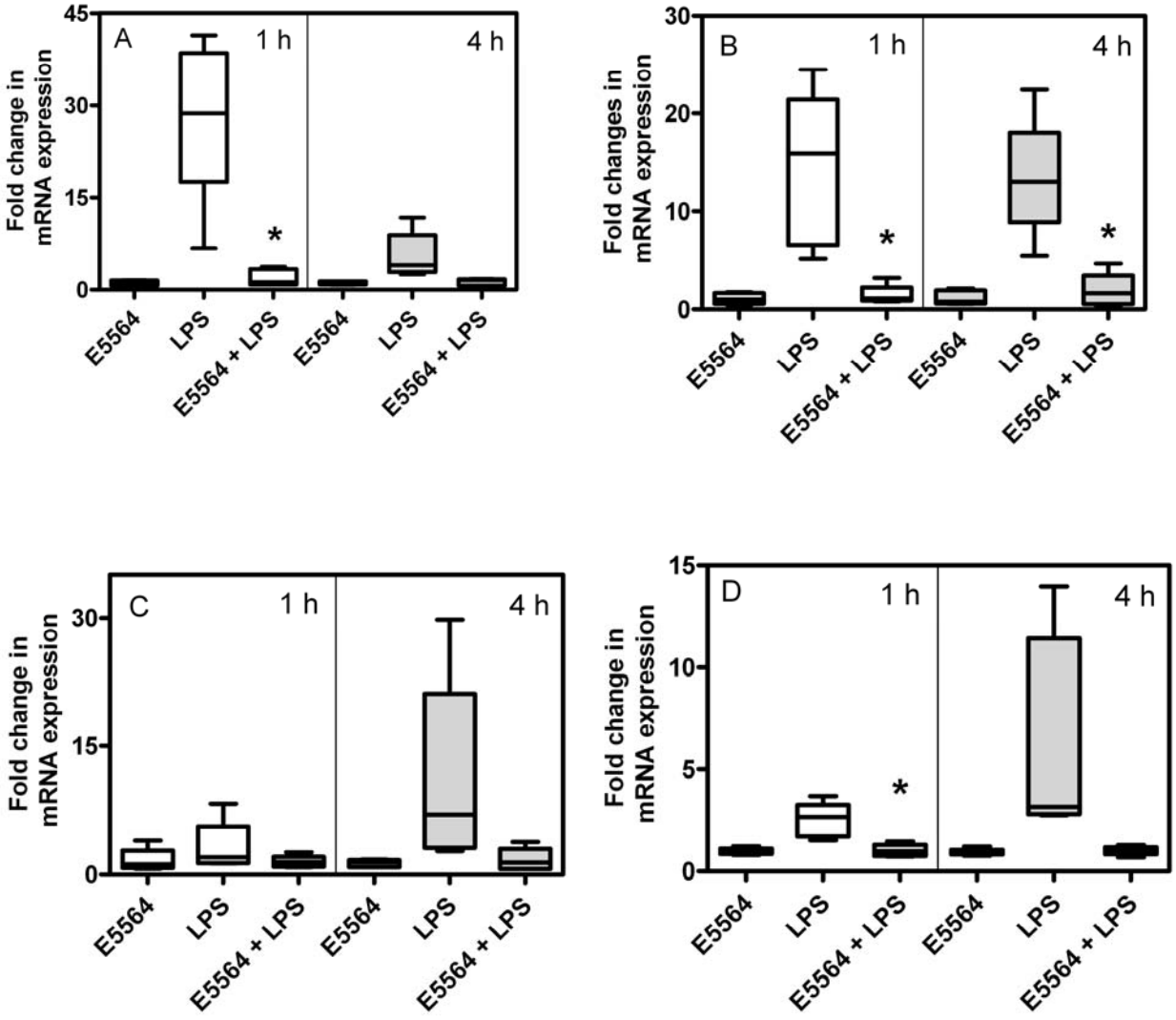

Figure 7.5 Effects of E5564 on LPS-induced expression of genes for TNF-α (A), IL-1ß (B), IL-6, (C), and IL-10 (D) at 1 and 4 hours of incubation in samples obtained from 5 horses. Results are expressed as the fold change in mRNA expression from that for medium alone. The horizontal bar within each box represents the median value; the bottom and top of each box represent the first and third quartiles, respectively; and the bars represent the range of the data. *Within a time point, the value differs significantly ($P < 0.05$) from the value for LPS.

CHAPTER 8

CONCLUSION

The main purposes of the studies in this dissertation project were: (1) to identify an optimal source of lipopolysaccharide-binding protein (LBP) for incubation of equine monocytes with lipopolysaccharide (LPS); (2) to validate a reliable set of primer pairs to measure gene expression by real-time quantitative RT-PCR in equine monocytes; (3) to investigate the responses and the differential signaling pathways utilized by equine monocytes incubated with specific Toll-like receptor (TLR) ligands; (4) to investigate the responses of equine monocytes to four structurally distinct *E. coli* lipid *A* compounds, and (5) to evaluate the effects of a specific TLR4 antagonist on responses of equine monocytes and whole blood to LPS.

Lipopolysaccharide-binding protein is an acute phase protein that binds the lipid A moiety of LPS and transfers LPS monomers to soluble CD14 in plasma or membrane bound CD14 on mononuclear phagocytes.[1,2] The end result of these interactions is activation of the TLR4 receptor complex, and the synthesis and release of inflammatory mediators. Inclusion of LBP purified from acute phase serum in cellular assays increases the sensitivity of cells expressing CD14 to LPS by more than 100-fold.[3] Although a previous study performed in our laboratory determined that equine monocytes respond strongly to fetal bovine serum by expressing procoagulant activity, this response can be circumvented in most situations by heat-inactivating the serum.[4] However, because monocytes from some horses are activated by the heat-inactivated serum, we compared five different sera to identify an ideal source for LBP

activity for studies on the effects of LPS on equine monocytes. The five serum preparations tested were: heat-inactivated fetal bovine serum, pooled commercial equine serum, heat-inactivated pooled commercial equine serum, autologous equine serum, and heat-inactivated autologous equine serum. The results of this study indicated that pooled commercial and autologous equine sera are excellent sources of LBP activity for studies being performed to elucidate the effects of LPS on equine monocytes. Furthermore, these sera do not stimulate equine cells in the absence of LPS, even when the sera are not heat-inactivated. The latter finding is in contradiction to the effects of fetal bovine serum, which significantly increases expression of procoagulant activity by equine mononuclear cells when not heat-inactivated.[4] An unexpected finding in the present study was that heat inactivation of autologous equine serum completely eliminated its effectiveness as a source of LBP activity, precluding its further use. Additional experiments were performed to identify the lowest concentration of LPS at which LBP activity would be evident. Results from this experiment indicated that significant LBP activity was evident at concentrations of LPS as low as 30 pg/mL. This finding has direct relevancy to clinical situations as similar plasma concentrations of LPS have been detected in horses with naturally occurring gastrointestinal diseases.[5,6] In concordance with the results of other studies, the incubation time required for maximal expression of PCA by LPS stimulation of monocytes with CES was six hours.[7]

In the second study in this dissertation, we established a straight-forward system to validate equine primer pairs for measuring gene expression by real-time reverse transcription quantitative PCR assay using the DNA binding dye, SYBR Green as a detection method. The steps included in our validation system were the following: (1) design primer pairs; (2) optimize primer concentration; (3) detect a single amplicon and amplification; (4) document PCR

efficiency of approximately 98%; and (5) generate equivalent efficiencies of target gene and housekeeping gene. In this study, we validated twenty-nine equine primer pairs for genes known to be involved in inflammation and immune responses (Table 8.1). All primer pairs were designed using computer software, with equine sequences available from the GenBank database. The optimal primer concentration established for the forward and reverse primers was 300 nM. Each primer pair yielded a single specific PCR product as determined by dissociation curve analysis and gel electrophoresis. For the majority of the primer pairs, the PCR standard curve efficiencies derived from LPS-stimulated monocytes generated a PCR efficiency of approximately 100%. However, for genes with low expression in response to LPS, such as ß-defensin, IFN-ß, IFN-γ, and TLR3, the apparent efficiencies ranged from 175 to 472%. Therefore, monocytes were incubated with PMA to yield increased expression of ß-defensin and IFN-γ, and with Poly I:C for IFN-ß and TLR-3. These agents, acting through different pathways than LPS, increased the level of gene expression and yielded good amplification efficiencies for these genes. Because it is essential that the efficiencies of target gene and housekeeping gene are equivalent for a valid quantitative assay using the comparative C_T method, we selected the eukaryotic 18S rRNA as our housekeeping gene. In agreement with other studies, there was limited change in expression of this housekeeping gene under different experimental conditions, and the PCR efficiencies for the majority of the target genes were equivalent to that of the housekeeping gene in all experiments. The availability and the reliability of these validated primer pairs were pivotal for monitoring responses of equine monocytes to pro-inflammatory stimuli in the other studies comprising this dissertation.

In the third study, we investigated the differential induction of the MyD88- and TRIF-dependent signaling pathways by TLR agonists in equine monocytes. Since the discovery of

TLRs less than 10 years ago, considerable progress has been made regarding their importance in the innate immune system. The TLRs provide a system for sensing dangerous pathogens, and initiating inflammatory and immune responses to protect the host.[8] These receptors do this by recognizing conserved molecular motifs of microbes and by eliciting effective defensive responses through specific adaptor molecules, most notably MyD88 and TRIF.[9] The recruitment of MyD88 is essential for the production of pro-inflammatory cytokines such as TNF-α, IL-1ß and IL-6 and the anti-inflammatory cytokine IL-10.[10] Genes that are dependent on TRIF activation include IFN-ß, CCL5, IP-10, and TRAF1.[11] The main objective of this study was to characterize responses of equine monocytes to TLR2, TLR3 and TLR4 ligands, and to determine the specificity of TLR ligand-induced gene expression via the MyD88-dependent pathway, the TRIF-dependent pathway, or a combination of the two pathways.

The results of this study indicated that the TLR4 (*E. coli* LPS) and TLR2 (Pam_3CSK_4) ligands reported to signal via MyD88, induced similar gene expression profiles, except for TNF-α which was significantly increased at 1hr in cells incubated with LPS but not with Pam_3CSK_4. In contrast, the TLR3 ligand (Poly I:C) reported to signal through TRIF, induced significantly higher expression of IFN–ß, IP–10 and CCL5 than either the TLR2 or TLR4 ligands (Table 8.2). Although MyD88 is a prominent adaptor molecule in TLR4 signaling, MyD88-independent pathways, in particular those that utilize TRIF, also have been described for TLR4 ligands.[11] Therefore, additional experiments were performed using high concentrations of *E. coli* LPS to determine if equine TLR4 could induce TRIF-dependent gene expression. However, incubation of equine monocytes with high concentrations of LPS did not induce significant expression of TRIF-dependent genes. These findings suggest that LPS is at best weakly coupled to TRIF mediated gene expression events in equine monocytes. The results of this study suggest that

equine monocytes utilize a common intracellular pathway in response to TLR2 and TLR4 ligands, but a distinct pathway in response to TLR3 ligands (Figure 8.1). Furthermore, ligand-induced expression of IL-1ß, TNF-α, IFN-ß, IP-10 and RANTES can be used to discriminate between MyD88 and TRIF dependent cellular responses in horse monocytes. Additional experiments were performed to investigate the effects of two-aminopurine (2-AP) on Poly I:C-induced TRIF dependent genes in equine monocytes. Studies have shown that 2-AP inhibits LPS-induced IFN-ß production by preventing TRIF-dependent signaling.[12] Although 2-AP significantly inhibited Poly I:C induced expression of TRIF dependent genes, additional studies are warranted to determine whether 2-AP is a specific inhibitor of the TRIF-dependent pathway in equine monocytes.

After characterizing the responses of equine monocytes to specific TLR2, 3 and 4 agonists, we further investigated the response of equine monocytes to four structurally distinct TLR4 agonists. The four lipid A compounds investigated, which were derived from *E. coli* LPS, were natural lipid A and three synthetic compounds lacking the KDO moiety of natural lipid A. The three synthetic compounds were lipid A with long fatty acids, lipid A with short chain fatty acids and lipid A with only one phosphate group. The results of this study indicated that natural lipid A and the three synthetic lipid A compounds all signal through the TLR4 receptor complex and utilize the MyD88-dependent pathway in equine monocytes. The natural lipid A, which contained KDO, induced significantly greater expression of inflammatory cytokines than the synthetic compounds that lacked KDO. Furthermore, differences in the length of the fatty acid chains attached to the lipid A backbone induced different levels of biological responses by equine monocytes. Collectively, these findings indicated that components of the lipid A core and the acylation pattern are important factors in initiation of biological responses by monocytes.

Finally, the fifth study in this dissertation project evaluated the effects of a specific TLR4 antagonist, E5564, on responses of equine monocytes and whole blood to LPS. Although endotoxemia is a leading cause of morbidity and fatalities in adult horses and foals, current methods of treatment that address the cause of endotoxemia are limited. The treatment options include use of antiinflammatory drugs, antibodies directed against specific regions of the LPS molecule, or endotoxin-binding drugs such as polymyxin B. Treatment with synthetic TLR4 antagonists provides another level of inhibition, by inhibiting the interaction of LPS with the TLR4 receptor complex, and preventing initiation of intracellular signaling pathways.[13] Although other studies failed to demonstrate antagonistic effects of other lipid A molecules on LPS stimulated equine monocytes,[14,15] we determined that the synthetic lipid A analogue, E5564, inhibited LPS-induced procoagulant activity and TNF-α production in a concentration-dependent manner, while inducing minimal pro-inflammatory effects in equine whole blood or monocytes by itself. Furthermore, E5564 significantly inhibited LPS-induced mRNA expression of TNF-α, IL-1ß and IL-10 and decreased LPS-induced expression of IL-6. The results of this study support the conclusion that E5564 is the first lipid A analogue that has potential as an effective therapeutic agent in horses with endotoxemia. Further studies using *ex vivo* and *in vivo* models are warranted to further investigate the therapeutic potential of E5564 in equine endotoxemia.

In conclusion, differential gene expression is induced by distinct TLR ligands in equine monocytes, and can serve as an excellent model for dissecting the effects of ligands that function via the MyD88 or TRIF dependent pathways. Furthermore, the results of the studies performed in this dissertation project suggest that TLRs and their associated adapter molecules should be considered potential targets in the development of agonists and antagonists for the treatment of various diseases, and as potential adjuvants in vaccine development. The emerging research

within the field of TLRs will increase our ability to fully understand the complex TLR signaling and its significant role in host immune responses and disease pathogenesis.

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Table 8.1. Validated equine genes for real-time quantitative PCR

Gene	Function
ß-defensin	Antimicromial peptide
Cathelicidin 2	
L-selectin	Cell adhesion molecule
MD-2 (myeloid differentiation factor-2)	Cell surface receptor
CD44	
TLR2 (Toll-like receptor 2)	
TLR3	
TLR4	
CCL5 (RANTES)	Chemokine
IL-8 (interleukin-8)	
IP-10 (interferon-inducible protein 10)	
MCP-3 (macrophage chemoattractant protein-3)	
IFN-ß (interferon-ß)	Cytokine
IFN-γ	
IL-1ß	
IL-6	
IL-10	
IL-12	
IL-1RA (interleukin-1 receptor antagonist)	
TNF-α (tumor necrosis factor-alpha)	
COX-2 (cyclooxygenase-2)	Enzyme
HYAL 1 (hyaluronidase)	
HYAL 2	
MMP9 (matrix metalloproteinase 9),	
IFIT1 (interferon-induced protein with tetratricopeptide repeats 1)	Mediator of signal transduction
TRAF1 (TNF receptor-associated factor 1)	
PAI1 (plasminogen activator inhibitor-1)	Serine protease inhibitor
SOD2 (superoxide dismutase 2)	Superoxide dismutase
TGF-ß (transforming growth factor beta)	Growth factor

Table 8.2. The peak and level of mRNA expression of TNF-α, IL-1ß, IL-6, IL-10, IFN-ß, IP-10, RANTES and TRAF1 in equine monocytes incubated with incubated with *E. coli* LPS (100 pg/ml), Pam_3CSK_4 (30 ng/ml) and Poly I:C (250 μg/ml) for 1, 4, 8, 12 and 20 hours.

	Ligands		
Genes	**TLR2 (Pam_3CSK_4)**	**TLR4 (*E. coli* LPS)**	**TLR3 (Poly I:C)**
MyD88-dependent			
TNF-α	+ 1 h	+ + 1 h	+ 20 h
IL-1ß	+ + 20 h	+ + 20 h	+ 20 h
IL-6	+ + 20 h	+ + 20 h	+ + 20 h
IL-10	+ + 4 h	+ + 4 h	+ + 20 h
TRIF-dependent			
IFN-ß	+ 1 h	+ 1 h	+ + 20 h
IP-10	+ 4 h	+ 1 h	+ + 20 h
RANTES	+ 1 h	+ 8 h	+ + 20 h
TRAF1	+ 1 h	+ + 20 h	+ 20 h

+: less than 1 log fold change; + +: greater than 1 log fold change

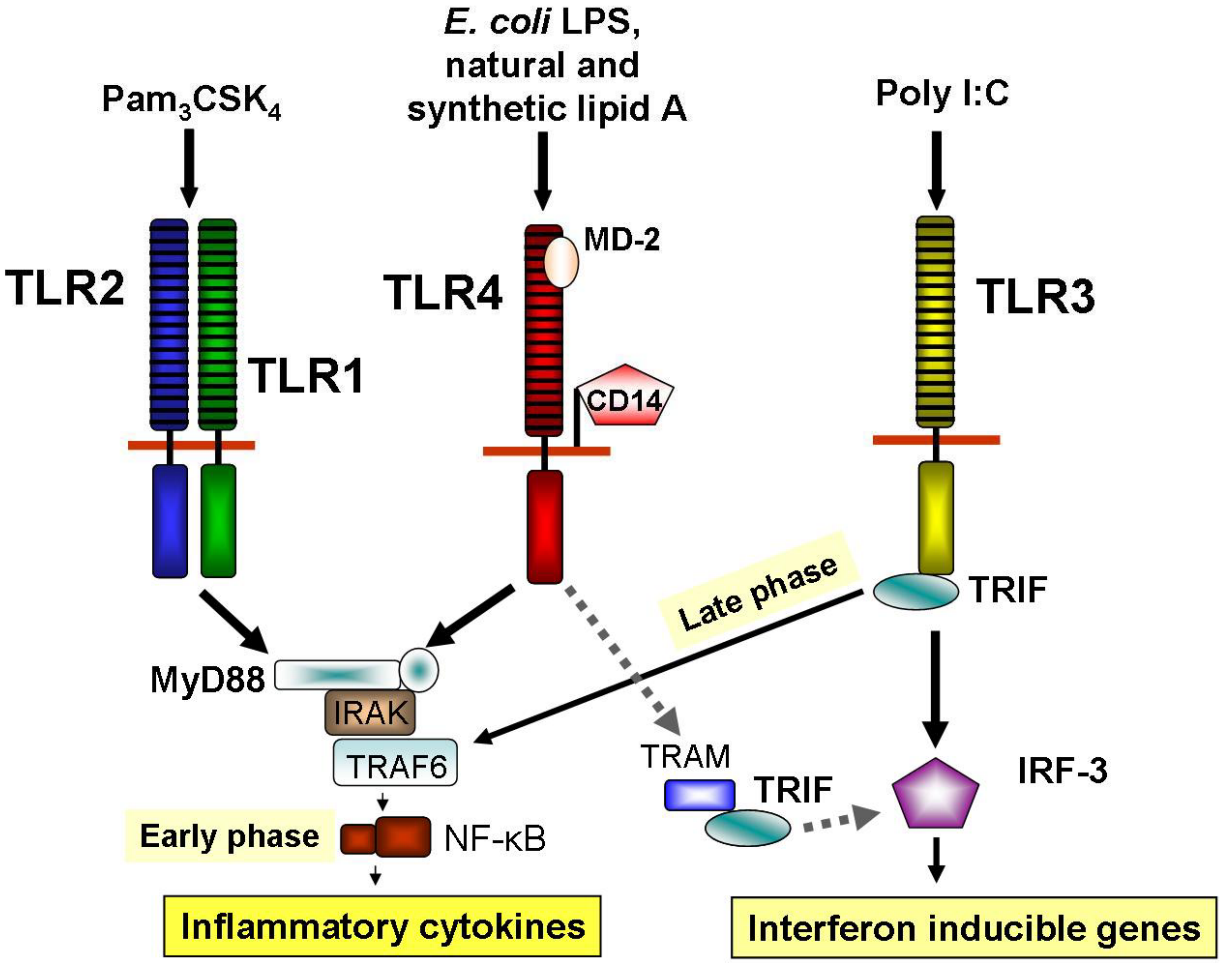

Figure 8.1 Proposed TLR intracellular signaling in equine monocytes. The solid black line represents the pathways induced by TLR ligands in equine monocytes. The dashed grey line represents a pathway that does not seem to be utilized in equine monocytes.

APPENDIX A

INHIBITION OF TRIF-DEPENDENT GENE EXPRESSION BY 2-AMINOPURINE

INTRODUCTION

Two-aminopurine (2-AP) is a specific inhibitor of double stranded RNA dependent protein kinase (PKR), a serine/threonine kinase induced by type I IFNs, viruses and double-stranded RNA.[1] Upon binding to double-stranded RNA generated by viruses, PKR is activated by dimerization and autophosphorylation. This results in inhibition of protein synthesis, which blocks virus multiplication, induction of type I IFN genes and may ultimately lead to cell death by apoptosis (Figure 1).[2,3] The results of one study indicated that 2-AP also inhibits LPS-induced production of nitric oxide by RAW cells by preventing the production of IFN-ß.[4] The results of that study suggest that 2-AP inhibits LPS-induced IFN-ß production by preventing TRIF-dependent signaling rather than by altering MyD88-dependent signaling.[4] The objective of this pilot study was to determine the effects of 2-AP on TRIF-dependent alterations in gene expression induced by Poly I:C.

MATERIALS AND METHODS

Peripheral blood monocytes isolated from five horses were incubated in the presence or absence of 2-AP (4 mM) for 30 min before being stimulated with Poly I:C for 20 h. Real time

quantitative RT-PCR using SYBR Green as the detector was performed to measure mRNA expression for IFN–ß, IP-10, CCL5 and IL-1ß. Gene expression was measured using an Applied Biosystems 7900HT sequence detection system, with 18S ribosomal RNA used as anendogenous control. Changes in mRNA expression were calculated by relative quantification using the ΔΔCT method, with RPMI media being used as the calibration sample. Fold change for each sample were calculated as $2^{-\Delta\Delta CT}$, and data were analyzed using a one-way ANOVA and Bonferroni post-hoc test. Significance was set at $P<0.05$.

RESULTS

Pre-incubation of monocytes with 2-AP significantly reduced Poly I:C induced expression of IFN-ß, IP-10 and CCL5 (Figure 2), but did not significantly change Poly I:C induced expression of IL-1ß. Incubation of monocytes with 2-AP appeared to increase expression of the gene for IL-1β (fold change 7.8 ± 3.2), as values obtained for 2-AP in the presence or absence of Poly I:C were higher than those obtained after incubation of cells with Poly I:C alone (Figure 3). Incubation of cells with 2-AP did not induce expression of the other three genes.

DISCUSSION

Detection of double-stranded RNA via PKR recently has been described as a TLR-independent pathway for dendritic cell activation by viruses.[5] Upon binding to double-stranded RNA, PKR is activated by autophosphorylation, after which it phosphorylates the alpha subunit

of eukaryotic protein synthesis initiation factor 2. Phosphorylation of this factor results in rapid inhibition of translation and limited production of virions.[6] While it is widely recognized that 2-AP inhibits the autophosphorylation of PKR, a step that is necessary for its activation,[7] 2-AP has also been reported to be an inhibitor of TRIF-dependent intracellular signaling.[4]

This study demonstrated that 2-AP significantly inhibited Poly I:C induced expression of the TRIF dependent genes, IFN-β, IP-10 and CCL5 in equine monocytes. Although pre-incubation with 2-AP did not result in significant changes in Poly I:C induced IL-1ß expression, the apparent increase in expression of this gene in cells incubated with 2-AP warrant additional studies to determine if 2-AP can be used as a specific TRIF inhibitor.

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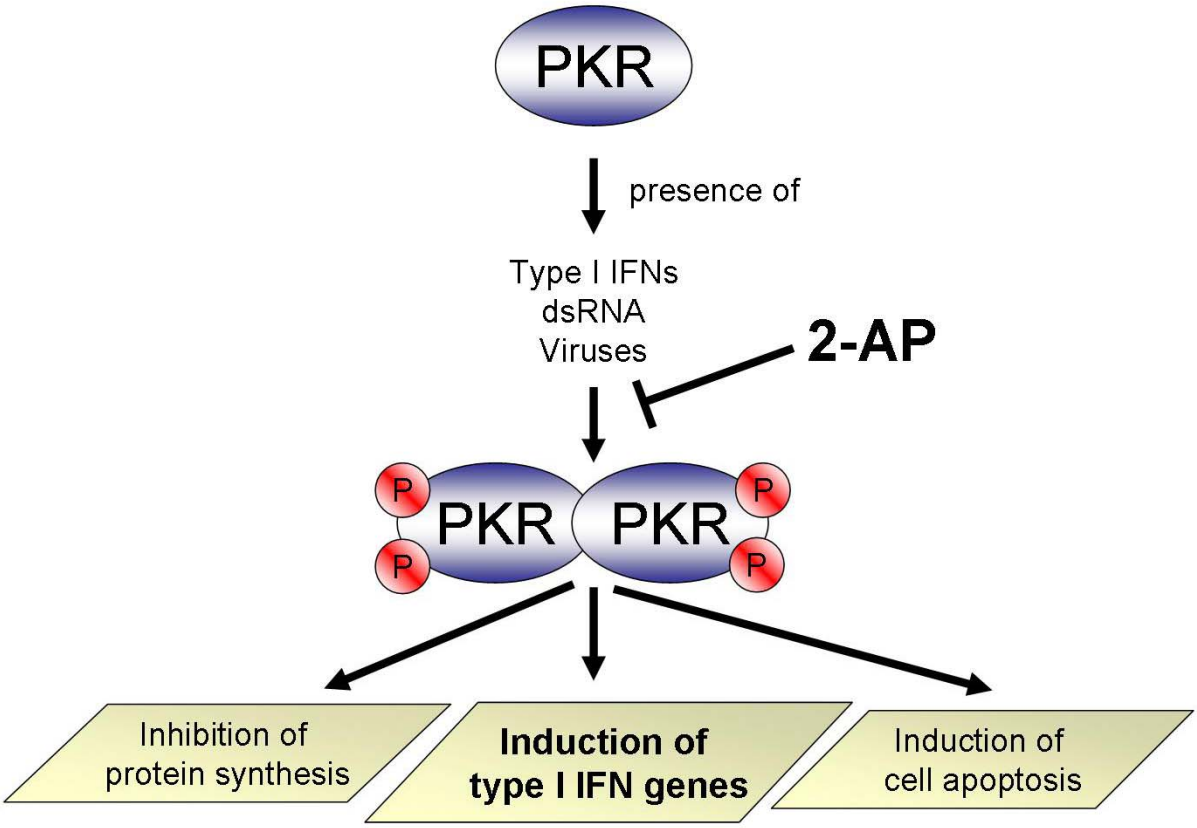
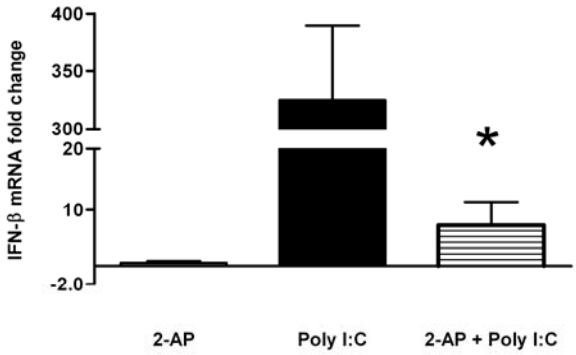


Figure A.1 The activation, function and inhibition of PKR. The intracellular presence of type I IFNs, dsRNA and viruses promotes the dimerization, autophosphorylation and activation of PKR. Activated PKR has been implicated in the regulation of the cellular antiviral response by preventing protein synthesis by phosphorylation of the translation initiation factor eIF2α, upregulating type I IFN gene transcription and inducing cellular apoptotis. PKR activation can be inhibited by 2-aminopurine (2-AP).

(A)



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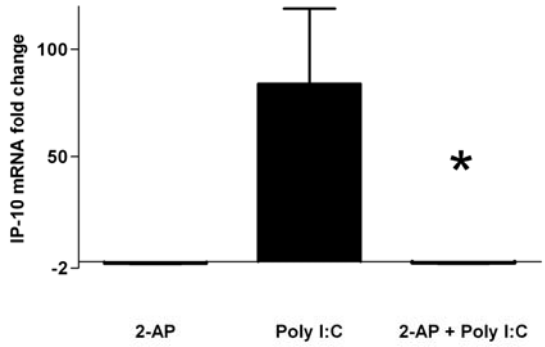

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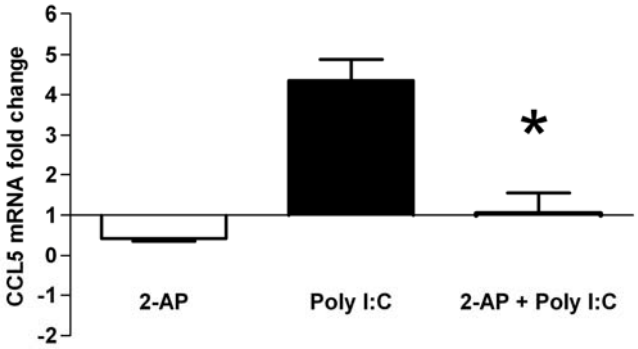

Figure A.2 The mean (± SEM) fold difference mRNA expression of IFN–ß (A), IP-10 (B) and CCL5 (C) in monocytes incubated with 2-AP (4 mM) (clear bars), Poly I:C (250 µg/mL) (dark bars), and 2-AP (4 mM) + Poly I:C (250 µg/mL) (dashed bars) for 20 hours; n= 5 horses.

* Denotes that 2-AP significantly inhibited IFN-ß, IL-1ß and CCL5 induced expression by Poly I:C ($P < 0.05$).

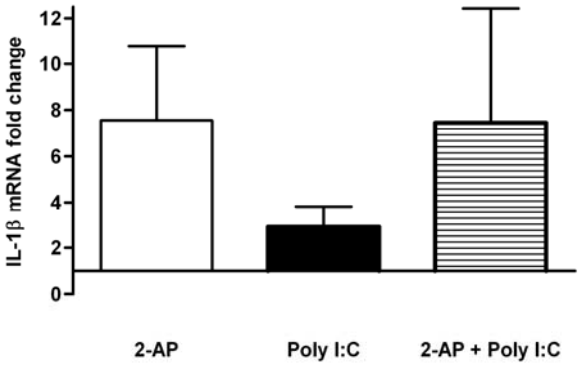

Figure A.3 The mean (± SEM) fold difference mRNA expression of IL–1ß in monocytes incubated with 2-AP (4 mM) (clear bars), Poly I:C (250 μg/mL) (dark bars), and 2-AP (4 mM) + Poly I:C (250 μg/mL) (dashed bars) for 20 hours; n= 5 horses.