DIFFERENTIAL SENSITIVITY OF EQUINE LEUKOCYTE POPULATIONS TO TOLL-LIKE RECEPTOR LIGANDS

by

SO YOUNG KWON

(Under the Direction of Thomas P. Robertson and James N. Moore)

ABSTRACT

Systemic inflammatory responses to microbial molecules contribute to disease pathogenesis and the development of life-threatening complications such as sepsis. Toll-like receptors function as sentinels for the innate immune system, detecting microbial ligands during infection and inflammation. In naturally occurring infections, multiple pathogen-associated receptors are likely to be activated during the initiation of the host response to infection. Therefore, it is important to understand the regulation of TLR signaling and interactions between the TLRs and their ligands. In the first study in this dissertation, we investigated the effects of ligands recognized by TLR 2, TLR 3 or TLR 4 upon expression of the genes encoding these receptor proteins by equine monocytes. We determined that incubation of monocytes with TLR 2 and 4 ligands, which signal through the intracellular

adaptor protein MyD88, induces expression of the TLR 2 and 4 genes, but not the TLR 3 gene. Conversely, incubation with a TLR 3 ligand, which recruits the TRIF adaptor protein, selectively induces expression of the TLR 3 gene, but not TLR 2 or 4 genes. Furthermore, incubation of these cells with TNF-α, the pro-inflammatory cytokine that is a hallmark of TLR activation, does not affect expression of any of the three TLR genes. In the second study, we investigated the responses of equine leukocytes to bacterial flagellin, the ligand recognized by TLR 5. Although equine neutrophils responded to both LPS and flagellin by producing reactive oxygen species and expressing mRNA for pro-inflammatory cytokines, flagellin had no stimulatory effect on monocytes or macrophages. While both neutrophils and monocytes expressed mRNA for TLR 5, the message appeared to be translated into protein only by the neutrophils. Incubation with neither LPS nor IFN-γ altered TLR 5 expression by the monocytes. These findings indicate that flagellin has disparate effects on neutrophils and mononuclear phagocytes isolated from horses, a species that is exquisitely sensitive to the TLR 4 ligand, LPS. Furthermore, equine mononuclear phagocytes, unlike corresponding cells of other mammalian species, lack surface expression of TLR 5 and do not respond to flagellin. Finally, we validated in-house ELISA assays to detect flagellin and antibodies directed against flagellin in serum samples obtained from hospitalized horses and foals. We determined that flagellin enters the circulation of horses with gastrointestinal diseases and septic foals, most likely due to intestinal barrier dysfunction. Furthermore, adaptive immune responses to flagellin were detected in horses with gastrointestinal disease that were reflected by increased levels of antibody directed against flagellin during hospitalization.

INDEX WORDS : Equine, Bacterial translocation, Colic, Sepsis, Leukocytes, Flagellin, Lipopolysaccharide, Toll-like receptors, Gene expression

DIFFERENTIAL SENSITIVITY OF EQUINE LEUKOCYTE POPULATIONS TO TOLL-LIKE RECEPTOR LIGANDS

by

SO YOUNG KWON

Doctor of Veterinary Medicine, Kon-Kuk University, Seoul, Korea, 2008

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2011

DIFFERENTIAL SENSITIVITY OF EQUINE LEUKOCYTE POPULATIONS TO TOLL-LIKE RECEPTOR LIGANDS

by

SO YOUNG KWON

Major professor	Thomas P. Robertson
Co-major professor	James N. Moore
Committee	Michel L. Vandenplas
	David J. Hurley
	Michelle H. Barton
	John F. Peroni

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
December 2011

DEDICATION

To my parents, for believing in me and supporting me to accomplish my dreams

To my husband, Byoung-Yun, for standing by me despite my imperfections

To my cat, Gonyang

ACKNOWLEDGEMENTS

I am grateful to many people for helping, in one way or other, with the writing of this dissertation. I realized I could not put into words how much I thank to those whom I owe the most.

I have been very fortunate with my mentors, Drs. Tom Robertson and Jim Moore. It would be hard to overstate how much I benefited from their deep insight, invariably correct intuitions, and untiring support. I truly cannot thank both of them adequately for all the effort they made to understand me and to bring out the best in me.

I am also very grateful to Dr. Michel Vandenplas for his incredible support on all dissertation and my graduate work. He also has been friend who smoothed the way for many of my adventures in academia. I owe a lot to Dr. David Hurley for helping me to understand principles of various assays as well as giving me invaluable practical tips to perform experiments. I am also very happy to have the opportunity to express my deep appreciation to Dr. Michelle Barton. She was constant source of inspiration, feedback, and support in and out of the academic process. During the writing she responded to my twists and turns with valuable suggestions. Special thanks also go to Dr. John Peroni for his practical suggestions during my graduate work. All my committee members's contributions made a significant difference to the overall shape of dissertation.

I am also grateful to Drs. Monica Figueiredo, Erik Noschka, and Wanchun Sun for their help in my earlier training and sincere friendship.

Warm thanks and sincere appreciation go to all building 11 family; Natalie Norton, Londa Berghaus, Kate Hurley, and Tracy Sturgill for providing me with invaluable help, encouragement, and friendship. It was always good time at a lab, barn, field, or waffle house at midnight because I was with funny and sweet friends like them. I also have been fortunate to come across my friend, Stephen Chelko. He walked with me throughout the last four years of this journey. I thank him for keeping me afloat in rough seas of classes and research during this graduate study. Special thanks also go to Sarah Ricco for her friendship.

I would like to also thank my mentors in Korea; Drs. Yun-Seo Park and Kye-Myung An for teaching me enthusiasm for clinical medicine, as well as their endless support.

On a personal note, I must of course thank my parents for all support beyond measure. My husband, Byoung-Yun, we went this adventure through together, and it would have been impossible to finish without his love. Also, special thanks go to my cat, Gonyang.

I would also like to acknowledge the financial, academic and technical support provided by both Physiology and Large Animal Medicine departments of College of Veterinary Medicine, University of Georgia.

TABLE OF CONTENTS

TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION

Among domestic animal species, horses suffer most commonly from colic, a general term indicating abdominal pain. The anatomy of the horse's digestive tract, its digestive physiology, and the management practices imposed by man contribute to the high occurrence of this disease. Very often, diverse forms of colic as well as other important equine clinical conditions, such as bacterial pneumonia, endometritis, and neonatal sepsis, have the potential to progress to sepsis, multi-organ dysfunction syndrome, and septic shock. These latter complications cause substantial morbidity and mortality despite advances in prevention and treatment. Although sepsis is defined as the systemic inflammatory response to infection, an emerging body of evidence indicates that widespread systemic inflammation can be triggered by many disease states, including infection. The clinical state of an uncontrolled or incongruous widespread inflammation is referred to as the systemic inflammatory response syndrome (SIRS).

Furthermore, the detection of endotoxin (lipopolysaccharide; LPS), a part of Gram-negative bacterial cell walls, in the bloodstream of septic patients, and the identification of the interactions between LPS and Toll-like receptor 4 that induce inflammatory responses have made endotoxin the gold standard in studies of the basic mechanism of sepsis. Consequently, in equine medical practice, the term ‘endotoxemia’

is often misused to describe conditions that are caused by systemic inflammatory responses rather than documented presence of endotoxins in the systemic circulation. Because it is likely that other bacterial products that are recognized by the innate immune system also contribute to development of systemic inflammation, the studies summarized in this dissertation were conducted to broaden our understanding of the horse's response to Toll-like receptor ligands. This dissertation begins with Chapter 2, a literature review comprised of four sections. Section I covers the mechanism of bacterial translocation from the intestine into the circulation. Section II introduces the TLRs, including their structure, ligands, signaling pathways, and function in the immune system. Studies that are conducted on TLRs in equine diseases or the equine immune system are reviewed in Section III. Section IV contains details on TLR5 and its ligand, bacterial flagellin. Chapters 3, 4 and 5 each consist of manuscripts describing the results of studies comprising my dissertation research. Chapter 3 contains studies performed to compare microbial ligand regulation of TLR expression in equine monocytes. Chapter 4 summarizes studies of disparities in TLR5 expression and responsiveness to flagellin in equine leukocytes. Chapter 5 contains flagellin and antibodies directed to flagellin in adult horses and foals. The dissertation ends with Chapter 6 that provides an overview of all of my studies in this area.

CHAPTER 2

LITERATURE REVIEW

I. Translocation of pathogen associated molecular patterns in sepsis

The gut is a reservoir of a variety of microbes that provide beneficial effects to the host by defending against pathogenic bacteria, metabolizing nutrients, and stimulating the host immune system.[1] This is especially true in horses, herbivorous animals that rely greatly on commensal microbes for their role in the digestion of polysaccharides and uptake of nutrients. In healthy immunocompetent hosts, the symbiosis between the host and commensal microbes is maintained by tight immune surveillance mechanisms including mucosal immunity (secretory immunoglobulins), cell-mediated immunity (macrophages and T-cells) and humoral immunity (serum immunoglobulins). However, under abnormal conditions, such as trauma, surgery, severe infection, disruptions in the intestinal microvasculature in situations such as shock, and immunosuppression, the structure and function of intestinal epithelium are compromised, allowing the passage of bacteria from the gut into the systemic circulation.[2-4] Therefore, bacterial translocation is defined as the movement of viable intestinal bacteria through the gut mucosa into the blood stream or tissues, causing disease. Furthermore, translocation of bacterial components from the gut might be responsible for the systemic injuries that have broadened the definition of bacterial translocation in current research.

Because the chemical makeup of microbial components is unique to these organisms, they have been termed "pathogen associated molecular patterns (PAMPs)".[5] Translocation of bacteria exposes PAMPs such as endotoxin, a structural component of the outer wall of gram-negative microbes to the systemic circulation. Subsequently, endothelial and host immune cells are activated to produce inflammatory mediators and cytokines that result in the systemic inflammatory response syndrome (SIRS) that can lead to multiple organ dysfunction and death.[6] Accordingly, the translocation of viable microbes and endotoxin from the gut to mesenteric lymph nodes and other organs has been described as a key feature of sepsis.[2] In recent studies, translocation of other bacterial PAMPs, such as peptidoglycan and microbial DNA, has been proposed to contribute to the development of SIRS.[7, 8] Similarly, various bacterial PAMPs, including endotoxin, peptidoglycan, flagellin, and bacterial DNA, were detected in the circulation in septic clinical patients or in experimental rodent sepsis models.[9, 10, 11] Furthermore, a positive correlation between various PAMPs and disseminated inflammatory responses ("endotoxin shock") has been reported for many in vivo studies performed in a variety of animals including horses.[12] Similarly, bacterial translocation from organs other than the intestinal lumen has also been reported. For example, circulating LPS has been detected in dogs with pyometra and cows with gangrenous mastitis.[13-16]

Bacterial translocation also occurs to a small extent in healthy animals where it may contribute to the development of protective mechanisms by allowing the immune system to have contact with external antigens. In healthy animals, bacteria/bacterial components that are translocated from the gut either interact with cells in the gut-associated lymphoid tissues or are cleared by phagocytes before reaching the circulation.[2]

Collectively, these tissues and cells play key roles in limiting the deleterious effects of bacterial translocation. Alterations in immune dysfunction, such as occurs in human immunodeficiency virus (HIV) infected human patients, results in increased levels of bacterial translocation reflected by increased concentrations of LPS and flagellin in the circulation, as well as massive deletion of $CD4^+$ T-cells from the gut.[17] Taken together, these findings suggest that PAMPs contribute to the pathogenesis of sepsis by inducing SIRS and organ injury that are triggered by immune cells and effector cells in the tissue.

II. Toll-like receptor family

Toll-like receptor

The innate immune system recognizes PAMPs that are present on microbes but not on the host via various pattern recognition receptors (PRRs) including the best-characterized PRRs, the Toll-like receptors (TLRs).[18] TLRs are highly conserved from *Drosophila* to humans and both share structural and functional similarities. Recognition of PAMPs by these receptors leads to stimulation of intracellular signaling pathways that results in the production of inflammatory mediators, such as cytokines, and up-regulation of co-stimulatory molecules that ultimately activate innate immunity.[19,20] By being expressed both in immune and parenchymal cells, TLRs serve as modulators of inflammatory responses for the innate immune system.

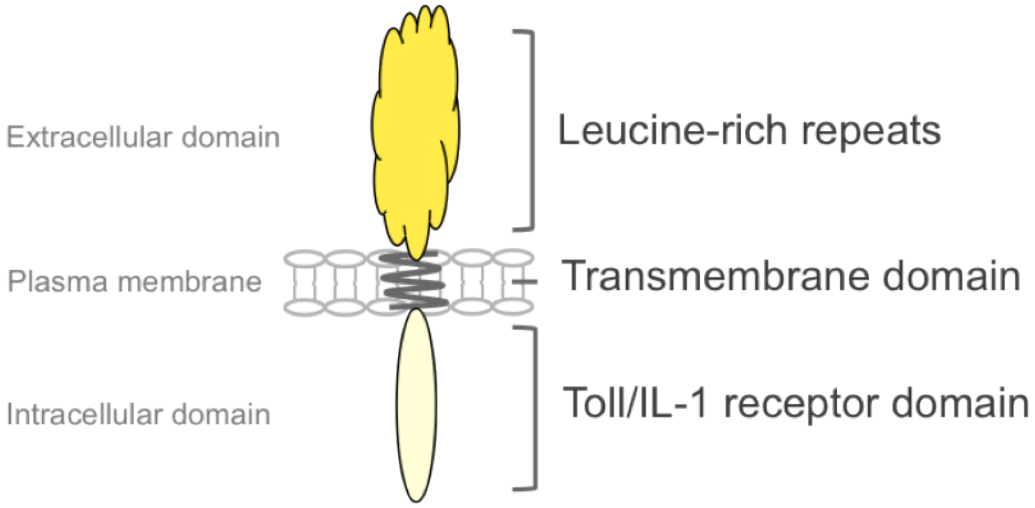

Figure 2.1. Schematic diagram of a Toll-like receptor

Currently, twelve members of the TLR family have been identified in mammals, with each TLR detecting a specific PAMP (Table 2-1). TLRs are type I glycoproteins that are composed of extracellular, transmembrane and intracellular domains (Fig. 2-1). The extracellular domains have leucin-rich repeat (LRP) modules and are responsible for recognition of specific PAMPs. Ligand-specific interactions lead to dimerization of TLRs in a homodimer or heterodimer fashion and recruitment of adaptor proteins to the intracellular Toll/interleukin-1 receptor (TIR) domains. TLRs initiate shared, but distinct signaling pathways by recruiting different combinations of TIR domain-containing adaptor molecules, such as myeloid differentiation primary response gene 88 (MyD88), toll-interleukin 1 receptor domain containing adaptor protein (TIRAP), TIR-domain-containing adapter-inducing interferon-β (TRIF), and TRIF-related adaptor molecule (TRAM).[21] MyD88, the first identified member of TIR family, is the main adapter protein for all TLR signal transduction except for TLR3, which recruits TRIF, the adaptor protein for a different signaling pathway.[18] TIRAP and TRAM function as sorting adaptors that recruit MyD88 to TLR2 and TLR4 and TRIF to TLR4, respectively. Therefore, TLR

signaling pathways can be mainly classified as either the MyD88-dependent pathway that drive the induction of inflammatory cytokines, or the TRIF-dependent pathway (or MyD88-independent pathway) that induces the synthesis of type I interferon as well as inflammatory cytokines.

MyD88 and TRIF signaling pathways

Activation of the MyD88-dependent pathway results in the induction of many inflammatory cytokine genes. Upon engagement of TLRs by their cognate PAMPs, the cytosolic TIR domain of the TLR recruits adaptor protein, MyD88, which then propagates the signal by phosphorylation and activation of multiple signaling molecules including IL-1 receptor associated kinases IRAK4, IRAK1, and IRAK2. IRAK activation results in an interaction with downstream signaling molecules including TNF receptor associated factor (TRAF) 6, IRF5, transforming growth factor β–activated kinase 1 (TAK1), and inhibitor kappa B kinase (IKK). Activated IKK then phosphorylates inhibitor-κB (IκB), which masks the nuclear localization signal of nuclear factor kappa-B (NFκB), leading to the translocation of NFκB from cytosol to the nucleus.[18] NF-κB is one of the most rapid acting transcription factors because it is present in cytosol as an inactive form and can be activated without new protein synthesis. When IKK is activated, IkB is phosphorylated and ubiquitinated which results in degradation of IκB in a proteosome. This dissociation exposes the nuclear localization signal of NF-κB, allowing NF-κB to enter the nucleus where it can induce gene expressions. NF-κB also stimulates IκB gene expression, and newly synthesized IκB prevents excessive NF-κB activation by

binding to it and returning it to the cytosol. This mechanism could lead to rapid functional changes in the host's cells. In addition to NF-κB activation, various mitogen-activated protein kinases (MAPKs), including MAPK3 (=Erk1), MAPK1 (=Erk2), p38, and Jnk, are activated, which then activate other transcription factors, including AP-1. Ultimately, these MyD88-dependent signaling cascades lead to the expression of pro-inflammatory genes that induce inflammation.

Conversely, activation of the TRIF pathway in TLR signaling primarily results in activation of interferon regulatory factor 3 (IRF-3), which then induces type-I interferon (IFN) gene expression for antiviral defense. Concomitantly, the TRIF pathway also leads to NF-κB activation to regulate the expression of various cytokine genes. TRIF recruits TRAF6 and activates TAK1, thereby resulting in activation of NF-κB and MAPK in a manner that is similar to that of the MyD88-dependent pathway.[19] The TRIF-dependent pathway is also used in TLR4 signaling in most mammalian species studied.[22] Indeed TLR4 is the only TLR that uses all four adaptor proteins mentioned above and activates both MyD88 and TRIF pathways. TLR4 activation at the plasma membrane firstly recruits TIRAP and MyD88 to trigger the initial activation of NF-κB and MAPK. TLR4 then undergoes endocytosis and trafficking to the endosome, where it forms a signaling complex with TRAM and TRIF. This TRIF-dependent pathway then leads to IRF3 activation as well as the 'late-phase' NF-κB and MAPK activation.[18,19,23]

Recruitment of adaptor proteins triggers distinct cascade of events in the signaling pathways that ultimately result in activation of NF-κB and IRF-3. Genes that encode various inflammatory cytokines, type I IFN, and chemokines are the targets of these transcription factors. The products of these genes have key roles in the initiation of

immediate immune responses and ultimately shape the adaptive immune response via chemoattraction and activation of immune cells, and up-regulation of co-stimulatory molecules for antigen presentation.[24] As a result, activation of the MyD88 and TRIF pathways result in distinct biological effects.

Differential expression of Toll-like receptors in cells

The TLRs exhibit different patterns of expression. For example, TLR 1, TLR 2, TLR 4, TLR 5, TLR 6, and TLR 11 are expressed on the cell membrane and serve as sensors for PAMPs that primarily originate from microbial membrane components; lipoprotein and lipopetide are recognized by TLR 2 in complex with TLR 1 or TLR 6, and lipopolysaccharide (LPS) and flagellin are recognized by TLR 4 and TLR 5, respectively. In addition, TLR 3, TLR 7, TLR 8, and TLR 9 are present intra-cellularly, where they detect PAMPs within intracellular vesicles such as endosomes, lysosomes, and compartments of the endoplasmic reticulum; double-stranded viral RNA is recognized by TLR 3, single-stranded RNA is recognized by TLR 7 and TLR 8, and TLR 9 mediates cellular response to unmethylated CpG dinucleotides that are present in microbial DNA.[18,19,23]

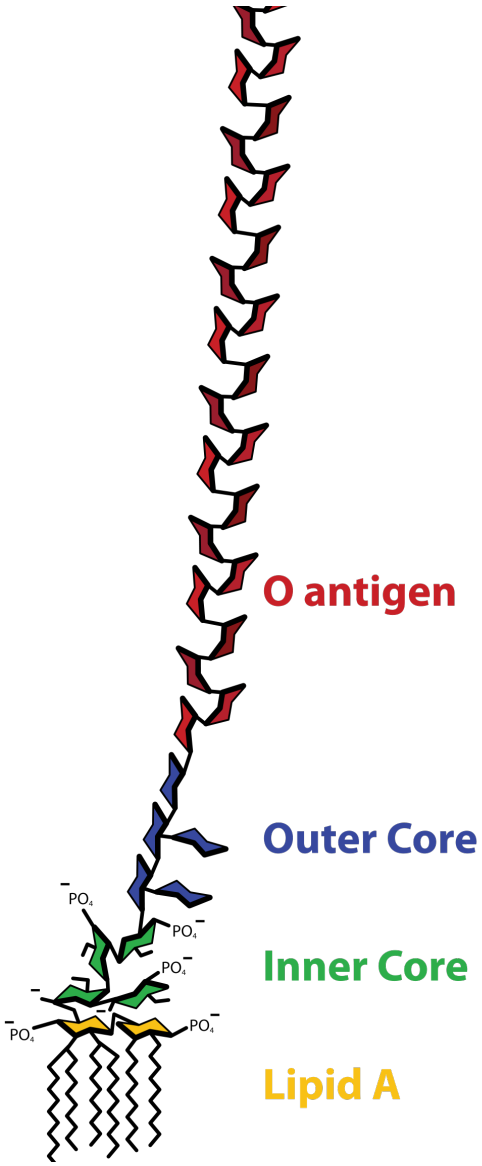

Figure 2.2. Schematic diagram of lipopolysaccharide [1]

LPS is the major component of the outer membrane of Gram-negative bacteria. LPS is composed of three parts; O polysaccharide (= O antigen) is a repetitive glycan polymer that varies from strain to strain. It comprises the outermost domain of the LPS molecule, and is a target for recognition by host antibodies. The core domain of LPS contains oligosaccharides. Lipid A is a phosphorylated glucosamine disaccharide attached to hydrophobic fatty acids that anchor the LPS molecule into the bacterial membrane. Lipid A is responsible for much of the toxicity of Gram-negative bacteria.

1 - The image was downloaded from Wikimedia Commons

When Gram-negative bacteria are destroyed by the immune system, fragments of bacterial membrane containing lipid A from LPS are released into the circulation (Fig. 2. 2). Five of the six lipid chains of LPS bind MD2 and the remaining lipid chain associates with TLR4. Additional proteins, such as LPS-binding protein (LBP) and CD14, are also involved in LPS binding. The formation of a receptor multimer composed of the TLR4/MD2-LPS complex initially transmits signals for the early-phase activation of NF-κB by recruiting TIRAP and MyD88 adaptor molecules (MyD88-dependent pathway, Fig. 2.3). After LPS binds to the TLR4/MD2 complex, the complex is internalized and retained in the endosome, where it triggers signal transduction by recruiting TRAM and TRIF, which in turn lead to activation of IRF3 and late-phase activation of NF-κB that result in the induction of type I interferon (TRIF-dependent pathway; Fig. 2. 3). The primary role of each pathway is different; the MyD88-dependent pathway induces the expression of inflammatory cytokines, including TNF-α, whereas the TRIF-dependent pathway regulates the expression of co-stimulatory molecules and type-I IFNs.[18,19,23] Both early- and late-phase activation of NF-κB are required for maximal induction of inflammatory cytokines.

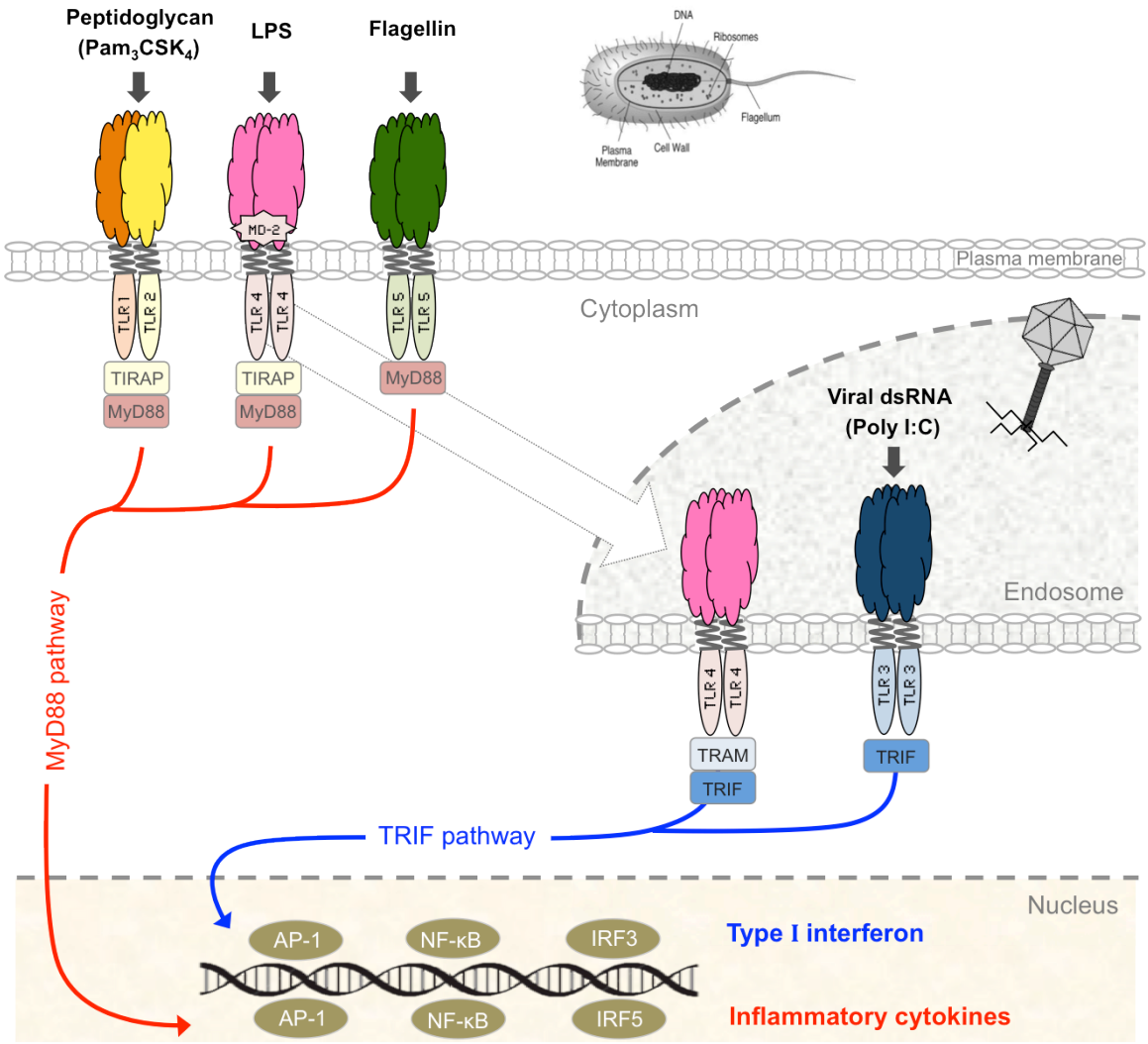

Figure 2.3. Pathogen-associated molecular patterns recognition by Toll-like receptors

The TLRs recognize PAMPs that are expressed on infectious agents, and mediate the production of cytokines necessary for the development of effective immunity. LPS recognition by TLR4 initiates both the MyD88-dependent and TRIF-dependent pathways. The MyD88-dependent pathway activates NF-κB, AP-1, and IRF5 that are responsible for inducing inflammatory cytokine expression. Activation of the TRIF-dependent pathway in TLR4-mediated responses requires recruitment of TRAM and TRIF to activate NF-κB and AP-1, and IRF3, which induce type I IFN expression. TLR3 utilizes a similar pathway, but without the involvement of TRAM. TLR2-TLR1 heterodimers recognize triacylated lipopeptide, and utilize TIRAP and MyD88 to induce production of inflammatory cytokines. Classically, TLR5 recognizes flagellin and activates NF-κB through MyD88, however, the results of a recent study of human colonic epithelial cells indicate a role for TRIF in TLR5 signaling. [27] Some TLRs are localized in intra-cellular compartments, where they detect internalized microbial components. For example, TLR3 in the endoplasmic reticulum recognizes double-stranded RNA derived from viruses or virus-infected cells, after which it traffics to the endolysosomes, where it engages its ligand. Activated TLR3 utilizes the TRIF-dependent pathway to induce type I interferon and inflammatory cytokines.

TLR2 is involved in the recognition of a wide range of PAMPs derived from bacteria, fungi, parasites and viruses. These include lipopeptides from bacteria, peptidoglycan and lipoteichoic acid from Gram-positive bacteria, lipoarabinomannan from mycobacteria, and zymosan from fungi (Table 2.1). Unlike TLR4, TLR2 forms heterodimers with TLR1 (triacylated lipopeptides from Gram-negative bacteria and mycoplasma, and Pam3CSK4) or TLR6 (diacylated lipopeptides from Gram-positive bacteria and mycoplasma). TLR2 activation primarily induces the production of inflammatory cytokines and increases its recognition capacity by collaborating with other PRRs. Consequently, TLR2 is an important TLR responsible for modulating signaling outcomes mediated by TLRs. TLR5 recognizes the flagellin protein component of bacterial flagella, a function that will be reviewed in greater detail in a later section of this literature review.

As mentioned previously, some TLRs are located in intra-cellular compartments where they recognize viral RNA and initiate antiviral responses. For example, TLR 3 recognizes double-stranded RNA derived from viruses or virus-infected cells. TLR 3 activates the TRIF-dependent pathway to induce anti-viral immune responses by promoting production of type-I interferon and inflammatory cytokines. Such activation is mimicked by polyinosinic-polycytidylic acid (poly(I:C)), a synthetic analogue of double-stranded RNA. By detecting genomic RNA that is produced during the replication of viral RNA inside the host cell, TLR 3 plays a key role in preventing viral infection. For this reason, TLR 3 deficient humans are highly susceptible to herpes simplex visrus type 1 infection.[25] Similarly, TLR 7 and TLR 9 recognize single stranded RNA derived from RNA viruses and DNA derived from both DNA viruses and bacteria, respectively. The

delivery of internalized nucleic acids to the endolysosomes is pivotal to interaction with these TLRs. For example, TLR 7 and TLR 9 are exclusively sequestered in the endoplasmic reticulum in unstimulated cells and rapidly traffic to endolysosomes after ligand stimulation. As a result, blockade of endolysosome acidification prevents TLR 7- and TLR 9-induced responses.[26]

III. TLR ligand responses in equine studies

Role of monocytes and neutrophils in the innate immune responses of horses

Monocytes/macrophages and neutrophils are key agents of the innate immune system that play a central role in the defense against microbial infections.[28] Activation, extravasation, and degranulation of neutrophils are central features of inflammation and may contribute to organ injury in clinical sepsis. Neutrophils are equipped with proteolytic enzymes and their ability to produce reactive oxygen and nitrogen species make them suitable for elimination of extracellular pathogens. Therefore, it is not surprising that neutrophils are the first and most abundant leukocytes to arrive at sites of infection. Neutrophils also contribute to the release of inflammatory cytokines. For example, neutrophil depletion before burn injury prevented early microvascular injury in the intestine.[29] In addition, monocytes/macrophages are pivotal cells in the production of various pro-inflammatory cytokines (e.g., IL-1, IL-2, IFN, TNF) that propagate the disease process.[30] The results of several studies indicate that severe trauma, burn injury and sepsis can each induce an imbalanced expression of these inflammatory mediators, followed by increased expression of anti-inflammatory cytokines.[31-33] The contribution of monocytes/macrophages to the production and release of these mediators has been

documented in many studies.[30,34,35] The recognition of PAMPs by tissue macrophages is mediated in part by TLRs and cytosolic PRRs, Nod-like receptors (NLRs). Tissue macrophages and dendritic cells present foreign antigens to the cells of adaptive immune system, thereby integrating responses of the innate and adaptive immune to pathogens. The imbalance between pro- and anti-inflammatory cytokine levels due to non-specific activation of monocytes/macrophages by PAMPs during infection may play a crucial role in the initiation of systemic inflammatory responses that characterize sepsis. Thus, these two leukocyte populations are important not only in the innate immune response to microbial infection, but also to the pathophysiology of SIRS.

TLR ligand responses in equine studies

Recent studies have documented the role of TLRs in various diseases of horses. Among the twelve mammalian TLRs that have been identified, TLRs 1-10 has been investigated in horses (Table 2.2). For example, the expression of TLR 4 and its ability to recognize LPS and initiate transcription of pro-inflammatory cytokines have been reported for equine peripheral blood leukocytes,[36,37] lungs and pulmonary macrophages,[38] bronchial epithelial cells,[39] and corneal epithelial cells.[40] Importantly, equine leukocytes are highly sensitive to LPS stimulation *in vitro* and produce pro-inflammatory cytokines, with EC_{50} values similar to those reported for humans or mice.[37,41,42] Similarly, in vivo LPS challenge in horses has documented the deleterious effects of this bacterial component, including alterations in coagulation, tissue perfusion, blood pressure, and organ function.[43, 44, 45] Because similar changes in cytokine production have been reported in septic human patients and horses,[46, 30] the TLRs appear to play an important

role in mediating host inflammatory responses. For that reason, several studies have been conducted to characterize the responsiveness of equine leukocytes to various microbial ligands. In those studies, equine monocytes were exposed to Pam_3CSK_4, Poly I:C or LPS, ligands for TLRs 2, 3 and 4, respectively, and expression of mRNAs for pro-inflammatory cytokines were quantified. The results of that study indicated that ligands for TLR2 and TLR4 increased mRNAs for TNF-α, IL-1β, IL-6, and IL-10, cytokines linked to the MyD88-pathway.[41] As mentioned above, TLR 4 activation also induces activation of the TRIF-dependent pathway in other mammalian species studied.[22] Therefore, TLR 4 activation by LPS leads to recruitment of TRIF, a response that is reflected by up-regulation of type-I IFN, IFN-β and its inducible chemokines, regulated upon activation, normal T-cell expressed (RANTES) and interferon gamma-induced protein 10 kDa (IP-10). However, LPS stimulation of equine monocytes resulted in minimal expression of type-I IFN regulated genes, which suggests that MyD88 is a dominant signaling pathway in equine monocytes for TLR4/LPS engagement.[41] On the other hand, Poly I:C stimulation of equine leukocytes induced expression of IFN-β, IP-10, RANTES, and TRAF1, molecules that are dependent on the TRIF pathway.[41]

In subsequent studies, LPS was shown to induce production of TNF-α and reactive oxygen species by equine neutrophils, a response that was antagonized by adenosine A_{2A} receptor agonists in a cAMP-dependent manner.[37] In addition, unmethylated CpG, the ligand for TLR 9, activated equine neutrophils, as evidenced by enhanced production of reactive oxygen species and expression of mRNAs for IFN-γ, IL-8 and IL-12.[47] These results are similar to those obtained with cells from other species, including humans and mice. It is important to note, however, that there are species

differences in responses to LPS, depending on the origin of this PAMP. For example, while LPS from *S. typhimurium* or *E. coli* act as agonists in all mammal species, LPS or its lipid A component from *Rhodobacter sphaeroides* are agonists in cells from horses and hamsters, but antagonists in cells from human and mice. This phenomenon is believed to be largely due to structural differences in either TLR4 or its co-stimulatory molecule MD2 that are important for ligand recognition.[48, 49]

The majority of the studies performed to evaluate the role of the TLRs in innate immune responses involved monitoring cellular responses to bacterial LPS. The results of those studies documented the role of TLR4/MD-2 in inflammatory responses of cells from humans and animals to this PAMP.[6,48] However, the results of several studies indicate that TLR 4 or MD-2 knock-out mice are resistant to LPS challenge but remain highly susceptible to Gram-negative bacterial infection and develop septic shock.[50, 51] These findings suggest that PAMPs other than LPS have potent pro-inflammatory effects and important roles during clinical SIRS. As an example of this, septic shock induced by bacterial lipopeptides was dependent on the presence of TLR 2 in mice[52] and similar effects can be elicited by bacterial DNA and its mimic CpG-DNA.[53] These studies support the concept that multiple microbial ligands are able to stimulate the host immune system, and are involved in the pathogenesis of SIRS.

IV. TLR 5 complex and bacterial flagellin

Bacterial flagellin

Bacterial flagellum consists of approximately 20,000 flagellin molecules that are encoded by the *fliC* gene (Fig. 2. 4). Flagellin is well known as a motor unit of bacteria, but is also important in bacterial adherence to epithelial cells and invasion of host tissues.[54] Flagellin is a 55-kDa protein monomer, which is composed of four domains; D0, D1, D2, and D3. D0 and D1 are buried in the center of the flagellin filament, and are conserved among flagellins from different bacterial species. In contrast, the highly variable D2 and D3 domains are exposed on the surface of the flagellar filament, and are targets for antibody responses.[55]

TLR 5 and Ipaf detect flagellin

One unique aspect of TLR 5 is that it recognizes a protein as a PAMP. Although flagellin does not undergo post-translational modification that will discriminate it from host cellular proteins, its amino and carboxy termini are extremely conserved, which allows TLR 5 to recognize different sources of flagellin as a PAMP. Furthermore, the conserved amino acid residues in the D1 domain of flagellin are released from the flagellin filament as it is depolymerized in order to interact with TLR 5.[56]

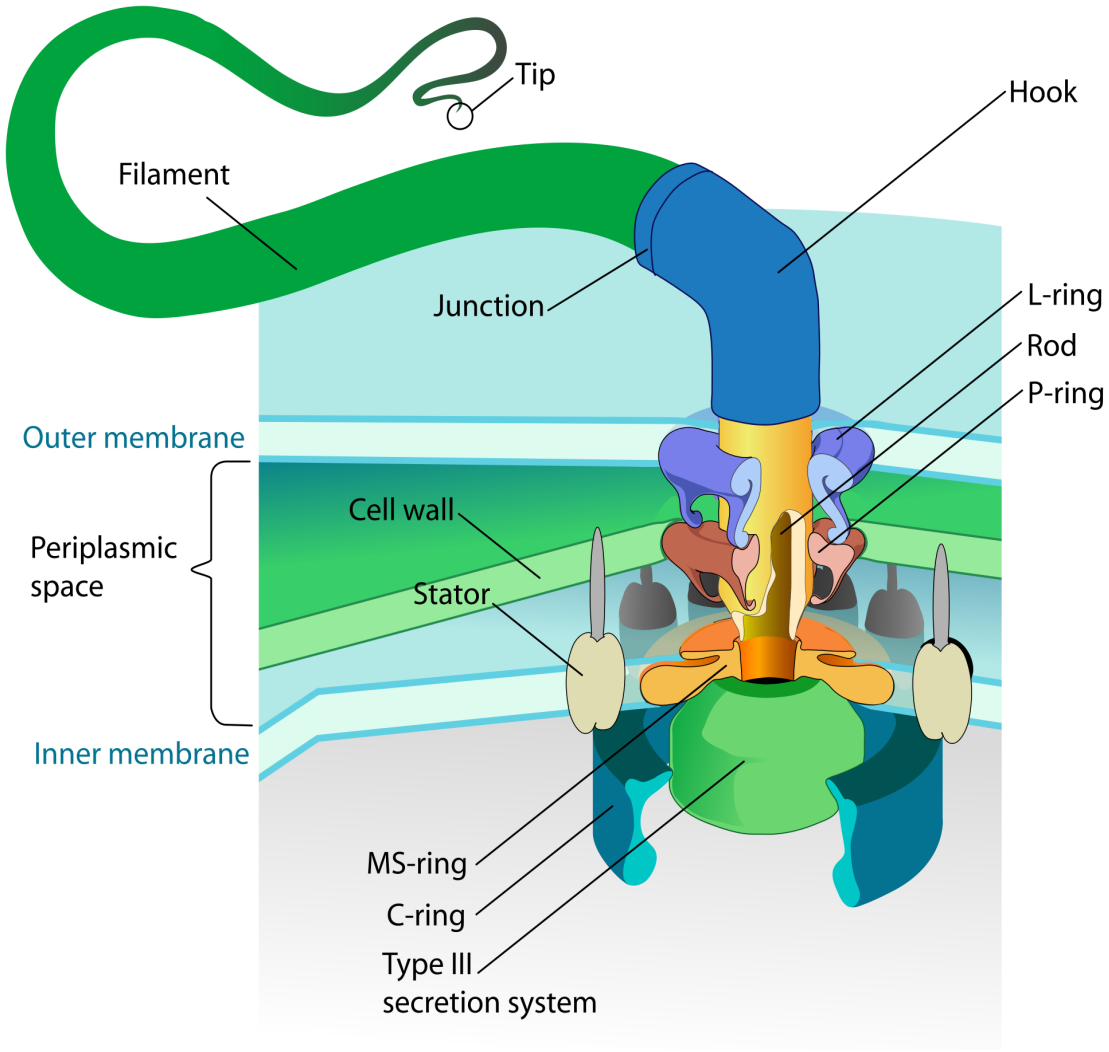

Figure 2.4. Schematic diagram of bacterial flagellin [2]

Flagellin protein is the major component of flagella. Flagella are composed of a helical rigid filament, a torsion adapter or hook, and a rotator known as the basal body. The basal body and hook structure anchor the flagella to the bacterial cell wall.

2 - The image was downloaded from Wikimedia Commons

Upon recognition of flagellin, TLR 5 dimerization recruits the MyD88 adaptor, which in turn leads to expression of pro-inflammatory cytokine genes via the typical MyD88-dependent pathway activation (Fig. 2. 5).[57] Further, cytosolic flagellin is recognized by an intracellular PRR, *I*CE protease activating factor (Ipaf). Some pro-inflammatory cytokines, such as IL-1β and IL-18, are synthesized as inactive precursors and are then activated by proteolytic processing by activated caspase-1 protease.[58] Therefore, Ipaf activation plays an important role in cytokine processing via activation of caspase-1 in cells responding to the presence of flagellin. In addition, this cytosolic PRR is important for detection of bacteria that invade the host's cells. For example, *S. typhimurium* uses the type-3 secretion system (T3SS) to invade epithelial cells or enter macrophages in order to replicate in the cytosol. This system is normally used for translocation of bacterial molecules into host cells, and flagellin can be introduced inside the cells in a similar way.[59] Flagellin monomers translocated into the cytosol of infected cells are then recognized by Ipaf. In the same context, *S. typhimurium or L. pneumophila* mutants that do not express flagellin are unable to activate caspase-1 during macrophage infection.[58,60] Together, cytokines produced as a result of TLR 5 and Ipaf activation could promote local inflammation and adaptive immune system activation. Although other TLRs expressed on the plasma membrane and other cytosolic PRRs activations could result in similar consequences, this dual monitoring system that detects flagellin suggests the importance of flagellin in the activation of the immune system.

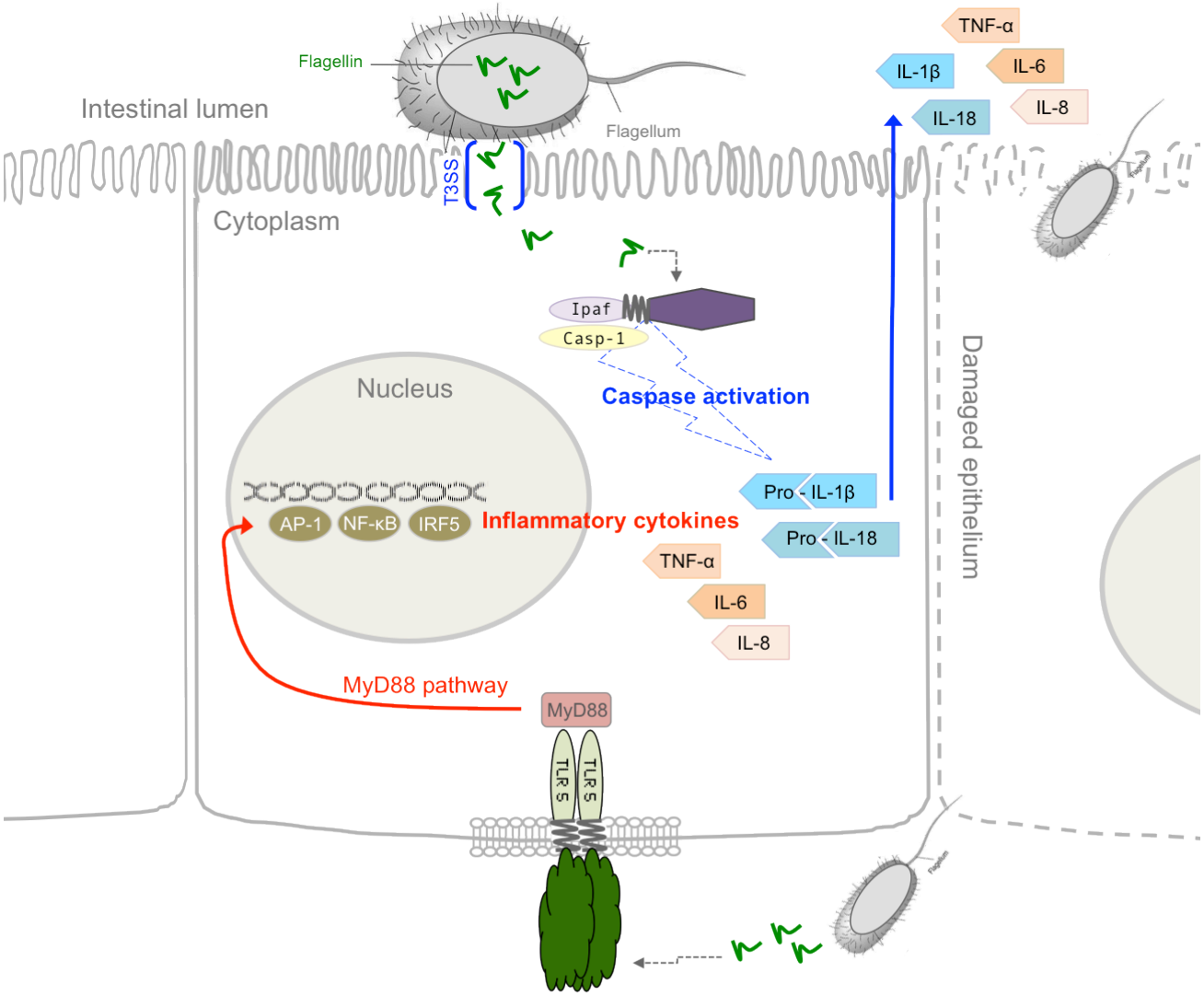

Figure 2. 5. Dual recognition and activation of PRRs by flagellin [3]

TLR5 has limited expression on the basolateral surface of intestinal epithelial cells. Once microbes breach the intestinal epithelium by crossing the damaged epithelial barrier, flagellin can activate TLR5 and result in pro-inflammatory cytokines gene translation via MyD88 pathway signaling. In addition, when flagellin enters the host cell via the type-3 secretion (T3SS) system that transports bacterial products into these cells, it is recognized by a cytosolic receptor, Ipaf. Ipaf signaling activates caspase-1 that contributes to the maturation of some pro-inflammatory cytokines, including Il-1β and Il-18. Taken together, the polarization of TLR 5 and dual recognition of flagellin by TLR5 and Ipaf allows for the initiation of selective inflammatory responses against invading microbes.

3 - Image was adopted and modified from Mia at al. 2007 [61]

Potential role for flagellin in equine immune responses

Recent investigations have revealed an important role for flagellin in the induction of host proinflammatory responses. Flagellin, at low concentrations, activates macrophages, monocytes and intestinal and pulmonary epithelial cells in vitro, and induces the release of a host of pro-inflammatory mediators in vitro and in vivo.[59,62] Also, increased levels of circulating flagellin protein and its antibody were detected in septic human patients and in a murine sepsis model.[11,59] In vitro studies demonstrated the ability of flagellin to induce production of pro-inflammatory mediators by human monocytes and epithelial cells, resulting from the activation of NF-κB.[63,64] In vivo flagellin administration in mice resulted in increased production of nitric oxide, a potent vasodilator, by epithelial cells and the development of hypotension and vascular hypocontractility that characterize septic shock.[59] In this study, evidence of widespread oxidative stress was noticed especially in lung and liver where TLR5 is highly expressed.

Expression of functional TLR 5 in cells that are continuously challenged by pathogenic and non-pathogenic microbes, such as intestinal, corneal and conjunctival epithelium, is limited to the basolateral face, but absent on the apical side.[65,66] This anatomical polarization of TLR 5 allows it to selectively distinguish between invasive flagellated bacteria and commensal microbes. Isolated cells from these tissues strongly respond to flagellin by secreting pro-inflammatory cytokines suggesting that flagellin-induced activation of TLR 5 allows effective recognition of invading pathogens.[65]

There is convincing evidence for a role for bacterial flagellin in the pathogenesis of some gastrointestinal diseases. For example, Crohn's disease, which is associated with imbalanced interactions between the microflora and the mucosal immune system in the intestine, that leads to the loss of tolerance of the gut to microfloral antigens and the initiation of inflammation. In this disease, flagellin is the dominant antigen recognized by antibody and T-cell responses, and increased titers of flagellin antibody have been detected in human patients with this disease.[67] There is also evidence for the involvement of antibodies against flagellin in recognition of self-antigens in patients with Crohn's disease, suggesting that adaptive immune response to flagellin may be involved in the pathogenesis of this disease by molecular mimicry between flagellin and auto-antigens.[64] In addition, flagellin has been identified as a dominant antigen capable of inducing colitis in TLR4-/- mice.[68] These findings suggest that flagellin is an important factor for pathogenesis of intestinal disease. TLR 5-mediated recognition of flagellin in pulmonary inflammation has also been studied extensively. For example, flagellin potently stimulates murine alveolar macrophages and epithelial cells to produce pro-inflammatory cytokines,[69] and injection of flagellin into mice results in a severe form of acute lung inflammation[11] Therefore, recognition of the flagellin-TLR 5 pathway may offer novel opportunities for the treatment of various forms of shock, sepsis, bacterial inflammation and infection. The aim of the present study was to characterize the activity of flagellin on isolated equine leukocytes.

V. Endogenous TLR ligands

In recent years, it is becoming increasingly evident that, in addition to responding microbial PAMPs, TLRs respond to several endogenous molecules and their degradation products and trigger inflammatory responses. These molecules have been termed "danger-associated molecular patterns (DAMPs)" and, therefore, have been compared to PAMPs.[70] During ischemia-reperfusion injury or septic shock, increased production of reactive oxygen species and pro-inflammatory cytokines, and activation of complement are commonly observed.[71] Consequently, immune cells are activated and migrate to the site of injury, thereby promoting additional local tissue injury and initiating damage to other organs via a variety of inflammatory mediators that are produced and released from immune and tissue cells. As a result of injury or inflammation, components of the extracellular matrix are cleaved by cellular proteases, such as metalloproteinase and hyaluronidase, and degradation products, such as hyaluronic acid, fibrinogen, and heparan sulfate, are released. For example, hyaluronic acid, which is the most abundant extracellular matrix molecule in connective tissues, degrades into fragments and is released as a result of increased activity of enzymes during inflammation, infection, or tissue injury. In addition, some microbes, such as *Streptococcus* and *Staphylococcus,* express hyaluronic acid lyases that facilitate their ability to invade the host's tissues by enhancing vascular permeability during infection or sepsis.[72] As a result, hyaluronic acid fragments, especially those of low-molecular weight, accumulate locally at the site of injury.[73,74] Furthermore, serum concentrations of hyaluronic acid increase in certain hepatic diseases, especially in patients with cirrhosis, in which clearance of hyaluronic acid is impaired. Rapid increases in circulating concentrations of hyaluronic acid also

occur in patients with massive burns, blood loss, shock, ischemic stroke, and major surgical procedures. [75, 76, 77] Therefore, serum hyaluronic acid has the potential to be a biomarker to detect injuries in aforementioned clinical conditions. In addition to extracellular matrix degradation products, other cellular components, such as heat shock proteins, and high-mobility group box 1 proteins, stimulate cell surface TLRs and have been detected in various disease conditions including sepsis.[78-80] Recognition of endogenous TLR ligands is mediated by cell surface TLRs, primarily TLR 2 and TLR 4, that leads to inflammation as well as repair responses. For example, low molecular weight hyaluronic acid can bind to the TLR 4-CD44 complex on macrophages, dendritic cells, and endothelial cells, and result in phosphorylation of MAPK and activation of NF-κB. These latter effects result in the release of various mediators that initiate and mediate inflammation.[81-83] Furthermore, low-molecular weight hyaluronic acid up-regulates expression of TLR 4 mRNA and inflammatory mediators, such as TNF-α and IL-1ß, in cultured murine chondrocytes and enhances cellular pro-inflammatory responses when co-incubated with LPS.[84] Therefore, it has been suggested that hyaluronic acid functions as a danger signal that alerts the host to the presence of tissue damage before fulminant infection or necrotic cell death develop can occur.

It is important to note that self-derived nucleic acids, DNAs and RNAs that are released by dying cells, can be recognized by TLR 7 and TLR 9 located in endosome. However, these nucleic acids do not activate innate immune responses under normal conditions, and the intracellular localization of TLR 7 and TLR 9 is important for avoiding contact with these nucleic acids in the extracellular space. A second safeguard against inappropriate responses to self-derived nucleic acids is the proteolytic maturation

process required for TLR 9. However, TLR 9 trafficking from the endoplasmic reticulum to the endosome is inducible by LPS stimulation, which suggests that inflammation could potentially increase the accessibility of self-derived nucleic acids to intracellular TLR during inflammation.[85]

In summary, the TLR family of receptors is a critical component of the innate immune system that detects invasion of the host by microbial pathogens or movement of PAMPs into the general circulation. Recognition of PAMPs by the TLRs initiates signal transduction pathways, which trigger the expression of genes that control innate immune responses and further instruct the development of antigen-specific acquired immunity. Differential recognition of PAMPs and utilization of different adaptor molecules provides specificity to the host's response. While appropriate and balanced activation of TLRs is required to respond to PAMPs and microbial pathogens, over-reactivation of the innate immune system can lead to fatal conditions. With this in mind, the following studies focus on activation and regulation of TLR-mediated innate immune responses in equine leukocytes, with the goal of more fully understanding the horse's response to pro-inflammatory stimuli.

Table 2.1. Mammalian Toll-like receptors and there ligands

TLRs	Microbial ligands (or synthetic activators)	Location	Signaling pathway
TLR 1	Triacyl lipopeptides from bacteria and mycobateria	Cell surface	MyD88/TIRAP
TLR 2	LTA from gram-positive bacteria, yeast, zymosan, lipopetides (Pam_3CSK_4, MALP-2), lipoarabinomanna from mycrobacteria	Cell surface	MyD88/TIRAP
TLR 3	Viral dsRNA (Poly I:C)	Intracellular compartment	TRIF
TLR 4	LPS from gram-negative bacteria (synthetic Lipid A molecules), mannan from candida albicans, Hsp, HA oligossacharides	Cell surface	MyD88/TIRAP TRAM/TRIF
TLR 5	Bacterial flagellin	Cell surface	MyD88
TLR 6	Diacyl lipopetides from Mycoplasma, LTA from gram-positive bacteria, yeast, (zymosan)	Cell surface	MyD88
TLR 7	Viral ssRNA, (Imiquimod), (synthetic polyU RNA)	Intracellular compartment	MyD88
TLR 8	Viral ssRNA	Intracellular compartment	MyD88
TLR 9	Bacterial and viral CpG DNA, (synthetic unmethylated CpG DNA)	Intracellular compartment	MyD88
TLR 10	Unknown	Cell surface	Unknown
TLR 11	Profin-like molecules from Toxoplama gondii, unknown ligand from uropathogenic bacteria	Cell surface	Unknown

Table 2.2. Expression of Toll-like receptors in equine cells and tissues

† mRNA expression, ‡ protein expression, §cellular activity by ligand stimulation

TLRs	Ligands	Tissue/cell type	References
TLR 1	Triacyl lipopeptides	Respiratory epithelium †	Quintana et al. 2011[86]
TLR 2	Diacyl lipopeptide Pam_3CSK_4	Peripheral blood monocytes †§	Kwon et al. 2010[87]
		Peripheral blood neutrophils §	Kwon et al. (unpublished data)
		Cornea, conjunctiva, limbus of the eye †	Gornik et al. 2011 [40]
		Lungs † : No basal expression, but TLR2 expression was detected in LPS administered horses	Singh Suri et al. 2006 [38]
		Respiratory epithelium †	Quintana et al. 2011[86]
		Liver †	McGeachy et al. [88]
TLR 3	dsRNA Poly I:C	Peripheral blood monocytes †§	Kwon et al. 2010[87]
		Cornea, conjunctiva, limbus of the eye †	Gornik et al. 2011 [40]
		Respiratory epithelium †‡	Quintana et al. 2011[86]
TLR 4	LPS	Peripheral blood monocytes †§	Figueiredo et al. 2009[41],Gold et al.[46] Kwon et al. 2010[87], Kwon et al. 2011 [89]
		Peripheral blood neutrophils †§	Sun et al. 2007[90], Kwon et al. 2011 [89]
		Peritoneal macrophages †§	Kwon et al. 2011 [89]
		Alveolar macrophages †§	Kwon et al. 2011 [89]
		Lungs †‡§	Singh Suri et al. 2006 [38]
		Bronchial epithelial cells	Berndt et al. 2007[39]
		Cornea, conjunctiva, limbus of the eye †	Gornik et al. 2011 [40]
		Respiratory epithelium †	Quintana et al. 2011[86]
		Keratinocytes (skin) §	Leise et al. 2010[91]
		Liver †	McGeachy et al. [88]
TLR 5	Flagellin	Peripheral blood monocytes †‡§	Kwon et al. 2011 [89]
		Peripheral blood neutrophils †‡§	Kwon et al. 2011 [89]
		Peritoneal macrophages †‡§	Kwon et al. 2011 [89]
		Alveolar macrophages †‡§	Kwon et al. 2011 [89]
		Keratinocytes §	Leise et al. 2010[91]
TLR 6	Diacyl lipopetides	Cornea, conjunctiva, limbus of the eye †	Gornik et al. 2011 [40]
		Respiratory epithelium †	Quintana et al. 2011[86]
TLR 7	ssRNA	Respiratory epithelium †	Quintana et al. 2011[86]
TLR 8	ssRNA	Respiratory epithelium †	Quintana et al. 2011[86]
TLR 9	CpG DNA	DCs †§, PMBC derived Mϕ †, PMNs ‡, T-cells ‡	Flamino et al. 2007[92] Zhang et al. 2008[93]
		Cornea, conjunctiva, limbus of the eye †	Gornik et al. 2011 [40]
		Respiratory epithelium †‡	Quintana et al. 2011[86]
TLR 10	Unknown	Respiratory epithelium†	Quintana et al. 2011[86]

VI. References

1. Williams RE. Benefit and mischief from commensal bacteria. *J Clin Pathol.* Nov 1973;26(11):811-818.
2. Naaber P, Smidt I, Tamme K, et al. Translocation of indigenous microflora in an experimental model of sepsis. *J Med Microbiol.* May 2000;49(5):431-439.
3. Brenchley JM, Price DA, Schacker TW, et al. Microbial translocation is a cause of systemic immune activation in chronic HIV infection. *Nat Med.* Dec 2006;12(12):1365-1371.
4. Vetuschi A, Latella G, Sferra R, Caprilli R, Gaudio E. Increased proliferation and apoptosis of colonic epithelial cells in dextran sulfate sodium-induced colitis in rats. *Dig Dis Sci.* Jul 2002;47(7):1447-1457.
5. O'Mahony DS, Pham U, Iyer R, Hawn TR, Liles WC. Differential constitutive and cytokine-modulated expression of human Toll-like receptors in primary neutrophils, monocytes, and macrophages. *Int J Med Sci.* 2008;5(1):1-8.
6. Palsson-McDermott EM, O'Neill LA. Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4. *Immunology.* Oct 2004;113(2):153-162.
7. Kane TD, Alexander JW, Johannigman JA. The detection of microbial DNA in the blood: a sensitive method for diagnosing bacteremia and/or bacterial translocation in surgical patients. *Ann Surg.* Jan 1998;227(1):1-9.
8. Ammori BJ. Role of the gut in the course of severe acute pancreatitis. *Pancreas.* Mar 2003;26(2):122-129.

9. Barton MH, Collatos C. Tumor necrosis factor and interleukin-6 activity and endotoxin concentration in peritoneal fluid and blood of horses with acute abdominal disease. *J Vet Intern Med.* Sep-Oct 1999;13(5):457-464.

10. Tabata T, Tani T, Endo Y, Hanasawa K. Bacterial translocation and peptidoglycan translocation by acute ethanol administration. *J Gastroenterol.* 2002;37(9):726-731.

11. Liaudet L, Szabo C, Evgenov OV, et al. Flagellin from gram-negative bacteria is a potent mediator of acute pulmonary inflammation in sepsis. *Shock.* Feb 2003;19(2):131-137.

12. Fessler JF, Bottoms GD, Coppoc GL, Gimarc S, Latshaw HS, Noble JK. Plasma endotoxin concentrations in experimental and clinical equine subjects. *Equine Vet J Suppl.* Jun 1989(7):24-28.

13. Hagman R, Kindahl H, Lagerstedt AS. Pyometra in bitches induces elevated plasma endotoxin and prostaglandin F2alpha metabolite levels. *Acta veterinaria Scandinavica.* 2006;47:55-67.

14. Okano S, Tagawa M, Takase K. Relationship of the blood endotoxin concentration and prognosis in dogs with pyometra. *The Journal of veterinary medical science / the Japanese Society of Veterinary Science.* Nov 1998;60(11):1265-1267.

15. Hakogi E, Tamura H, Tanaka S, Kohata A, Shimada Y, Tabuchi K. Endotoxin levels in milk and plasma of mastitis-affected cows measured with a chromogenic limulus test. *Veterinary microbiology.* Jul 1989;20(3):267-274.

16. Silva E, Leitao S, Henriques S, et al. Gene transcription of TLR2, TLR4, LPS ligands and prostaglandin synthesis enzymes are up-regulated in canine uteri with cystic endometrial hyperplasia-pyometra complex. *Journal of reproductive immunology.* Jan 2010;84(1):66-74.

17. Douek D. HIV disease progression: immune activation, microbes, and a leaky gut. *Topics in HIV medicine : a publication of the International AIDS Society, USA.* Aug-Sep 2007;15(4):114-117.

18. Uematsu S, Akira S. Toll-like receptors and innate immunity. *J Mol Med.* Sep 2006;84(9):712-725.

19. Akira S, Takeda K. Toll-like receptor signalling. *Nat Rev Immunol.* Jul 2004;4(7):499-511.

20. Muzio M, Bosisio D, Polentarutti N, et al. Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells. *J Immunol.* Jun 1 2000;164(11):5998-6004.

21. Beutler BA. TLRs and innate immunity. *Blood.* Feb 12 2009;113(7):1399-1407.

22. Kawai T, Takeuchi O, Fujita T, et al. Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. *Journal of immunology.* Nov 15 2001;167(10):5887-5894.

23. Lee MS, Kim YJ. Signaling pathways downstream of pattern-recognition receptors and their cross talk. *Annual review of biochemistry.* 2007;76:447-480.

24. Kumar H, Kawai T, Akira S. Toll-like receptors and innate immunity. *Biochem Biophys Res Commun.* Oct 30 2009;388(4):621-625.

25. Zhang SY, Jouanguy E, Ugolini S, et al. TLR3 deficiency in patients with herpes simplex encephalitis. *Science.* Sep 14 2007;317(5844):1522-1527.

26. Kim YM, Brinkmann MM, Paquet ME, Ploegh HL. UNC93B1 delivers nucleotide-sensing toll-like receptors to endolysosomes. *Nature.* Mar 13 2008;452(7184):234-238.

27. Choi YJ, Im E, Chung HK, Pothoulakis C, Rhee SH. TRIF mediates Toll-like receptor 5-induced signaling in intestinal epithelial cells. *The Journal of biological chemistry.* Nov 26 2010;285(48):37570-37578.

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

29. Sir O, Fazal N, Choudhry MA, Goris RJ, Gamelli RL, Sayeed MM. Role of neutrophils in burn-induced microvascular injury in the intestine. *Shock.* Aug 2000;14(2):113-117.

30. Munoz C, Carlet J, Fitting C, Misset B, Bleriot JP, Cavaillon JM. Dysregulation of in vitro cytokine production by monocytes during sepsis. *J Clin Invest.* Nov 1991;88(5):1747-1754.

31. Remick DG, Bolgos GR, Siddiqui J, Shin J, Nemzek JA. Six at six: interleukin-6 measured 6 h after the initiation of sepsis predicts mortality over 3 days. *Shock.* Jun 2002;17(6):463-467.

32. Kretzschmar M, Pfeiffer L, Schmidt C, Schirrmeister W. Plasma levels of glutathione, alpha-tocopherol and lipid peroxides in polytraumatized patients; evidence for a stimulating effect of TNF alpha on glutathione synthesis. *Exp Toxicol Pathol.* Sep 1998;50(4-6):477-483.

33. Mokart D, Capo C, Blache JL, et al. Early postoperative compensatory anti-inflammatory response syndrome is associated with septic complications after major surgical trauma in patients with cancer. *Br J Surg.* Nov 2002;89(11):1450-1456.

34. Tamandl D, Bahrami M, Wessner B, et al. Modulation of toll-like receptor 4 expression on human monocytes by tumor necrosis factor and interleukin-6: tumor necrosis factor evokes lipopolysaccharide hyporesponsiveness, whereas interleukin-6 enhances lipopolysaccharide activity. *Shock.* Sep 2003;20(3):224-229.

35. Zughaier SM, Zimmer SM, Datta A, Carlson RW, Stephens DS. Differential induction of the toll-like receptor 4-MyD88-dependent and -independent signaling pathways by endotoxins. *Infect Immun.* May 2005;73(5):2940-2950.

36. Figueiredo MD, Salter CE, Andrietti AL, Vandenplas ML, Hurley DJ, Moore JN. Validation of a reliable set of primer pairs for measuring gene expression by real-time quantitative RT-PCR in equine leukocytes. *Vet Immunol Immunopathol.* Sep 15 2009;131(1-2):65-72.

37. Sun WC, Berghaus LJ, Moore JN, et al. Lipopolysaccharide and TNF-alpha modify adenosine A(2A) receptor expression and function in equine monocytes. *Vet Immunol Immunopathol.* Dec 11 2009.

38. Singh Suri S, Janardhan KS, Parbhakar O, Caldwell S, Appleyard G, Singh B. Expression of toll-like receptor 4 and 2 in horse lungs. *Vet Res.* Jul-Aug 2006;37(4):541-551.

39. Berndt A, Derksen FJ, Venta PJ, Ewart S, Yuzbasiyan-Gurkan V, Robinson NE. Elevated amount of Toll-like receptor 4 mRNA in bronchial epithelial cells is associated with airway inflammation in horses with recurrent airway obstruction. *Am J Physiol Lung Cell Mol Physiol.* Apr 2007;292(4):L936-943.

40. Gornik K, Moore P, Figueiredo M, Vandenplas M. Expression of Toll-like receptors 2, 3, 4, 6, 9, and MD-2 in the normal equine cornea, limbus, and conjunctiva. *Veterinary ophthalmology.* Mar 2011;14(2):80-85.

41. Figueiredo MD, Vandenplas ML, Hurley DJ, Moore JN. Differential induction of MyD88- and TRIF-dependent pathways in equine monocytes by Toll-like receptor agonists. *Vet Immunol Immunopathol.* Jan 15 2009;127(1-2):125-134.

42. Jackson JJ, Kropp H. Antibiotic-induced endotoxin release: Important parameters dictating responses. In: Brade H, Opal SM, Vogel SN, Morrison DC, eds. *Endotoxin in Health and Disease*. New York: Marcel Dekker Inc.; 1999:67-75.

43. Moore JN, Garner HE, Shapland JE, Schaub RG. Equine endotoxemia: an insight into cause and treatment. *J Am Vet Med Assoc.* Sep 1 1981;179(5):473-477.

44. Morris DD. Endotoxemia in horses. A review of cellular and humoral mediators involved in its pathogenesis. *J Vet Intern Med.* May-Jun 1991;5(3):167-181.

45. Duncan SG, Meyers KM, Reed SM, Grant B. Alterations in coagulation and hemograms of horses given endotoxins for 24 hours via hepatic portal infusions. *Am J Vet Res.* Jun 1985;46(6):1287-1293.

46. Gold JR, Perkins GA, Erb HN, Ainsworth DM. Cytokine profiles of peripheral blood mononuclear cells isolated from septic and healthy neonatal foals. *J Vet Intern Med.* May-Jun 2007;21(3):482-488.

47. Liu M, Liu T, Bordin A, Nerren J, Cohen N. Activation of foal neutrophils at different ages by CpG oligodeoxynucleotides and Rhodococcus equi. *Cytokine.* Dec 2009;48(3):280-289.

48. Lohmann KL, Vandenplas M, Barton MH, Moore JN. Lipopolysaccharide from Rhodobacter sphaeroides is an agonist in equine cells. *J Endotoxin Res.* 2003;9(1):33-37.

49. Qureshi N, Takayama K, Kurtz R. Diphosphoryl lipid A obtained from the nontoxic lipopolysaccharide of Rhodopseudomonas sphaeroides is an endotoxin antagonist in mice. *Infect Immun.* Jan 1991;59(1):441-444.

50. Poltorak A, He X, Smirnova I, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science.* Dec 11 1998;282(5396):2085-2088.

51. Nagai Y, Akashi S, Nagafuku M, et al. Essential role of MD-2 in LPS responsiveness and TLR4 distribution. *Nat Immunol.* Jul 2002;3(7):667-672.

52. Meng G, Rutz M, Schiemann M, et al. Antagonistic antibody prevents toll-like receptor 2-driven lethal shock-like syndromes. *J Clin Invest.* May 2004;113(10):1473-1481.

53. Sparwasser T, Miethke T, Lipford G, et al. Bacterial DNA causes septic shock. *Nature.* Mar 27 1997;386(6623):336-337.

54. Van Asten FJ, Hendriks HG, Koninkx JF, Van der Zeijst BA, Gaastra W. Inactivation of the flagellin gene of Salmonella enterica serotype enteritidis strongly reduces invasion into differentiated Caco-2 cells. *FEMS Microbiol Lett.* Apr 15 2000;185(2):175-179.

55. Brown A, Hormaeche CE. The antibody response to salmonellae in mice and humans studied by immunoblots and ELISA. *Microbial pathogenesis.* Jun 1989;6(6):445-454.

56. Smith KD, Andersen-Nissen E, Hayashi F, et al. Toll-like receptor 5 recognizes a conserved site on flagellin required for protofilament formation and bacterial motility. *Nature immunology.* Dec 2003;4(12):1247-1253.

57. Hayashi F, Smith KD, Ozinsky A, et al. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature.* Apr 26 2001;410(6832):1099-1103.

58. Franchi L, Amer A, Body-Malapel M, et al. Cytosolic flagellin requires Ipaf for activation of caspase-1 and interleukin 1beta in salmonella-infected macrophages. *Nature immunology.* Jun 2006;7(6):576-582.

59. Eaves-Pyles T, Murthy K, Liaudet L, et al. Flagellin, a novel mediator of Salmonella-induced epithelial activation and systemic inflammation: I kappa B alpha degradation, induction of nitric oxide synthase, induction of proinflammatory mediators, and cardiovascular dysfunction. *J Immunol.* Jan 15 2001;166(2):1248-1260.

60. Molofsky AB, Byrne BG, Whitfield NN, et al. Cytosolic recognition of flagellin by mouse macrophages restricts Legionella pneumophila infection. *J Exp Med.* Apr 17 2006;203(4):1093-1104.

61. Miao EA, Andersen-Nissen E, Warren SE, Aderem A. TLR5 and Ipaf: dual sensors of bacterial flagellin in the innate immune system. *Semin Immunopathol.* Sep 2007;29(3):275-288.

62. Ciacci-Woolwine F, Blomfield IC, Richardson SH, Mizel SB. Salmonella flagellin induces tumor necrosis factor alpha in a human promonocytic cell line. *Infection and immunity.* Mar 1998;66(3):1127-1134.

63. Gewirtz AT. Flag in the crossroads: flagellin modulates innate and adaptive immunity. *Curr Opin Gastroenterol.* Jan 2006;22(1):8-12.

64. Lunardi C, Bason C, Dolcino M, et al. Antiflagellin antibodies recognize the autoantigens Toll-Like Receptor 5 and Pals 1-associated tight junction protein and induce monocytes activation and increased intestinal permeability in Crohn's disease. *J Intern Med.* Feb 2009;265(2):250-265.

65. Kojima K, Ueta M, Hamuro J, et al. Human conjunctival epithelial cells express functional Toll-like receptor 5. *Br J Ophthalmol.* Mar 2008;92(3):411-416.

66. Gewirtz AT, Navas TA, Lyons S, Godowski PJ, Madara JL. Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression. *J Immunol.* Aug 15 2001;167(4):1882-1885.

67. Sitaraman SV, Klapproth JM, Moore DA, 3rd, et al. Elevated flagellin-specific immunoglobulins in Crohn's disease. *Am J Physiol Gastrointest Liver Physiol.* Feb 2005;288(2):G403-406.

68. Lodes MJ, Cong Y, Elson CO, et al. Bacterial flagellin is a dominant antigen in Crohn disease. *J Clin Invest.* May 2004;113(9):1296-1306.

69. Raoust E, Balloy V, Garcia-Verdugo I, Touqui L, Ramphal R, Chignard M. Pseudomonas aeruginosa LPS or flagellin are sufficient to activate TLR-dependent signaling in murine alveolar macrophages and airway epithelial cells. *PLoS One.* 2009;4(10):e7259.

70. Piccinini AM, Midwood KS. DAMPening inflammation by modulating TLR signalling. *Mediators Inflamm.* 2010;2010.

71. Tsan MF, Gao B. Endogenous ligands of Toll-like receptors. *J Leukoc Biol.* Sep 2004;76(3):514-519.

72. Sutherland IW. Polysaccharide lyases. *FEMS Microbiol Rev.* Jul 1995;16(4):323-347.

73. Laurent TC, Fraser JR, Laurent UB, Engstrom-Laurent A. Hyaluronan in inflammatory joint disease. *Acta Orthop Scand Suppl.* Oct 1995;266:116-120.

74. Teder P, Vandivier RW, Jiang D, et al. Resolution of lung inflammation by CD44. *Science.* Apr 5 2002;296(5565):155-158.

75. Berg S, Brodin B, Hesselvik F, Laurent TC, Maller R. Elevated levels of plasma hyaluronan in septicaemia. *Scand J Clin Lab Invest.* Dec 1988;48(8):727-732.

76. Engstrom-Laurent A. Changes in hyaluronan concentration in tissues and body fluids in disease states. *Ciba Found Symp.* 1989;143:233-240; discussion 240-237, 281-235.

77. Girish KS, Kemparaju K. The magic glue hyaluronan and its eraser hyaluronidase: a biological overview. *Life Sci.* May 1 2007;80(21):1921-1943.

78. Johnson GB, Brunn GJ, Platt JL. Cutting edge: an endogenous pathway to systemic inflammatory response syndrome (SIRS)-like reactions through Toll-like receptor 4. *J Immunol.* Jan 1 2004;172(1):20-24.

79. Cohen-Sfady M, Nussbaum G, Pevsner-Fischer M, et al. Heat shock protein 60 activates B cells via the TLR4-MyD88 pathway. *J Immunol.* Sep 15 2005;175(6):3594-3602.

80. Scheibner KA, Lutz MA, Boodoo S, Fenton MJ, Powell JD, Horton MR. Hyaluronan fragments act as an endogenous danger signal by engaging TLR2. *J Immunol.* Jul 15 2006;177(2):1272-1281.

81. Jiang D, Liang J, Fan J, et al. Regulation of lung injury and repair by Toll-like receptors and hyaluronan. *Nat Med.* Nov 2005;11(11):1173-1179.

82. Termeer C, Benedix F, Sleeman J, et al. Oligosaccharides of Hyaluronan activate dendritic cells via toll-like receptor 4. *J Exp Med.* Jan 7 2002;195(1):99-111.

83. Noble PW, McKee CM, Cowman M, Shin HS. Hyaluronan fragments activate an NF-kappa B/I-kappa B alpha autoregulatory loop in murine macrophages. *J Exp Med.* May 1 1996;183(5):2373-2378.

84. Campo GM, Avenoso A, Campo S, D'Ascola A, Nastasi G, Calatroni A. Molecular size hyaluronan differently modulates toll-like receptor-4 in LPS-induced inflammation in mouse chondrocytes. *Biochimie.* Feb 2010;92(2):204-215.

85. Saitoh S, Miyake K. Regulatory molecules required for nucleotide-sensing Toll-like receptors. *Immunological reviews.* Jan 2009;227(1):32-43.

86. Quintana AM, Landolt GA, Annis KM, Hussey GS. Immunological characterization of the equine airway epithelium and of a primary equine airway epithelial cell culture model. *Veterinary immunology and immunopathology.* Apr 15 2011;140(3-4):226-236.

87. Kwon S, Vandenplas ML, Figueiredo MD, et al. Differential induction of Toll-like receptor gene expression in equine monocytes activated by Toll-like receptor

ligands or TNF-alpha. *Veterinary immunology and immunopathology.* Dec 1 2010;138(3):213-217.

88. Alterations in Toll-like Receptor Regulation in the Liver from Horses with Laminitis. The FASEB Journal; 2008.

89. Kwon S, Gewirtz AT, Hurley DJ, Robertson TP, Moore JN, Vandenplas ML. Disparities in TLR5 expression and responsiveness to flagellin in equine neutrophils and mononuclear phagocytes. *Journal of immunology.* Jun 1 2011;186(11):6263-6270.

90. Sun WC, Moore JN, Hurley DJ, Vandenplas ML, Linden JM, Murray TF. Pharmacologic characterization of novel adenosine A2A receptor agonists in equine neutrophils. *Am J Vet Res.* Sep 2007;68(9):981-987.

91. Leise BS, Yin C, Pettigrew A, Belknap JK. Proinflammatory cytokine responses of cultured equine keratinocytes to bacterial pathogen-associated molecular pattern motifs. *Equine veterinary journal.* May 2010;42(4):294-303.

92. Flaminio MJ, Borges AS, Nydam DV, Horohov DW, Hecker R, Matychak MB. The effect of CpG-ODN on antigen presenting cells of the foal. *Journal of immune based therapies and vaccines.* 2007;5:1.

93. Zhang YW, Davis EG, Blecha F, Wilkerson MJ. Molecular cloning and characterization of equine Toll-like receptor 9. *Veterinary immunology and immunopathology.* Aug 15 2008;124(3-4):209-219.

CHAPTER 3

MICROBIAL LIGAND REGULATION OF TOLL-LIKE RECEPTOR EXPRESSION IN ISOLATED EQUINE MONOCYTES

Kwon S, Vandenplas ML, Figueiredo MD, Salter CE, Andrietti AL, Robertson TP, Moore JN, Hurley DJ.

2010. *Vet Immunol Immunopathol.* 138(3):213-7
www.sciencedirect.com/science/article/pii/S0165242710002667

I. Abstract

Toll-like receptors (TLRs) function as sentinels for the innate immune system, detecting microbial ligands during infection and inflammation. Previous studies indicate that activation of these receptors on equine monocytes leads to discrete pro- and anti-inflammatory responses that are mediated through the induction of specific cytokine genes. However, less is known regarding the regulation of TLR gene expression in these cells. Therefore, we investigated the effects of ligands recognized by TLR 2, 3 or 4 upon TLR 2, 3 and 4 gene expression by equine monocytes. We determined that incubation of monocytes with TLR 2 and 4 ligands, which signal through the intracellular adaptor protein MyD88, induces expression of the TLR 2 and 4 genes, but not the TLR 3 gene. Conversely, incubation with a TLR 3 ligand, which recruits the TRIF adaptor protein, selectively induces expression of the TLR 3 gene, but not TLR 2 or 4 genes. Furthermore, incubation of these cells with TNF-α, the pro-inflammatory cytokine that is a hallmark of TLR activation, does not affect expression of the three TLR genes. These findings suggest that exposure of equine monocytes to microbial ligands but not to endogenous inflammatory mediators may initiate responses that alter the horse's sensitivity to other microbial components during infections.

II. Introduction

Bacterial pathogen-associate molecular patterns (PAMPs) and pattern recognition receptors (PRRs) are often intensively related to the initiation of the host response during infection and inflammation.[1] These innate receptors that function in immune cells and various parenchymal cells including intestinal epithelium in recognition of microbes, and initiation of the innate immune response and regulation of the adaptive immune response have been studied extensively.[2] [3] Toll like receptors (TLRs) are one of the key PRRs and in mammals, 12 members of TLR family have been identified.[4] With respect to microbial recognition, TLR 2, 4, 5, and 9 function to detect common bacterial and fungal structures, whereas TLR 3, 7, and 8 are aimed primarily at viral detection.[5] These receptors signal through activation of specific adaptors, which include signaling proteins, protein kinases, and transcription factors.

Each TLR utilizes a different set of intracellular adaptor proteins, and two major adaptor proteins to initiate inflammatory mediator expression are myeloid differentiation primary response gene 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF).[6] Both the MyD88 and TRIF pathways are activated by TLR 4 ligand such as LPS, whereas ligands for TLR 2 like bacterial lipoteichoic acid, peptidoglycan, and Pam_3CSK_4 activate only the MyD88 pathway. In contrast, TLR 3 ligands such as viral dsRNA or synthetic polyinosinic:polycytidylic acid (poly I:C) trigger activation of the TRIF pathway alone.[7] Eventhough different TLRs recruit a different set of adaptor proteins upon recognition of specific pathogenic products, their downstream signaling cascade merge into an activation of transcription factor nuclear factor-kappa B (NF-κB), Activated NF-κB plays a key role in regulating the innate immune response to infection

by binding to transcription sites and inducing activation of an array of genes for acute phase proteins, inducible nitric oxide synthase, coagulation factors, pro-inflammatory cytokines such as TNF-α , as well as enzymatic activation of cellular proteases.[8]

PRRs including TLRs are often called 'innate receptors' because there is no rearrangement of genetic information encoding them as occurs with immunoglobulin and T-cell receptor genes. Consequently they do not provide a specific memory response as seen in the adaptive immune system.[9] Thus so far, extensive research has been done to uncover the fundamental interactions and processes that lead to primary inflammatory activation by microbial products, and far less is known about the regulation of the receptors that sense these microbial triggers after primary activation. However, it has also become apparent that there is complex positive and negative regulation of the PRRs as a result of crosstalk with other signals.[10] Also, there is limited and contradictory evidence available about secondary activation of monocytes and macrophages by these microbial products. Some evidence suggests that secondary activation is inhibited and dampened, and other evidence supports that up regulation of one or several of the receptors involved in microbial ligand recognition is enhanced.[9,10]

III. Materials & methods

General materials:

Equine serum was purchased from Hyclone (Logan, UT), RPMI-1640, and phosphate buffered saline (PBS) and penicillin/streptomycin were from Mediatech, Inc. (Herndon, VA). *Escherichia coli* 055:B5 LPS was from List Biological Laboratories, Inc. (Campbell, CA), and 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 obtained from InvivoGen (San Diego, CA). Recombinant equine TNF-α was purchased from Thermo Scientific (Rockford, IL), and reconstituted as recommended 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 Universal 18S rRNA Taqman kits were purchased from Applied Biosystems (Foster City, CA). Histopaque-1077 and all other high-grade chemicals were from Sigma–Aldrich (St. Louis, MO).

Animals:

Twelve healthy adult horses, owned and managed by the University of Georgia, were used for this study. Peripheral blood samples were drawn from the jugular vein of each horse using a pyrogen-free syringe containing EDTA as the 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 collected after density-gradient centrifugation over lymphocyte separation solution (Histopaque 1077), suspended and then incubated as previously described to allow for isolation of monocytes.[11,12] After incubation, non-adherent cells were removed by washing, and adherent monocytes were incubated in medium containing 10% equine donor serum, supplemented with 100 units/ml penicillin and 100 mg/ml streptomycin.

Incubation of monocytes with TLR ligands:

The monocytes were incubated either in RPMI-1640 medium containing 10% equine serum alone or in this medium containing serum and one of the following: *E.coli* LPS (100 pg/ml), Pam_3CSK_4 (30 ng/ml), Poly I:C (250 μg/ml), or recombinant equine TNF-α (10 ng/ml). These ligand concentrations were selected based on data in previous studies.[12,13] Incubations were performed at 37°C in a humidified 5% CO_2 atmosphere for various time intervals. Time-matched, unstimulated cells were utilized as basal expression controls. After incubation, the cells were washed with cold phosphate buffered saline, scraped from the plates in RNA cell lysis solution, and stored at –80°C.

RNA extraction and cDNA synthesis for mRNA expression analysis:

After the cell lysates were thawed, total RNA was extracted using the RNeasy kit according to the manufacturer's protocol and incubated with DNase at 24°C for 30 min to remove any contaminating genomic DNA. 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) using 100 ng of RNA as a template. [12]

Real-time quantitative RT-PCR:

Real-time quantitative reverse transcription PCR (qRT-PCR) assays, using SYBR Green as a detector, were performed in an Applied Biosystems 7900HT (Foster City, CA) sequence detection system, with Taqman 18S ribosomal RNA assays serving as endogenous controls as previously described.[13] The TLR primer sets and their validation for qRT-PCR were described previously.[11] Changes in gene expression were calculated by relative quantification against 18S rRNA using the $\Delta\Delta C_T$ method; where $\Delta\Delta C_T$ = ([gene of interest C_T – 18S rRNA C_T]$_{sample}$ – [gene of interest C_T – 18S rRNA C_T]$_{calibrator}$) as previously described.[11,12] Results are 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 value of 1. All reactions were performed in triplicate.

Statistical analysis:

Changes in gene expression over time were compared by one way ANOVA followed by Dunnett's post hoc test where appropriate using GraphPad Prism Software (San Diego, CA). All data are reported as mean ± S.E.M. Values of $p < 0.05$ were considered to represent statistical significant differences, with > 2-fold changes in gene expression being regarded as biologically relevant.

IV. Results & Discussion

Ligand-induced modulation of TLR function (e.g., down-regulation and uncoupling from signaling pathways) and changes in TLR gene expression are important events in the regulation of cellular inflammatory responses. Given the critical role that inflammation plays in the development of complications associated with many equine diseases, including coagulopathy, laminitis and thrombophlebitis,[14] we investigated whether; 1) incubation of equine monocytes with TLR ligands alters TLR gene expression, 2) individual TLR ligands alters the expression of genes for TLRs that recognize other microbial ligands, 3) preferential utilization of one of the two primary TLR-recruited adaptor proteins, MyD88 or TRIF, determines the profile of TLR gene expression, and if 4) TNF-α, a potent pro-inflammatory cytokine produced in response to TLR activation, induces similar changes in TLR gene expression, and potentially increase the sensitivity of these cells to microbial ligands.

Incubation of monocytes with the TLR 4 ligand, LPS, significantly increased expression of mRNA for both TLR 4 (3-fold) after 20 hours, and for TLR 2 after both 4 hours (3.7-fold) and 12 hours (3-fold; Fig. 3.1 and 2). Similarly, incubation of monocytes with the TLR 2 ligand, Pam_3CSK_4, significantly increased expression of mRNA for TLR 2 that peaked at 4 hours (4.9-fold), and of mRNA for TLR 4 that peaked at 20 hours (2.5-fold; Fig. 3.1)

In contrast to the ligands that signal through the MyD88-dependent pathway, incubation of monocytes with the TLR 3 ligand (Poly I:C), which signals exclusively via the TRIF-dependent pathway, did not alter the expression of mRNA for either TLR 2 or TLR 4 during the 20 hours of the study (Fig. 3.1 and 3.2). Incubation of equine

monocytes with the TLR 3 ligand, poly I:C, significantly increased TLR 3 mRNA after 12 hours (9-fold) and 20 hours (26-fold; Fig. 3. 3). Furthermore, neither LPS nor Pam_3CSK_4 caused a significant change in expression of TLR 3 mRNA during the 20-hour period of the study (Fig. 3. 3).

TNF-α, a pro-inflammatory cytokine secreted primarily by macrophages and monocytes, contributes to many deleterious clinical consequences of inflammation (e.g., fever, leukopenia and tachycardia) and is strongly induced by TLR activation.[7, 15] Stimulation of TNF type 2 receptors also results in activation of NF-κB and the subsequent induction of genes associated with inflammation.[16] Consequently, cellular activation by TNF-α could potentially induce TLR gene expression and provide a means for enhancing cellular responsiveness to microbial ligands recognized by those TLRs. After having confirmed that cross talk (i.e. the ability of one to regulate the other) between MyD88 and TRIF dependent pathways does not occur in terms of regulating TLR gene expression, we examined whether incubation of equine monocytes with recombinant equine TNF-α altered expression of TLR genes. To do so, peripheral blood monocytes isolated from 6 horses were incubated with equine recombinant TNF-α (10 ng/ml) for 1, 4, 8, 12 and 24 hours, and expression of TLR2, 3 and 4 was examined by qRT-PCR. At all time points, mRNA expression for TLR2, 3 and 4 was unaltered when compared to that of cells incubated in medium alone (Fig. 3.4). These findings are in contrast to the transient TNF-induced decrease in expression of mRNA for TLR4 that occurred at 6 hours in human monocytes[17], and the increase in TLR4 expression reported for human leukocytes incubated with TNF-α for 3 hours in another study.[18] The observed differences could be explained in four possible ways. First, there may be species

differences in the effects of TNF-α stimulation on TLR expression, with expression of TLRs increasing in response to TNF-α in human leukocytes, but not in equine monocytes. Second, it is possible that TNF-α induced changes in expression of TLRs in equine monocytes may require more than 24 hours to be evident. Third, the recombinant equine TNF-α may lack the ability to induce TLR gene expression because it lacks critical glycosylation or other post-translational modifications present in endogenous TNF. However, we were able to detect a significant increase in the expression of TNF-α and IL-6 genes by qRT-PCR evaluation of the RNA isolated from the same monocytes (data not shown). Finally, it is possible that expression of TLRs is not altered by TNF-α (or not modulated by TNF-α alone). This seems to be a less likely reason as modulation of the expression of TLR genes has been documented in recent *in vitro* studies in which pro-inflammatory cytokines were used to mimic an inflammatory environment.[19,20] In these latter studies, however, mixtures of cytokines were used, suggesting that TNF-α alone may not be sufficient to induce TLR gene expression.

During naturally occurring infections, it is likely that the host will be exposed to a variety of PAMPs, and that several innate receptors will be activated. Indeed, increased expression of TLR 4 and cytokine genes has been documented to occur in septic foals and human patients.[21,22] Therefore, it is possible that interaction between signaling pathways distal to different PRRs could lead to additive, synergistic, or inhibitory effects on the expression of genes that are involved in the immune response. For example, activation of both TLR and NOD-like receptors are required to produce and process mature IL-1β.[23]

In a previous study, we determined that LPS and Pam_3CSK_4 induced expression of MyD88-dependent genes such as IL-1ß, IL-6 and TNF-α in equine monocytes, while the viral RNA mimetic, poly I:C, induced expression of TRIF-dependent genes such as IFN-γ, RANTES (CCL5) and IP-10.[12] In other mammalian species studied to date, activated TLR 4 recruits both MyD88 and TRIF, but TLR 3 utilizes only TRIF, and TLR 2 ligands only signal through MyD88. Our findings with equine monocytes were consistent with minimal recruitment of TRIF after activation of TLR 4 by LPS.[12] One consequence of TLR activation in other species is the up-regulation of TLR gene expression. For example, stimulation of cells with LPS has previously been shown to induce expression of the TLR 4 gene in mice and humans.[18,22] Presumably, this increases the responsiveness of immune cells to subsequent microbial insults. Similarly, the results of the current study indicated that LPS and Pam_3CSK_4 induced the expression of both TLR 2 and TLR 4. Each of these ligands utilizes the MyD88-dependent pathway that culminates in activation of NF-κB, and the subsequent expression of inflammatory genes. In contrast, Poly I:C, a mimic of viral infection, selectively induced expression of TLR 3, a receptor known to signal though recruitment of TRIF.

Based on the results of the current study, there appear to be temporal differences in TLR 2 and TLR 4 gene expression after incubation with LPS and PAM_3CSK_4, with expression of TLR 2 preceding that of TLR 4 by approximately 8 hours (Fig. 3.1 and 3.2). Further, neither of these ligands, which recruit MyD88, induces expression of TLR3, a receptor that signals through TRIF. This pattern of TLR expression may represent an energetically economical strategy for the cells, as TLR 2 and TLR 4

recognize bacterial components while TLR 3 senses viral dsRNA. In addition, the increase in expression of the TLR 3 gene induced by Poly I:C appears to occur later and to a relatively greater degree than the increases in expression of the TLR 2 and TLR 4 genes in response to their respective bacterial components (i.e., PAM_3CSK_4 and LPS; Figs. 3.1 - 3.3). These temporal differences may reflect the longer period of time (often 8-24 hours) required for viral replication within cells than for optimal bacterial growth (as short as 30 minutes) as part of the host-invader relationship.

In summary, the results of the current study indicate that activation of equine monocytes by TLR 2 and TLR 4 ligands increases the expression of genes for these two TLRs, but not for TLR 3. Conversely, cellular activation via TLR 3 selectively causes induction of the TLR 3 gene. These differences in receptor gene profile expression segregate with the different adaptor proteins utilized by TLR 2 and TLR 4, namely MyD88, and TLR 3, namely TRIF. Furthermore, incubation of equine monocytes with the pro-inflammatory cytokine, TNF-α, at a concentration that increased expression of inflammatory genes failed, to induce the expression of any of the three TLRs examined here.

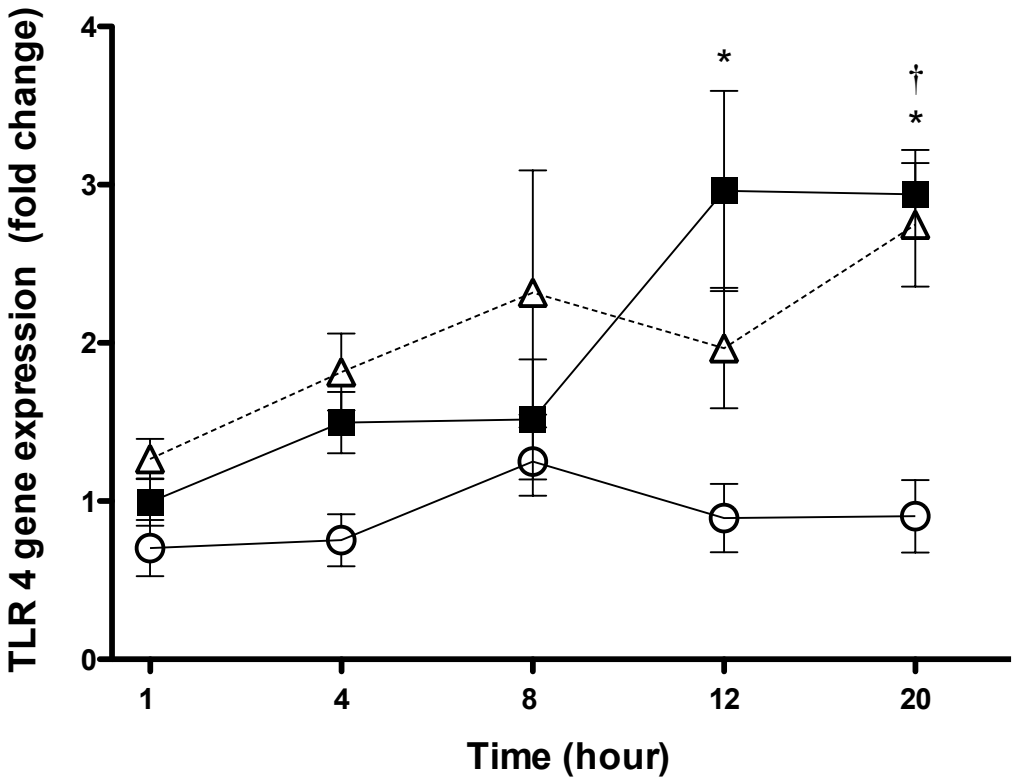

Figure 3.1. Differential induction of TLR 4 response to TLR ligands

Relative expression of mRNA for TLR 4 in isolated equine peripheral blood monocytes incubated with 100 pg/ml *E. coli* LPS (■), 30 ng/ml Pam_3CSK_4 (△), or 250 μg/ml Poly I:C (⊖) for 1, 4, 8, 12, and 20 hours. Stimulatory concentrations of TLR ligands were based on the results of previous studies.[12] Values (mean ± SD) are expressed as fold change. *P<0.05 *E. coli* LPS versus non-stimulated cells at corresponding times. †P<0.05 Pam_3CSK_4 versus non-stimulated cells at corresponding times. (n = 6 horses)

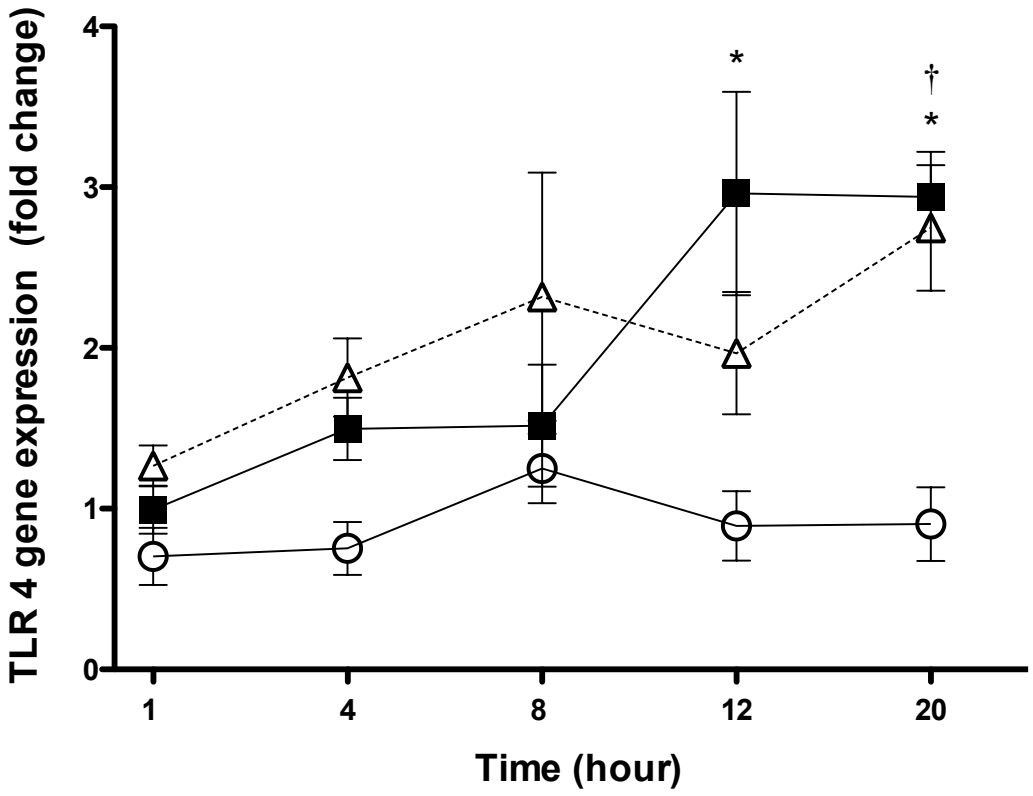

Figure 3. 2. Differential induction of TLR 2 in response to TLR ligands

Relative expression of mRNA for TLR 2 in isolated equine peripheral blood monocytes incubated with 100 pg/ml *E. coli* LPS (■), 30 ng/ml Pam_3CSK_4 (△), or 250 μg/ml Poly I:C (⊖) for 1, 4, 8, 12, and 20 hours. Stimulatory concentrations of TLR ligands were based on the results of previous studies.[12] Values (mean ± SD) are expressed as fold change. *P<0.05 *E. coli* LPS versus non-stimulated cells at corresponding times. †P<0.05 Pam_3CSK_4 versus non-stimulated cells at corresponding times. (n = 6 horses)

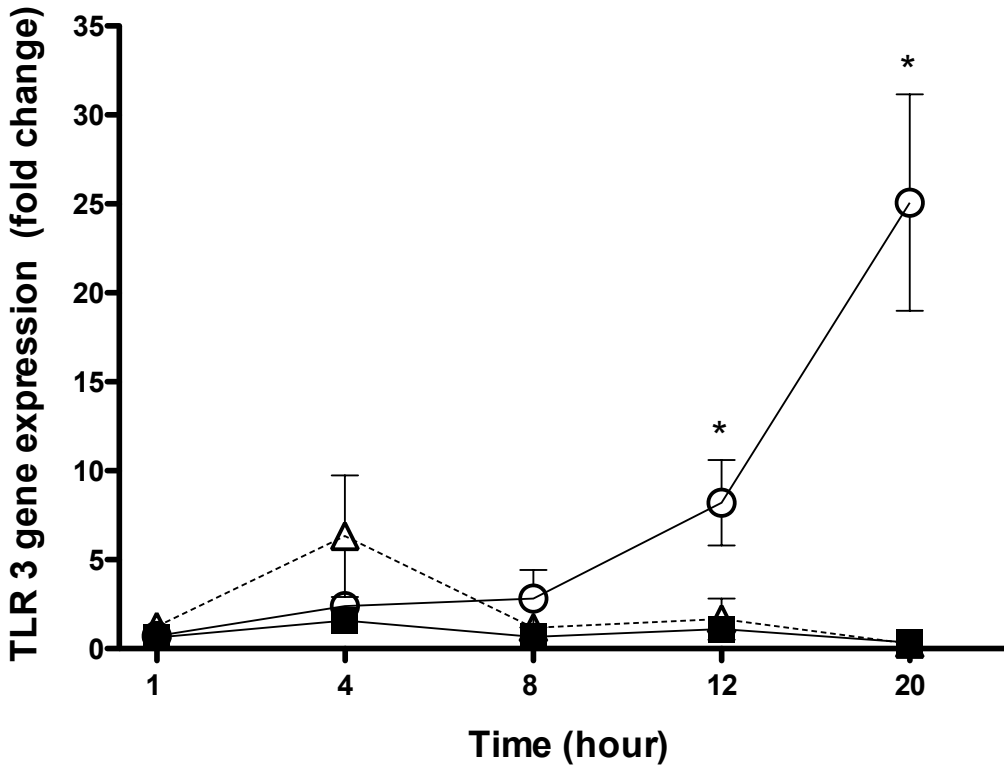

Figure 3. 3. Differential induction of TLR 3 in response to TLR ligands

Relative expression of mRNA for TLR 3 in isolated equine peripheral blood monocytes incubated with 100 pg/ml *E. coli* LPS (■), 30 ng/ml Pam_3CSK_4 (⧋), or 250 μg/ml Poly I:C (⊖) for 1, 4, 8, 12, and 20 hours. Stimulatory concentrations of TLR ligands were based on the results of previous studies.[12] Values (mean ± SD) are expressed as fold change. *P<0.05 Poly I:C versus non-stimulated cells at corresponding times. (n = 6 horses)

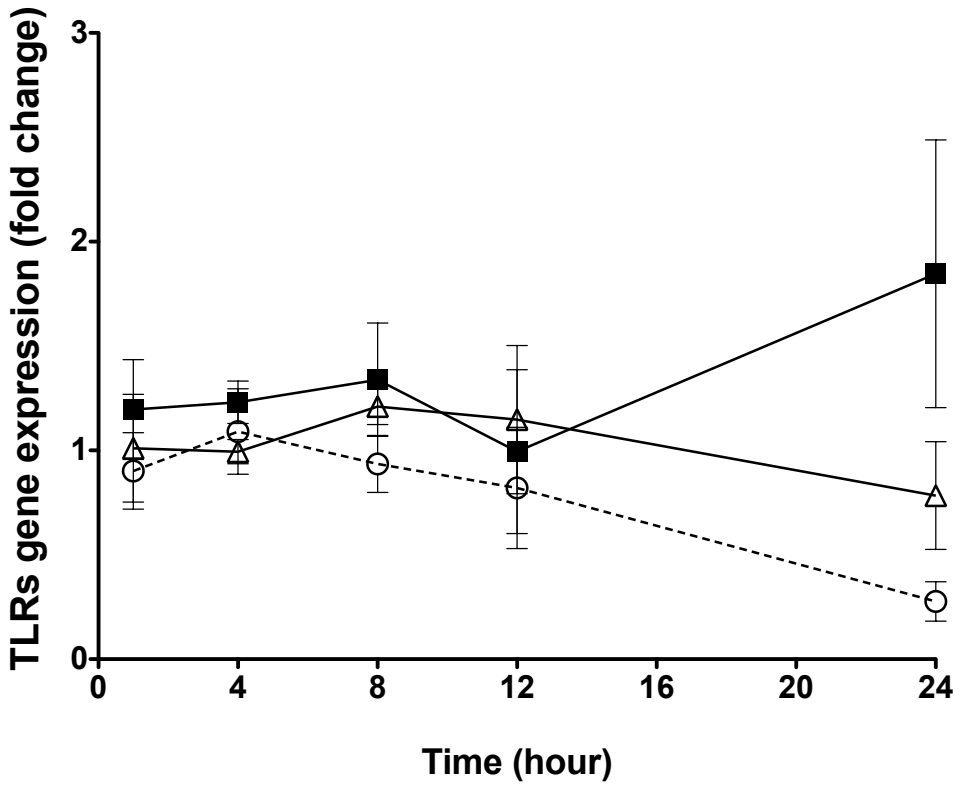

Figure 3. 4. TNF-α does not induce TLR gene expression in equine monocytes

Relative expression of TLR 2 (△), TLR 3 (⊖), and TLR 4 (■) mRNA in isolated equine peripheral blood monocytes incubated with 10 ng/ml recombinant equine TNF-α for 1, 4, 8, 12, and 24 hours. Values (mean ± SD) are expressed as fold increase over control (non-stimulated) cells. (n = 6 horses)

V. References

1. Uematsu S, Akira S. Toll-like receptors and innate immunity. *J Mol Med.* Sep 2006;84(9):712-725.
2. Shuang C, Wong MH, Schulte DJ, Arditi M, Michelsen KS. Differential expression of Toll-like receptor 2 (TLR2) and responses to TLR2 ligands between human and murine vascular endothelial cells. *J Endotoxin Res.* 2007;13(5):281-296.
3. Parker LC, Whyte MK, Dower SK, Sabroe I. The expression and roles of Toll-like receptors in the biology of the human neutrophil. *J Leukoc Biol.* Jun 2005;77(6):886-892.
4. Beutler BA. TLRs and innate immunity. *Blood.* Feb 12 2009;113(7):1399-1407.
5. Wells JM, Loonen LM, Karczewski JM. The role of innate signaling in the homeostasis of tolerance and immunity in the intestine. *Int J Med Microbiol.* Jan 2010;300(1):41-48.
6. Zughaier SM, Zimmer SM, Datta A, Carlson RW, Stephens DS. Differential induction of the toll-like receptor 4-MyD88-dependent and -independent signaling pathways by endotoxins. *Infect Immun.* May 2005;73(5):2940-2950.
7. Akira S, Takeda K. Toll-like receptor signalling. *Nat Rev Immunol.* Jul 2004;4(7):499-511.
8. Cinel I, Opal SM. Molecular biology of inflammation and sepsis: a primer. *Crit Care Med.* Jan 2009;37(1):291-304.
9. O'Neill LA. When signaling pathways collide: positive and negative regulation of toll-like receptor signal transduction. *Immunity.* Jul 18 2008;29(1):12-20.

10. Palazzo M, Gariboldi S, Zanobbio L, et al. Cross-talk among Toll-like receptors and their ligands. *Int Immunol.* May 2008;20(5):709-718.

11. Figueiredo MD, Salter CE, Andrietti AL, Vandenplas ML, Hurley DJ, Moore JN. Validation of a reliable set of primer pairs for measuring gene expression by real-time quantitative RT-PCR in equine leukocytes. *Vet Immunol Immunopathol.* Sep 15 2009;131(1-2):65-72.

12. Figueiredo MD, Vandenplas ML, Hurley DJ, Moore JN. Differential induction of MyD88- and TRIF-dependent pathways in equine monocytes by Toll-like receptor agonists. *Vet Immunol Immunopathol.* Jan 15 2009;127(1-2):125-134.

13. Sun WC, Berghaus LJ, Moore JN, et al. Lipopolysaccharide and TNF-alpha modify adenosine A(2A) receptor expression and function in equine monocytes. *Vet Immunol Immunopathol.* Dec 11 2009.

14. Moore JN, Morris DD. Endotoxemia and septicemia in horses: experimental and clinical correlates. *J Am Vet Med Assoc.* Jun 15 1992;200(12):1903-1914.

15. Kumar H, Kawai T, Akira S. Toll-like receptors and innate immunity. *Biochem Biophys Res Commun.* Oct 30 2009;388(4):621-625.

16. Ihnatko R, Kubes M. TNF signaling: early events and phosphorylation. *Gen Physiol Biophys.* Sep 2007;26(3):159-167.

17. Tamandl D, Bahrami M, Wessner B, et al. Modulation of toll-like receptor 4 expression on human monocytes by tumor necrosis factor and interleukin-6: tumor necrosis factor evokes lipopolysaccharide hyporesponsiveness, whereas interleukin-6 enhances lipopolysaccharide activity. *Shock.* Sep 2003;20(3):224-229.

18. Muzio M, Bosisio D, Polentarutti N, et al. Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells. *J Immunol.* Jun 1 2000;164(11):5998-6004.

19. O'Mahony DS, Pham U, Iyer R, Hawn TR, Liles WC. Differential constitutive and cytokine-modulated expression of human Toll-like receptors in primary neutrophils, monocytes, and macrophages. *Int J Med Sci.* 2008;5(1):1-8.

20. Raicevic G, Rouas R, Najar M, et al. Inflammation modifies the pattern and the function of Toll-like receptors expressed by human mesenchymal stromal cells. *Hum Immunol.* Jan 5 2010.

21. Gold JR, Perkins GA, Erb HN, Ainsworth DM. Cytokine profiles of peripheral blood mononuclear cells isolated from septic and healthy neonatal foals. *J Vet Intern Med.* May-Jun 2007;21(3):482-488.

22. Lorenz E. TLR2 and TLR4 expression during bacterial infections. *Curr Pharm Des.* 2006;12(32):4185-4193.

23. Martinon F. Detection of immune danger signals by NALP3. *Journal of leukocyte biology.* Mar 2008;83(3):507-511.

CHAPTER 4

DISPARITIES IN TLR-5 EXPRESSION AND RESPONSIVENESS TO FLAGELLIN IN EQUINE NEUTROPHILS AND MONONUCLEAR PHAGOCYTES

Soyoung Kwon, Andrew T. Gewirtz, David J. Hurley, Thomas P. Robertson, James N. Moore, and Michel L. Vandenplas

J Immunol 2011 186:6263-6270

www.jimmunol.org/content/186/11/6263.full

I. Abstract

As sentinel cells of the innate immune system, neutrophils and mononuclear phagocytes utilize specific Toll-like receptors to recognize the conserved molecular patterns that characterize microbes. This study was performed to compare the responses of equine neutrophils and mononuclear phagocytes to LPS and flagellin, bacterial components that are recognized by TLR 4 and TLR 5, respectively. Neutrophils and mononuclear phagocytes isolated from healthy horses were incubated in vitro with LPS, flagellin or pronase-inactivated flagellin in the presence or absence of polymyxin B. Production of reactive oxygen species and expression of mRNA for proinflammatory cytokines were used as readouts for activation of neutrophils; production of TNFa was used for the mononuclear cells. Western blot analysis and flow cytometry were used to detect TLR 5 protein in both cell types. Although the neutrophils responded to both LPS and flagellin by producing reactive oxygen species and expressing mRNA for pro-inflammatory cytokines, flagellin had no stimulatory effect on monocytes or macrophages. While both neutrophils and monocytes expressed mRNA for TLR 5, it appeared to be translated into protein only by the neutrophils. Incubation with neither LPS nor IFN-γ altered TLR 5 expression by the monocytes. These findings indicate that flagellin has disparate effects on neutrophils and mononuclear phagocytes isolated from horses, a species that is exquisitely sensitive to the TLR 4 ligand, LPS, and that equine mononuclear phagocytes, unlike corresponding cells of other mammalian species, lack surface expression of TLR5 and do not respond to flagellin.

II. Introduction

Potential role for bacterial flagellin in equine immune responses

Many gastrointestinal diseases of humans and domestic animals have an inflammatory component to their pathogenesis. Of particular importance is the translocation of the structural components of enteric bacteria into the bloodstream, resulting in activation of the innate immune system and generation of the pro-inflammatory mediators that are responsible for patient morbidity and mortality.[1] These microbial components often referred to as "microbial molecular patterns", bind to specific pattern recognition receptors, of which the Toll-like receptor (TLR) family is the most extensively characterized. Of the mammalian species, horses and humans are exquisitely sensitive to LPS, a component of Gram-negative bacterial cell walls that binds to the TLR4/MD-2 complex and induces a pro-inflammatory response in leukocytes (e.g., monocytes and neutrophils).[2-4] LPS has been detected in the circulation of 35-45% of horses with various forms of gastrointestinal disease,[5] has been associated with increased morbidity and mortality, and induces the production of a variety of pro-inflammatory mediators by equine leukocytes.[6,7] Furthermore, administration of purified LPS to horses stimulates coagulation, reduces blood pressure and tissue perfusion and results in lactic acidosis.[8,9] Although several studies have been performed to evaluate the therapeutic potential of interventions directed against the deleterious effects of LPS in horses and other species, conflicting results have been obtained.[10-13, 14] Furthermore, while the results of several studies indicate that both TLR 4 and MD-2 knock-out mice are resistant to LPS challenge, these animals remain highly susceptible to gram-negative infection and develop septic shock.[10,11] Because the systemic inflammatory response is

secondary to the effects of proinflammatory mediators produced by the host in response to microbes and their components, these findings suggest that microbial molecular patterns other than LPS may have important roles in the development of clinical sepsis.

Flagellin, the principal component of the motor unit of bacteria, is important in bacterial adherence to epithelial cells and invasion of the host tissue.[12] Host cells recognize flagellin as a microbial associated molecular pattern through specific regions of TLR 5.[13] There is convincing clinical and experimental evidence that flagellin has an important role in disease pathogenesis. For example, both flagellin and antibodies recognizing flagellin have been detected in human patients and a murine model of sepsis.[15,16] Furthermore, concentrations of antibodies directed against flagellin are increased in human patients with Crohn's disease,[17] the results of in vitro studies indicate that flagellin induces the production of NF-κB dependent pro-inflammatory mediators by human monocytes, murine alveolar macrophages and epithelial cells,[18-20] and in vivo administration of flagellin to mice leads to the development of hypotension, acute pulmonary inflammation, and vascular hypocontractility that resemble findings in septic shock.[21] Finally, flagellin induces colitis in $TLR4^{-/-}$ mice.[22]

In addition to their similar levels of sensitivity to LPS, humans and horses develop severe, life-threatening diarrhea in response to infection with *Salmonella* organisms.[16,23] These flagellated bacteria invade intestinal epithelial cells, where they interact with mononuclear phagocytes (macrophages, dendritic cells, and monocytes) and neutrophils.[24,25] These interactions lead to the synthesis and release of a variety of pro-inflammatory mediators that result in rapid recruitment of additional neutrophils to the site of infection.[21,26] As evidence for the deleterious effects of salmonellosis in horses, a

single strain recently was responsible for a case fatality rate of 36.1% of infected animals and closure of an entire university veterinary teaching hospital.[27]

Due to the central roles played by neutrophils and mononuclear phagocytes in the development of the systemic inflammatory response syndrome initiated by microbial molecular patterns, the present study was performed to compare the responses of equine peripheral blood leukocytes, alveolar macrophages and peritoneal macrophages to flagellin and LPS.

The main hypothesis of this study is that similar to bacterial endotoxin, bacterial flagellin will stimulate isolated equine leukocytes.

III. Materials & methods

General materials:

Equine serum was purchased from Hyclone (Logan, UT). RPMI-1640, phosphate-buffered saline (PBS) and penicillin/streptomycin were from Mediatech, Inc. (Herndon, VA). *Escherichia coli* 055:B5 lipopolysaccharide (LPS) was from List Biological Laboratories, Inc. (Campbell, CA), and Bradford reagent was purchased from Biorad (Hercules, CA). RNeasy Mini Kits and the RNase-Free DNase set were purchased from Qiagen Inc. (Valencia, CA). Oligonucleotide primers were synthesized by Eurofins MWG Operon (Huntsville, AL). The High Capacity cDNA archive kit, SYBR Green PCR master mix, and universal 18S rRNA Taqman kit were purchased from Applied Biosystems (Foster City, CA). Pronase was from Roche (Nutley, NJ) and accutase was from eBioscience (San Diego, CA). Avidin–horseradish peroxidase was from BD

Biosciences (San Jose, CA). Anti-equine TNF-α polyclonal antibody and anti-equine biotin-labeled TNF-α polyclonal antibody were from Endogen (Woburn, MA). ECL reagent and HRP-conjugated secondary antibodies were purchased from GE Healthcare (Pittsburgh, PA). Primary antibodies used in the studies were as follows: MHC class I, MHC class II (VMRD, Pullman,WA), TLR5 (Imgenex, San Diego, CA), α-tubulin (Calbiochem, Gibbstown, NJ). Recombinant equine IFN-γ was purchased from R & D Systems (Minneapolis, MN) and the reticulocyte in vitro translation kit was purchased from Ambion Inc (Austin, TX). The FITC-labeled anti-mouse secondary antibody used for flow cytometry, Histopaque-1077 and all other high-grade chemicals were obtained from Sigma–Aldrich (St. Louis, MO).

Flagellin preparations:

Flagellin, purified from *S. typhimurium* (SL3201)-conditioned medium by sequential anion/cation exchange and polymyxin-B agarose chromatography, was obtained from Dr. Andrew Gewirtz (Emory University, Atlanta, GA). [28] Due to the sensitivity of equine leukocytes to LPS, the *Limulus* amebocyte lysate assay (Charles River, Wilmington, MA) was used to determine the level of LPS contamination in the flagellin preparation. To remove additional LPS from this preparation, an LPS removal column, ProteoSpin™ (Norgen Biotek Corp. Thorold, Cananda) was used according to the manufacturer's instructions. This procedure reduced the concentration of LPS to < 1 pg LPS/μg flagellin, well below the minimal concentration of LPS proven to stimulate equine monocytes and neutrophils.[29] [30]

Protein concentrations were measured by Bradford assay using BSA as the standard. For some experiments, flagellin was inactivated by incubating it with pronase (1 mg/ml) in 100 mM HEPES buffer, pH = 7.2. Briefly, 200 μg flagellin was incubated for 2-6 h with 20 μg pronase in a reaction volume of 200 μl at 40°C. The samples were then heated at 95°C for 1 h to inactivate the pronase.[31]

Horses and blood collection:

Ten adult horses owned and managed by the University of Georgia were used as blood donors for the studies described below. The horses were assessed to be healthy based on physical examination findings. The skin over the right jugular vein was aseptically prepared, and peripheral blood samples were collected into syringes containing EDTA as an anticoagulant. All experimental protocols using animals were reviewed and approved by the Animal Care and Use Committee at the University of Georgia.

Cell isolation and culture:

Isolation of equine neutrophils and monocytes

Neutrophils and monocytes were isolated by density-gradient centrifugation over Histopaque 1077 as previously described.[29] Neutrophils were suspended at a final concentration of 3×10^7 cells/ml in RPMI 1640 medium containing 10% fetal bovine serum, 2 mM L-glutamine, 2 mM sodium pyruvate, and 50 μg/ml gentamicin. Cells were then incubated in a humidified 5% CO_2 atmosphere at 37°C for 90 minutes to reduce

signal induced by exposure to the Histopaque 1077 solution. After this incubation period, cell viability determined by trypan blue dye exclusion exceeded 98%.

Mononuclear cells were suspended in RPMI-1640 at a final concentration 4×10^6 cells/ml, and 2×10^6 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. Non-adherent cells were removed and adherent monocytes were then incubated in RPMI-1640 containing 10% equine serum supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin. For TLR 4 and 5 mRNA and protein expression studies, approximately 4×10^7 mononuclear cells were incubated in sterile 150 × 15 mm Petri dishes for 2 h at 37 °C in a 5% CO_2 atmosphere in the aforementioned medium. After non-adherent cells were removed, adherent monocytes were released by adding accutase (10 ml/dish) for 10 min at 37 °C.

Preparation of equine alveolar and peritoneal macrophages

Bronchoalveolar lavage cells were obtained from two horses. Each horse was sedated with a combination of xylazine hydrochloride (1.0 mg/kg, IV) and butorphanol tartrate (0.07 mg/kg, IV). Bronchoalveolar lavage was performed using a 10 mm internal diameter, 2.4 m BAL catheter (Jorgenson Laboratories, Loveland, CO, USA). Warm saline solution (240 ml) was administered through the tube and retrieved into sterile bottles. The bronchoalveolar lavage fluid was centrifuged at 400 ×g for 10 min after which the cell pellet was washed twice with PBS at 20°C and the cells collected as described previously.[32] After washing, the cells were suspended in 10 ml PBS for viability assessment and cell counting in a hemacytometer. Cells were then suspended in RPMI-1640 medium at 4×10^6 cells/ml, and 2×10^6 cells were added to each well of a

sterile 24-well polystyrene plate and incubated for 1 h at 37 °C, in a 5% CO_2 atmosphere. Non-adherent cells were removed by washing, and adherent alveolar macrophages were incubated in the aforementioned medium. Peritoneal cells were obtained through abdominal paracentesis as previously described.[33] Briefly, the ventral abdomen of the horse was clipped, scrubbed, and intradermal and subcutaneous 2% lidocaine was injected 5 cm caudal to the xyphoid process. A stab incision was then made through the skin and abdominal fascia using a scalpel blade, followed by blunt insertion of a sterile teat canulla into the peritoneal cavity. 100 ~ 200 ml of peritoneal fluid was collected by gravity into sterile conical centrifuge tubes that contained EDTA as an anticoagulant. The peritoneal fluid was centrifuged, washed, and plated in a similar fashion to the alveolar cells. For isolated macrophage stimulations, washed cells were incubated in RPMI-1640 medium containing 10% equine serum containing *E.coli* LPS (1 ng/ml) or flagellin (200 ng/ml) with peritoneal macrophage LPS stimulations also done in the presence of polymyxin B (13 units/ml).

Isolation of leukocytes for flow cytometric analysis

For flow cytometric studies, total peripheral blood leukocytes were isolated from blood samples obtained from 6 donor horses. In brief, leukocyte-rich plasma was transferred to sterile 50 ml centrifuge tube, diluted with PBS solution, and cells collected by centrifugation at 400 ×g for 10 minutes at 20°C. The supernatant was removed and the residual erythrocytes in the cell pellet were lysed with distilled water and normal osmotic conditions restored by addition of an equal volume of 2 × PBS. After two additional washes with PBS, the leukocytes were counted in a hemocytometer and viability assessed by exclusion of trypan blue.

Measurement of reactive oxygen species (ROS) produced by isolated neutrophils:

Neutrophils were diluted by the addition of the aforementioned medium to achieve a final concentration of 3×10^5 cells/well in 96-well flat-bottom tissue culture plates. Cells were incubated with flagellin ranging from 100 pg/ml to 10 μg/ml in the absence or presence of 13 units/ml polymyxin B. For some experiments, inactivated flagellin was used to demonstrate the specificity of the reaction to flagellin. Reactive oxygen species were detected using the non-fluorescent dihydrorhodamine dye, as described previously. [30] Plates were incubated in a humidified atmosphere of 5% CO_2 at 37°C for 2 hours. Fluorescence was measured by use of a fluorescent plate reader with a 485-nm excitation filter and 538-nm emission filter and reported in arbitrary fluorescent units (AFU). The mean and standard deviation of the triplicate readings were calculated. The induced ROS activity was compared between experiments by generating a response ratio (RR: Average AFU of stimulation-induced reactive oxygen species activity / Average AFU of endogenous reactive oxygen species activity measured from unstimulated cells).[34]

Measurement of TNF-α protein produced by isolated monocytes and macrophages:

After being washed two times with RPMI-1640, the monocytes were incubated in RPMI-1640 medium containing 10% equine serum containing either *E.coli* LPS (100 pg/ml) or flagellin (300 ng/ml, 1 μg/ml, and 3 μg/ml) in the presence or absence of polymyxin B (13 units/ml). In certain experiments, pronase-digested flagellin was included as an additional control. For all experiments, after a 6 h incubation at 37 °C in 5% CO_2, cell supernatants were collected for determination of TNF-α concentration using

an equine specific ELISA as previously described. [30] Briefly, supernatant samples were incubated in a microtiter plate coated with anti-equine TNF-α polyclonal antibody. Subsequently, a biotin-labeled anti-equine TNF-α antibody was used for the detection of immobilized TNF-α protein. Then avidin-horseradish peroxidase was added, followed by color development with ABTS substrate. The spectrophotometric absorbance of the samples was determined at 405 nm using an automated microplate reader (Dynatech Laboratories, Chantilly, VA). TNF-α production was normalized and transformed to percentile change using negative control cells incubated with media only (0%) and positive control cells incubated with 100 pg/ml *E.coli* LPS (100%).

Identification of a partial equine TLR5 mRNA sequence required for RT-PCR primer design:

A BLASTN search of equine sequences in GenBank using the human TLR5 mRNA sequence (Accession #: NM 003268.) identified an equine EST (EST name: CT020002A20G09) with strong homology (E-value = 1e-137, score = 490 bits). The identified equine TLR 5 sequence is 454 bp in length and maps to nucleotides 2501-2947 of the human TLR 5 query sequence. RT-PCR primers (Forward: 5'-GGGCCTCTACTCTGTTTCCA) and (Reverse: 5'-CACCACCCGTGTCTAAGGAA) were designed with Primer3 software to generate an amplicon of 449 bp with equine neutrophil RNA as template in the Qiagen ONeStep RT-PCR kit. The amplicon (Figure 4) was purified after agarose gel electrophoresis using the QIAquick gel purification kit (Qiagen, Valencia, CA) and sequenced in both directions by Eurofins MWG Operon (Huntsville, AL) to confirm its identity by BLASTN homology search of GenBank

sequences. The high-quality sequence (Q > 20) was in concordance with the published equine TLR5 EST sequence. This primer set was also used for end-point RT-PCR assays. A second set of internal primers (Forward: 5'-TCCATGGAGGGTTGTGATGA and Reverse: 5' CCCCGGAACTTTGTGACAAT) for equine TLR5 was designed using PrimerExpress software and validated for SybrGreen qRT-PCR gene expression assays as previously described for equine gene expression studies.[35] Differences in TLR gene expression were calculated by relative quantification against 18S rRNA using the ΔC_T method; where ΔC_T = gene of interest C_T - 18S rRNA C_T. All reactions were performed in triplicate [35]. For these studies GAPDH expression served as a comparative house-keeping control gene between TLR neutrophil and monocyte expression profiles.

RNA isolation, cDNA synthesis, and real-time qRT-PCR assays for mRNA expression analysis:

The equine target genes of interest in this study were TNF-α, IL-1ß, and IL-10, with gene expression analysis done by qRT-PCR assay as previously described.[35] Approximately 1×10^7 isolated neutrophils in the aforementioned medium were added to sterile polystyrene tubes and incubated for 1 or 4 h at 37 °C in a 5% CO_2 atmosphere in the presence or absence of either 100 ng/ml LPS or 200 ng/ml flagellin. After incubation, cold PBS solution was added to tubes to stop cellular activity. The tubes were then centrifuged at 4°C for 5 min, the PBS solution removed, and cell pellets were lysed with RNA 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 to remove any genomic DNA. 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 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 control as previously described.[30,35] The validated primer sets for equine TLR4, TNF-α, COX-2 and IL-10 qRT-PCR assays have been previously reported.[35] Changes in cytokine gene expression over time were calculated by relative quantification against 18S rRNA using the $\Delta\Delta C_T$ method; where $\Delta\Delta C_T$ = [(gene of interest C_T - 18S rRNA C_T)sample - (gene of interest C_T - 18S rRNA C_T)calibrator], with the calibrator being template from time-matched, non-stimulated cells. Results were expressed as the mean fold change in gene expression at each time point. The $\Delta\Delta C_T$ of the time-matched, non-stimulated sample used as the calibrator was assigned a fold change in expression of 1, with > 2-fold changes in expression regarded as biologically relevant. All reactions were performed in.

Endpoint RT-PCR for TLR 4 and 5 mRNA expression analysis:

Non-stimulated neutrophils and accutase-released monocytes were used to monitor and compare basal expression of TLR 4 and 5 mRNA. For these studies, cells were obtained from 6 healthy horses. Equine glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal housekeeping gene for endpoint RT-PCR gene expression analysis. The primers for equine GAPDH were forward: 5'-AAGTGGATATTGTCGCCATCAAT and reverse: 5'-AACTTGCCATGGGTGGAATC

while the primers used for equine TLR4 endpoint RT-PCR detection were the same as those used for the qRT-PCR assays.[35] Briefly, for endpoint RT-PCR, 100 ng RNA template together with primers required to generate the 449 bp TLR5 amplicon, the TLR4 amplicon or the GAPDH amplicon were used in the OneStep RT-PCR assay kit (Qiagen) according to the manufacturer's protocol. The assays were run at 95 ^{o}C for 1 min, 50 ^{o}C for 30 min, 95 ^{o}C for 15 min followed by 35 cycles of 94^{o}C, 57^{o}C, and 72 ^{o}C for 1 min and a final polishing cycle of 10 min at 72 ^{o}C. Amplicons (12.5 μl of each reaction) were resolved by electrophoresis in 1.5% agarose 0.5X TBE gels and stained with ethidium bromide to visualize products for CCD camera image capture followed by densitometric analysis. These studies allowed for qualitative comparison of TLR5 mRNA expression in isolated equine neutrophils and monocytes.

Western blotting for TLR5:

Cell pellets from isolated equine monocytes and neutrophils were suspended and allowed to swell in hypertonic buffer solution (10mM HEPES, pH = 7.9, 10 mM KCl, 0.1 mM EGTA, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF) for 10 min on ice. The cell suspensions were lysed by the addition of NP-40 to 0.625% and the nuclei removed by centrifugation at 14,000 x*g* for 2 min at 4 ^{o}C. Total membrane and cytosol proteins from both leukocyte populations, adjusted to equivalent concentrations using the Bradford assay, were fractionated by 4 - 20 % SDS-PAGE and transferred onto nitrocellulose membranes for Western blotting. The blots were probed with antibody to TLR 5 (1:200) or α-tubulin (1:300) using a SNAP i.d. protein detection system (Millipore, Billerica,

MA), and processed with enhanced ECL reagent. Images were captured with a CCD camera and used for denisotmetric analysis.

Densitometric and data analysis:

All non-saturated images were captured using an Ultra-Lum™ (Claremont, CA) imaging system. The densitometric quantification of selected Western blots and PCR agarose gels images were performed using Ultraquant 6.0 software. Values were normalized with their respective internal controls (i.e., GAPDH mRNA or α-tubulin protein). Thereafter, densitometry values were averaged between samples obtained from 6 different horses and reported as mean ± S.E.M.

Immunostaining:

Total leukocytes were suspended in sterile FACS buffer solution (PBS supplemented with 0.2% bovine serum albumin and 0.1% sodium azide) at a concentration of 2 x10^7 cells/ml and 100 µl aliquots of the suspension added to wells of 96-well round bottom microtiter plate. After centrifugation (400 ×g for 10 min at 4°C) and removal of the FACS buffer, the cells were stained in separate wells with 20 µl of either primary antibodies for MHC class I (1:50), MHC class II (1:100) (used to discriminate among the cell populations) or TLR5 (serial dilutions) in FACS buffer. Antibody concentrations utilized had been optimized in preliminary studies (*data not shown*). The plates containing primary antibody were incubated for 20 min at 4°C and then each well was washed three times as previously described.[34] After the third wash,

the FACS buffer was removed and replaced with 100 µl of a FITC-conjugated goat anti-mouse IgG (1:200, Sigma, St. Louis, MO). The plates were incubated and washed as before. The cells were then suspended in 100 µl of cold FACS buffer, and added to a 1.5 ml microfuge tube containing 400 µl of cold FACS fixative (4% formalin in FACS buffer).

***TLR 5 flow cytometry*:**

For each sample, data from 10,000 cells were collected and analyzed using an Accuri C6 Cytometer (Accuri Cytometers, Inc., Ann Arbor, MI). Full list mode data were acquired and saved for each sample. Total leukocytes were chosen as the test population to reduce the non-specific antibody binding effects observed after monocytes were isolated by adherence. In preliminary studies it was determined that neutrophil, monocyte and lymphocyte populations within a total leukocyte sample could be gated based on forward and side scatter to yield clean populations for analysis. Further, the three gated populations had the same staining patterns as each as the isolated leukocytes, except that the monocyte population had a much lower non-specific staining level in the gated population from total leukocytes than in adherence isolated monocytes. Use of light scatter based leukocyte populations had previously been done with this TLR5 antibody for staining of canine leukocytes populations.[36] The samples were gated based on forward angle and side scatter into windows that had previously been established for equine lymphocytes, monocytes and neutrophils.[34] Each cell type was assessed for the percentage of cells having fluorescence staining that exceeded the second antibody background. MHC class I and II mean fluorescent brightness values were also

determined as a further check of the purity of each cell type. The gating and assessment of fluorescence was carried out using Accuri analysis software as previously described.[37]

Cell-free translation assay:

High quality RNAs from isolated equine monocytes and neutrophils obtained from three blood donors were used for *in vitro* reticulocyte lysate translation reactions according to the manufacturer's instructions (Rectic Lysate IVT™, Ambion). Briefly, 1 µg of RNA isolated from each cell preparation, was incubated with 17 µl of rabbit reticulocyte lysate in a total volume of 25 µl for 1 h at 30°C. Assay products were resolved by 4 - 20 % SDS-PAGE and transferred onto nitrocellulose membranes for Western blotting. TLR5 antibody (1:200) was used to detect TLR 5 protein as described above. Densitometry was used as before to record concentrations of TLR 5 protein in each reaction sample examined.

Data analysis:

The EC_{50} for flagellin was generated 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 pair-wise using F tests. Differences between treatments and time points were detected by repeated measures ANOVA. Values of $P < 0.05$ were considered to be significantly different and individual

P-values are reported in the figures, as appropriate. The data are presented as the mean values (mean ± S.E.M.), and the results presented in the figures are representative of at least three independent observations with cells from six different horses, except for the alveolar macrophage data that was generated with cells obtained from two horses.

IV. Results

Flagellin activates equine neutrophils but not monocytes

We have previously reported that equine peripheral blood monocytes are highly sensitive to activation by LPS, a TLR 4 ligand, and respond well and selectively to TLR 2 and TLR 3 ligands.[29] To explore the potential ability for bacterial flagellin to contribute to the inflammatory response in horses, we stimulated equine peripheral blood monocytes with increasing concentrations of highly purified flagellin. Unexpectedly, flagellin at concentrations as high as 3 μg/ml failed to induce TNF-α secretion from monocytes isolated from six horses (Fig. 4.1). In contrast, LPS, a known and potent activator of equine monocytes, induced TNF-α secretion from these cells to a similar extent as previously reported. Co-incubation of monocytes with polymyxin B reduced LPS-induced secretion of TNF-α by >95% while having no effect on the minimal responses of these cells to flagellin. Similarly, pre-incubation of the flagellin with pronase did not change this non-responsiveness (Fig. 4.1). This finding was unexpected as human monocytes and murine alveolar macrophages are sensitive to flagellin stimulation, producing cytokines upon flagellin treatment *in vitro*.[20,38] As a lack of response to flagellin could be due to degradation of the flagellin, we resolved the flagellin by SDS-

PAGE and demonstrated a single band of 55 kd indicative of an intact protein (*data not shown*). We next determined whether flagellin could induce ROS production in isolated equine peripheral blood neutrophils, as these cells contribute to innate immune response of horses to TLR ligands.[7] Interestingly, incubation of equine neutrophils with a range of concentrations of flagellin caused a significant production of ROS with an EC_{50} of 20 ng/ml with a response plateau at ~ 1 μg/ml (Fig. 4.2). Co-incubation of neutrophils from six different horses with polymyxin B to inhibit any residual contaminating LPS in the flagellin preparation did not cause a significant rightward shift of the dose response curve (EC_{50} = 40 ng/ml). Polymyxin B co-incubation did, however, significantly reduce ROS production in LPS stimulated neutrophils, as did pre-incubaton of flagellin with pronase (Fig. 4.3).

Both equine monocytes and neutrophils contain TLR 5 mRNA transcripts

To better understand the dramatic difference in the response of equine monocytes and neutrophils to flagellin, we investigated the role of TLR 5 in the two cell types. TLR 5 is the cell surface receptor for bacterial flagellin, and to date, no additional proteins have been shown to be required for flagellin/TLR5 interaction.[39] Therefore, we compared the expression pattern of TLR5 in equine monocytes and neutrophils. Real-time qRT-PCR primers were designed and validated, and the difference between TLR 5 and TLR 4 mRNA expression was compared in equine neutrophils and monocytes. Equine neutrophils expressed 1.8 times more TLR 5 mRNA than equine monocytes based on ratios of the ΔC_T values obtained from the RT-qPCR assays of TLR 5 expression (Table 4.1). Similar results were observed in endpoint RT-PCR assays using different TLR5

amplification primers (Fig. 4.4A); TLR 5 mRNA is expressed in both equine monocytes and neutrophils. However, after normalization of band intensity to that of GAPDH transcripts in the same samples, densitometry analysis revealed that TLR 5 transcripts were at least twice as abundant in equine neutrophils when compared to monocytes (Fig. 4.4B). Neutrophils also expressed 1.9 times more TLR 4 mRNA than monocytes (Table 4.1). Our finding that equine monocytes express 1.5 times more TLR 4 than TLR 5 may explain the greater responsiveness of these cells to LPS than to flagellin.[7,40] As maturation of peripheral blood monocytes to tissue macrophages could lead to a greater concentration of TLR 5 in macrophages, we compared expression of TLR 5 in these two populations. As shown in Table I, there is no significance difference in TLR 5 expression between equine monocytes and alveolar macrophages. Additionally, all three cell populations showed a similar degree of GAPDH gene expression.

Both TLR5 mRNAs from equine monocytes and neutrophils can be translated

After finding that equine monocytes do not respond to flagellin while having mRNA for TLR 5, we used an *in vitro* translation system to determine whether or not the TLR 5 mRNA in both cell types can be translated into protein. The results of these assays indicated that mRNAs from isolated monocytes and neutrophils yield TLR 5 protein that was detected by western blot analysis using antibody against TLR 5. However, twice the amount of monocyte RNA was needed to produce a similar amount of TLR 5 protein to that translated from RNA isolated from neutrophils (Fig. 4. 5).

Equine monocytes and neutrophils show differential expression of TLR 5

While the difference in responsiveness of equine neutrophils and monocytes to flagellin can be explained in part by the difference in TLR 5 mRNA expression, equine monocytes do express TLR 5 mRNA and could therefore have functional TLR 5 protein on their surface. We therefore performed flow cytometric analysis of cell surface TLR 5 expression on isolated equine leukocytes. Total leukocyte samples were used to minimize cell surface damage during the cell isolation process. To separate leukocyte populations, cells were gated according to their forward (FSC) and side scatter (SSC) profiles (data not shown). Samples stained with MHC class I and II were used to check the purity of cell populations; both neutrophils and mononuclear cells stained strongly for MHC class I while only monocytes were strongly positive for MHC class II (Table 4. 2). Strong signal for TLR 5 was detected on the neutrophil populations and weak signal was detected on the monocyte populations (Table 4. 2). It is possible that the gated monocyte population contained contaminating lymphocytes that contribute to the weak TLR 5 staining observed in the monocyte populations. However, similar staining trends were observed when isolated neutrophils and monocytes were studied, although, higher non-specific staining in the presence of secondary antibody alone was also observed particularly in the isolated monocytes (Table 4. 2). Whereas incubation of human monocytes with IFN-γ modulates TLR5 expression[41], surface expression of TLR 5 on neither equine monocytes nor neutrophils was altered by 3 or 20 hrs of incubation of peripheral blood leukocytes with either 100pg/ml *E.coli* LPS or 30 ng/ml equine recombinant IFN-γ (Table 4. 3).

To demonstrate that the flow cytometry data were due to specific binding to TLR 5 on neutrophils and also that TLR 5 in monocytes was not sequestered in the intracellular environment, we performed TLR5 western blot analysis on neutrophil and monocytes lysates enriched for cytosol and membrane proteins. As shown in Figure 4.6, neutrophil lysates have a clear ~95 kd band in agreement with the size of TLR 5 in other species. In contrast, isolated monocytes did not generate a similar signal. It would thus appear that equine monocytes do not express TLR 5 on either the cell surface or in the cytosolic compartment (Fig. 4.6). Taken together, the RT-PCR, flow cytometry and western blot data suggest that equine monocytes do not express TLR 5, which clarifies the unresponsive nature of this leukocyte population to flagellin.

Flagellin induces cytokine gene expression in equine neutrophils in a similar fashion to TLR 4 and TLR 2 ligands

Given the unique profile of TLR 5 expression in equine leukocytes, there is a remote possibility that equine TLR 5 signaling may differ to that of TLR 2 and TLR 4 that also signal predominately through MyD88.[39] We therefore compared the expression of mRNA for TNF-α, COX-2 and IL-10 in equine neutrophils stimulated with flagellin (TLR 5), LPS (TLR 4) or PAM_3CSK_4 (TLR2). EC_{90} concentrations (Sun et al.[30] and unpublished data) of the aforementioned TLR ligands induced a comparable degree of TNF-α and COX-2 gene expression at 1 h and of IL-10 at 4 h (Fig 4.7) without significant differences between the three ligands. It thus appears that equine TLR 5 signals in a similar fashion to other MyD88-coupled equine receptors.

Maturation of monocytes into tissue macrophage may increase TLR 5 gene expression and protein synthesis thereby allowing macrophage to respond to flagellin stimulation. Therefore, alveolar and peritoneal macrophages, two populations derived from monocytes, were investigated for their responsiveness to flagellin. Our results indicated that both cell types have only extremely weak responsiveness to flagellin stimulation, similar to that detected for equine monocytes (Fig. 4. 8).

V. Discussion

Microbes carry an array of structurally distinct PAMPs that are recognized by different TLRs and other PRRs. In this study, we compared the responses of equine neutrophils, monocytes and macrophages to LPS and flagellin. The importance of flagellin in the pathogenesis of infectious diseases of mammals is underscored by the finding that it circulates as a free protein in the plasma of rats with experimentally-induced gram-negative sepsis, has been detected in the sera of human patients with sepsis, [21] and antibodies directed against flagellin have been documented in the sera of human patients with Crohn's disease or short bowel syndrome.[16, 17, 42] Flagellin is expressed by a variety of motile bacterial species including *Salmonella*, *E. coli, Bacillus spp., Campylobacter, and Bordetella bronchiseptica* that are associated with equine diseases such as colitis and bronchopneumonia.[43]

In the relatively few studies published to date, flagellin activates mononuclear cells and neutrophils isolated from other species, and does so by interacting with TLR5.[20] For example, human peripheral blood monocytes express moderate amounts of TLR 5[44]

and flagellin induces marked pro-inflammatory cytokine gene expression in these cells.[45] Furthermore, there is evidence that flagellin, acting through TLR 5, delays apoptosis in human neutrophils, and thereby perpetuates inflammation.[46] In mice, TLR 5 mRNA expression appears to be restricted to dendritic cells and macrophages that originate from glomerular mesangial cells (SV40 MES-13), and is not detected in murine neutrophils.[47] Furthermore, murine alveolar macrophages respond strongly to flagellin in studies that illuminated the role of TLR5 in pulmonary infections.[20] Although bovine antigen presenting cells express relatively less mRNA for TLR 5 than for TLR 4[48], bovine macrophages respond to flagellin in a manner that is similar to responses of human macrophages.[24] Finally, while canine peripheral blood mononuclear cells express less TLR5 than other TLRs, they also respond significantly to flagellin by secreting TNF-α.[49]

Based on this information, we expected the responses of equine monocytes and neutrophils to flagellin to be at least qualitatively similar to those reported for other species. Because TLRs are expressed on the surface of circulating innate immune cells whose primary function is to identify and eliminate invading microorganisms, the lack of response of equine monocytes to bacterial flagellin was unexpected, particularly as these cells are highly sensitive to LPS with an EC_{50} of 30 pg/ml, through TLR 4 activation, and respond strongly to synthetic TLR 2 and TLR 3 ligands.[29] In contrast, the equine neutrophils in the present study responded robustly to flagellin; we have previously reported that they are far less sensitive to LPS (EC_{50} of 9 ng/ml) than equine monocytes.[7] To study the difference in responsiveness of equine monocytes and neutrophils to flagellin, we compared the concentrations of TLR 5 mRNA in these two cell types. While equine monocytes contain TLR 5 mRNA, it is not as abundant as it is in

neutrophils. Furthermore, although the results of cell-free translation assays indicate that monocyte TLR 5 mRNA can be translated into protein, this does not occur in intact monocytes.

Because equine neutrophils respond strongly to flagellin, TLR 5 is clearly a functional receptor in these cells. Based on our findings, it appears that the lack of response of equine monocytes to flagellin is related to the low density of TLR 5 in these cells. The reason for the lack of TLR 5 protein in equine monocytes remains unresolved, but may be due to a specific block on TLR 5 mRNA translation or to rapid turnover of the small amount of TLR 5 protein synthesized by these cells. The differences in expression of TLR 5 in equine monocytes and neutrophils may be important in the functional roles of these two cell types in innate immune responses of horses. For instance, early detection and removal of flagellated bacteria by neutrophils, the first leukocytes recruited into tissues after microbial invasion, may be an effective way to provide protection against a commonly present microbial molecule without initiating excessive stimulation of immune system in equine diseases associated with bacterial infection (e.g. colitis and sepsis in foals).[23, 50] Monocytes, on the other hand, are recruited later to sites of infection and play an important role in producing mediators that help restrict the spread of microorganisms and that promote removal of cellular debris. The lack of response of equine mononuclear phagocytes to flagellin may help account for this species propensity to suffer fatal infections caused by *Salmonella* organisms[23,50] that have evolved mechanisms to down-regulate their surface expression of flagellin[51] and thereby avoid host responses that otherwise might prevent tissue invasion.

In contrast to immune responses to intact bacteria, the responses of leukocytes to circulating microbial molecular patterns may be different, particularly during gastrointestinal diseases in which translocation of such molecular patterns from the gut into the bloodstream occurs. In this regard, equine monocytes respond to far lower concentration of LPS than neutrophils, emphasizing the fact that individual microbial molecular patterns can selectively activate different equine leukocyte populations.[7,40] Similarly, differences in concentrations of individual microbial molecular patterns may also direct the outcome of the equine innate immune response. For example, equine monocytes are highly sensitive to LPS (EC_{50} ~10-30 pg/ml), moderately sensitive to the synthetic TLR 2 ligand Pam_3CSK_4 (EC_{50} ~1-5 ng/ml), less sensitive to the extracellular TLR 3 ligand poly I:C (EC_{50} >100 μg/ml), and insensitive to flagellin. Thus, as greater concentrations of bacterial products enter the circulation during gastrointestinal diseases monocytes would be activated initially by LPS and subsequently by the other bacterial TLR ligands, leading to the release of discrete sets of innate immune mediators over time (e.g. TNF-α, IL-1β and prostaglandins). Presumably, as the concentrations of microbial molecular patterns in the circulation increase further, LPS and flagellin would then activate neutrophils and result in the production of other innate immune mediators, such as ROS.

The pronounced differences in the responses of monocytes and neutrophils to bacterial ligands identified in the current study appear to be unique to the horse, as similar observations have not been reported for other mammalian species. Therefore, there appear to be species-specific differences among mammalian leukocytes with regard

to TLR 5 expression that affects their responsiveness to flagellin and consequently the manner in which immune responses are regulated in different species of animals.

However, some studies indicate that intracellular sequestration of such receptors occurs to reduce the likelihood of unnecessary cellular activation by commensal bacteria. Thus, TLR 4 can be sequestered in the Golgi apparatus in intestinal or respiratory epithelial cells.[52, 53] Similarly, expression of TLR 5 is limited to the basolateral surface of human intestinal epithelial cells, which enables TLR 5 to be effective in detecting invading pathogens, and not commensal organisms in the gut.[54] In contrast, it is important for TLRs to be expressed on the surface of circulating innate immune cells, such as monocytes and neutrophils, whose primary function is to identify and eliminate invading microorganisms. Thus, our finding that equine monocytes do not respond to bacterial flagellin was unexpected, particularly as these cells are highly responsive to LPS, through TLR 4 activation, and respond strongly to synthetic TLR 2 and TLR 3 ligands.[29] In contrast, equine neutrophils respond robustly to flagellin, while being far less sensitive to LPS. To study the difference in responsiveness of equine monocytes and neutrophils to flagellin, we compared the concentrations of TLR 5 mRNA in these two cell types. While equine monocytes contain TLR 5 mRNA, it is not as abundant as it is in neutrophils. Furthermore, although the results of cell-free translation assays indicate that monocyte TLR 5 mRNA can be translated into protein, this does not occur in intact monocytes.

Table 4. 1. TLR 5 mRNA expression in equine neutrophils and monocytes

Cell type	ΔC_T value		
	TLR 4	TLR 5	GAPDH
Monocytes	8.82 ± 0.34	13.31 ± 0.76	4.94 ± 0.52
Neutrophils	4.69 ± 0.59**	7.59 ± 0.34††	5.78 ± 0.29
Alveolar macrophages	7.21 ± 0.12	14.21 ± 0.46	5.47 ± 0.05

The mean ± S.E. $_{\Delta}$CT [gene of interest CT - 18S rRNA CT] values for TLR 4, 5, and GAPDH of equine monocytes, neutrophils, and alveolar macrophages. **P<0.001 for mean $_{\Delta}$CT TLR4 of neutrophils versus monocytes and ††P<0.001 for mean ΔCT TLR5 of neutrophils versus monocytes. ; n = 6 for monocytes and neutrophils and n = 2 for alveolar macrophages.

Table 4. 2. Expression of TLR 5 on equine monocytes and neutrophils as determined by flow cytomtery

Cell population+	MHC I	MHC II	TLR 5 (dilutions)		
			1:10	1:50	1:100
Monocytes	84.0 ± 16.9	71.8 ± 15.9	10.1 ± 7.2	8.8 ± 7.4	8.3 ± 7.5
Neutrophils	98.0 ± 3.5	19.2 ± 12.2	70.7 ± 8.3†	30.0 ± 8.9*	14.4 ± 5.1

+Cell populations were gated according to their forward (FSC) and side scatter (SSC) profiles as well as MHC I and II expression patterns. Cells stained with FITC-labeled secondary antibody only were used as a background control. Values (mean ± SD) are expressed as the percent of neutrophils and monocytes stained positively to the primary antibodies indicated, and normalized using cells stained with FITC-labeled secondary antibody only. Ten thousand events were acquired. †P < 0.001 for monocytes versus neutrophils at 1:10 and *P < 0.05 for 1:50 dilutions of TLR5 primary antibody staining. n = 5 horses.

Table 4.3. Expression of TLR 5 on equine monocytes and neutrophils as determined by flow cytomtery after incubation with either LPS or IFN-γ.

Cell population	Time	Treatment	MHC I	MHC II	TLR5 (dilutions)		
					1:10	1:50	1:100
Monocytes	3 h	Control	66.1 ± 22.5	60.2 ± 21.5	7.8 ± 5.4	8.8 ± 4.8	7.0 ± 4.4
		LPS	67.2 ± 18.1	63.0 ± 14.6	10.6 ± 7.7	7.0 ± 6.4	11.5 ± 12.3
		IFN-γ	65.1 ± 19.4	61.1 ± 18.2	8.6 ± 6.6	8.5 ± 6.7	8.9 ± 7.1
	20 h	Control	59.2 ± 15.6	34.6 ± 22.9	9.6 ± 9.7	7.3 ± 9.5	4.7 ± 5.9
		LPS	62.6 ± 13.4	34.7 ± 21.7	5.2 ± 4.3	3.2 ± 3.0	2.0 ± 1.9
		IFN-γ	59.6 ± 17.1	27.9 ± 11.3	8.7 ± 7.8	4.9 ± 3.5	3.0 ± 2.2
Neutrophils	3 h	Control	98.0 ± 1.1	11.4 ± 1.5	53.6 ± 9.0	16.3 ± 5.2	5.4 ± 2.5
		LPS	97.5 ± 1.3	14.7 ± 2.9	61.1 ± 1.8	24.1 ± 5.6	9.2 ± 3.1
		IFN-γ	96.0 ± 2.2	14.5 ± 1.4	60.7 ± 8.3	25.1 ± 4.4	10.3 ± 4.7
	20 h	Control	69.2 ± 44.9	11.9 ± 8.4	24.5 ± 19.6	4.0 ± 3.6	2.1 ± 1.5
		LPS	80.3 ± 26.3	10.3 ± 7.7	24.5 ± 20.0	4.0 ± 3.2	1.7 ± 1.0
		IFN-γ	85.5 ± 17.1	10.1 ± 7.7	12.8 ± 9.9	1.9 ± 1.2	0.8 ± 0.4

Total leukocytes were incubated either with 100pg/ml *E.coli* LPS or 30 ng/ml equine recombinant IFN-γ for 3 or 20 hours. Cell populations were gated according to their forward (FSC) and side scatter (SSC) profiles as well as MHC I and II expression patterns. Cells stained with FITC-labeled secondary antibody only were used as a background control. Values (mean ± SD) are expressed as the percent of neutrophils and monocytes stained positively to the primary antibodies indicated, and normalized using cells stained with FITC-labeled secondary antibody only. Ten thousand events were acquired.

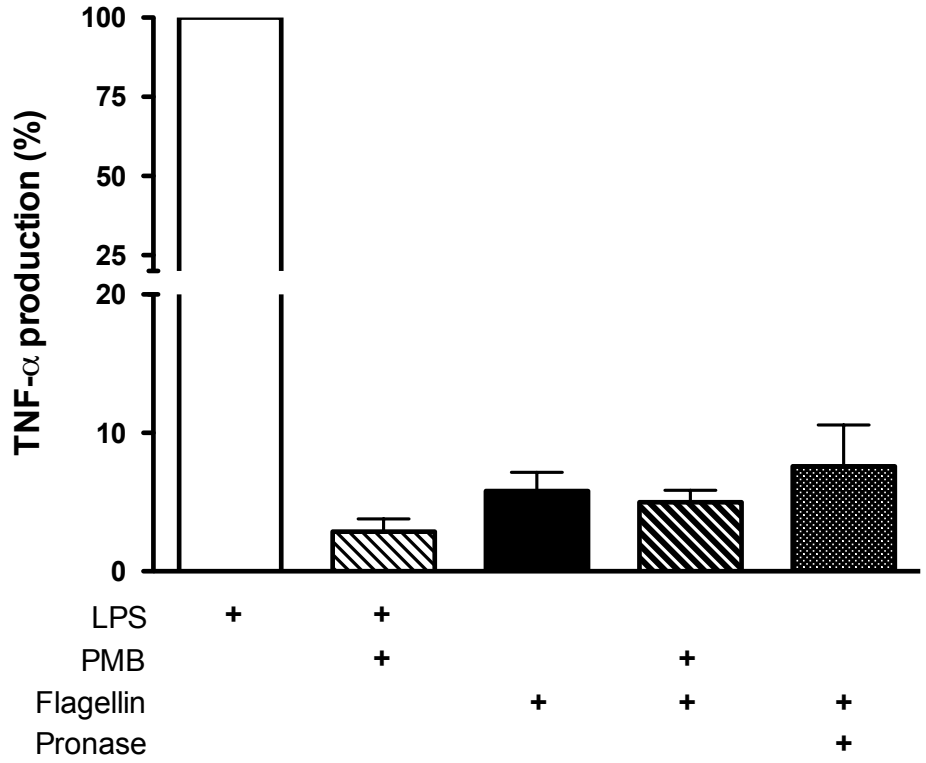

Figure 4. 1. Flagellin induces minimal TNF-α production by equine peripheral blood monocytes

Relative concentrations (mean ± S.E.M.) of TNF-α in supernatants of isolated equine monocytes incubated with *E. coli* 0111:B4 LPS alone (100 pg/ml) and in the presence of polymyxin B (PMB; 13 units/ml), flagellin (3 μg/ml) alone, and flagellin in the presence of PMB (13 units/ml) or deactivated with pronase (1 mg/ml) for 6 hours. TNF-α concentrations for unstimulated, control cells were set at 0 % and positive control cells incubated with 100 pg/ml *E.coli* LPS were set at 100 %. All other stimulations were calculated relative to the LPS stimulation.

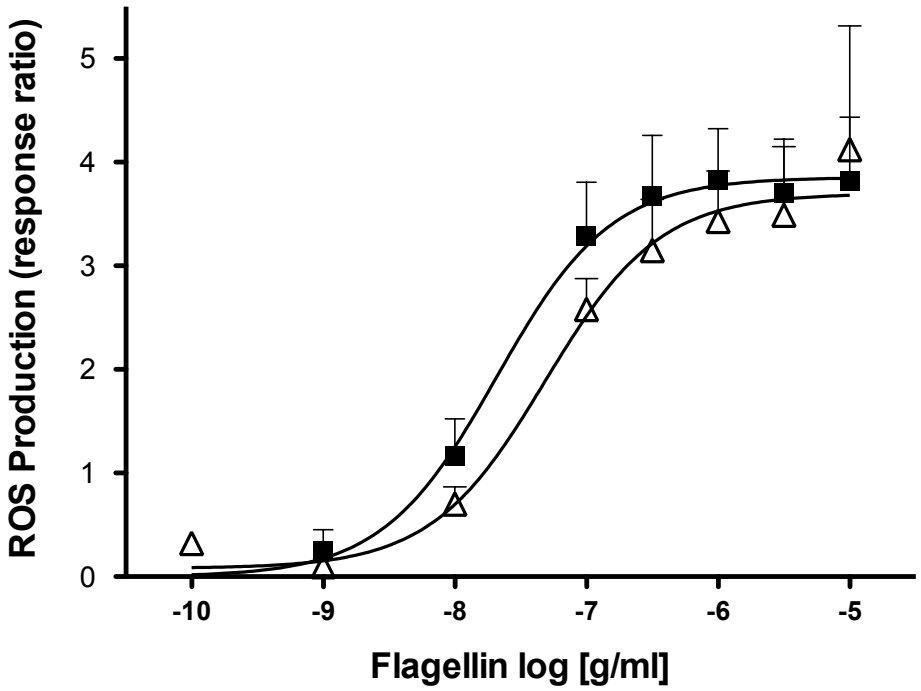

Figure 4. 2. Flagellin induces a dose dependent increase in production of radical oxygen species (ROS) by equine peripheral blood neutrophils

ROS production in isolated neutrophils incubated with flagellin alone (■) (100 pg/ml to 10 μg/ml) and in the presence of polymyxin B (Δ) (13 units/ml). Results are reported as the ratio of ROS production induced by flagellin compared to non-stimulated cells. Each point represents the mean ± S.E.M. for 6 horses.

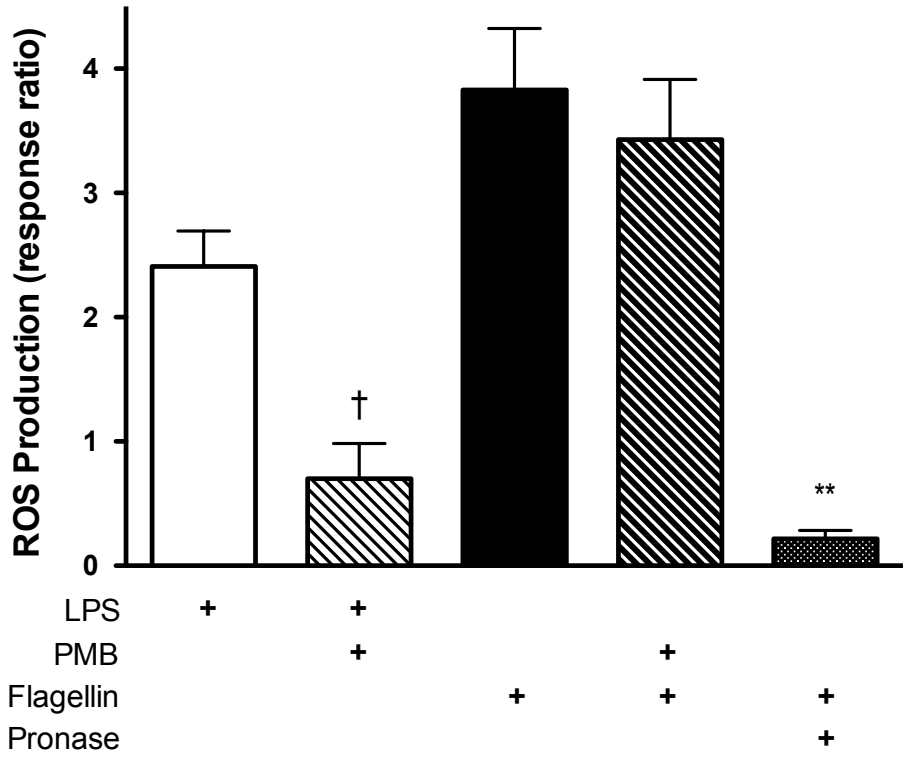

Figure 4. 3. Both flagellin and LPS induce ROS production in equine neutrophils

ROS production (mean ± S.E.M.) in isolated neutrophils incubated with *E. coli* 0111:B4 LPS alone (1 ng/ml) and in the presence of polymyxin B (PMB) (13 units/ml), flagellin (1 μg/ml) alone, flagellin in the presence of PMB (13 units/ml) and flagellin inactivated with pronase (1 mg/ml). Results are reported as the ratio of ROS production induced by flagellin compared to non-stimulated cells (stimulated ROS/non-stimulated ROS). $^{\dagger}P < 0.05$ *E. coli* LPS co-incubated with polymyxin B versus *E. coli* LPS alone. $^{**}P < 0.001$ inactivated flagellin by pronase treatment versus flagellin alone. Similar results were obtained with different concentrations of flagellin. (*data not shown*); n = 6 horses.

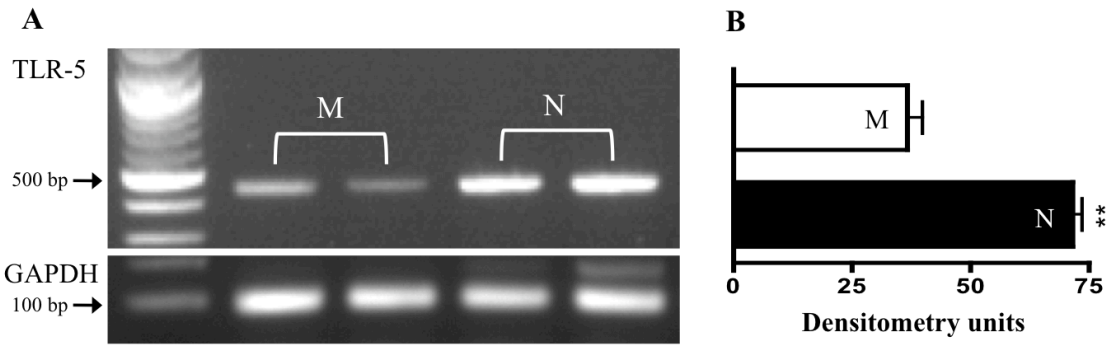

Figure 4. 4. TLR5 mRNA expression in equine neutrophils and monocytes

(A) Representative expression of Toll-like receptor 5 (top) and GAPDH (bottom) mRNA by unstimulated equine monocytes (M) and neutrophils (N). Shown are the agarose gel-resolved end-point RT-PCR amplicons generated with equivalent RNA template (100 ng) from cells obtained from 2 different horses.

(B) Densitometry was performed and normalized to the GAPDH band densities in the agarose gels after the endpoint RT-PCR for TLR 5 mRNA detection. TLR 5 band density was normalized against the density of GAPDH as an internal control. **$P < 0.001$; $n = 6$ horses

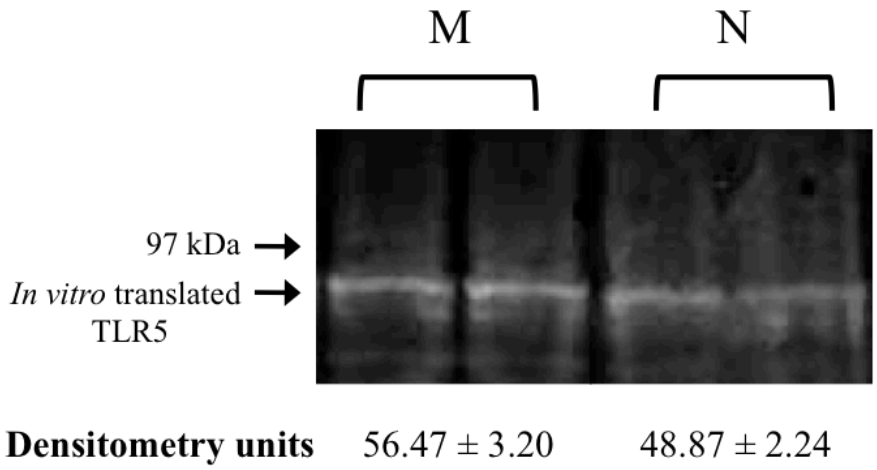

Figure 4. 5. Both equine monocyte and neutrophil RNAs can be translated into TLR 5 protein

RNA from monocytes (1 μg) or neutrophils (0.5 μg) was translated using *in vitro* reticulocyte lysate reactions for 1 hour at 30°C and proteins revolved by SDS-PAGE prior to performing TLR 5 immunoblots. Shown are monocyte (M) and neutrophil (N) translation products from the RNA of two horses. Similar results were obtained with RNA isolated from the cells of two additional horses. Densitometry units are presented as mean ± S.D. from four horses.

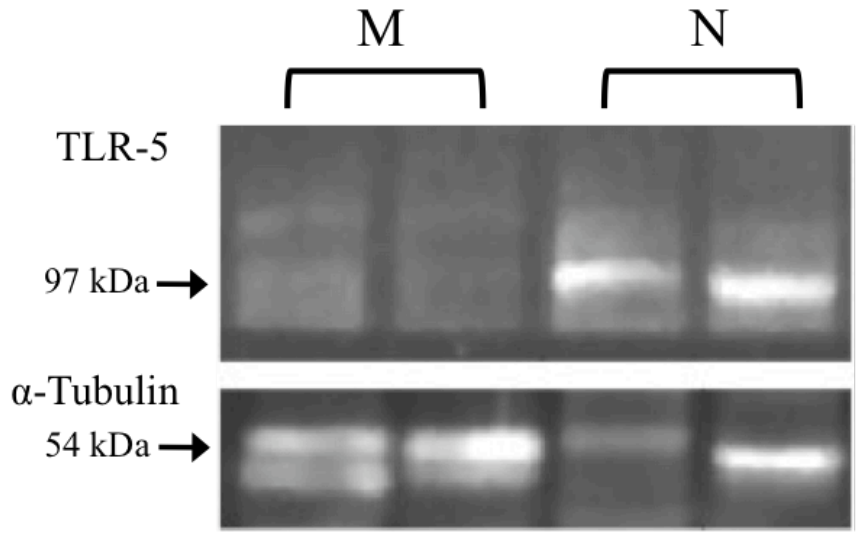

Figure 4. 6. Toll-like receptor 5 protein expression in equine monocytes and neutrophils

Equivalent concentrations (10 μg) of enriched cytosol/membrane protein preparations from equine monocytes and neutrophils were used for the Western blot detection of TLR 5. This experiment was repeated twice with similar results.

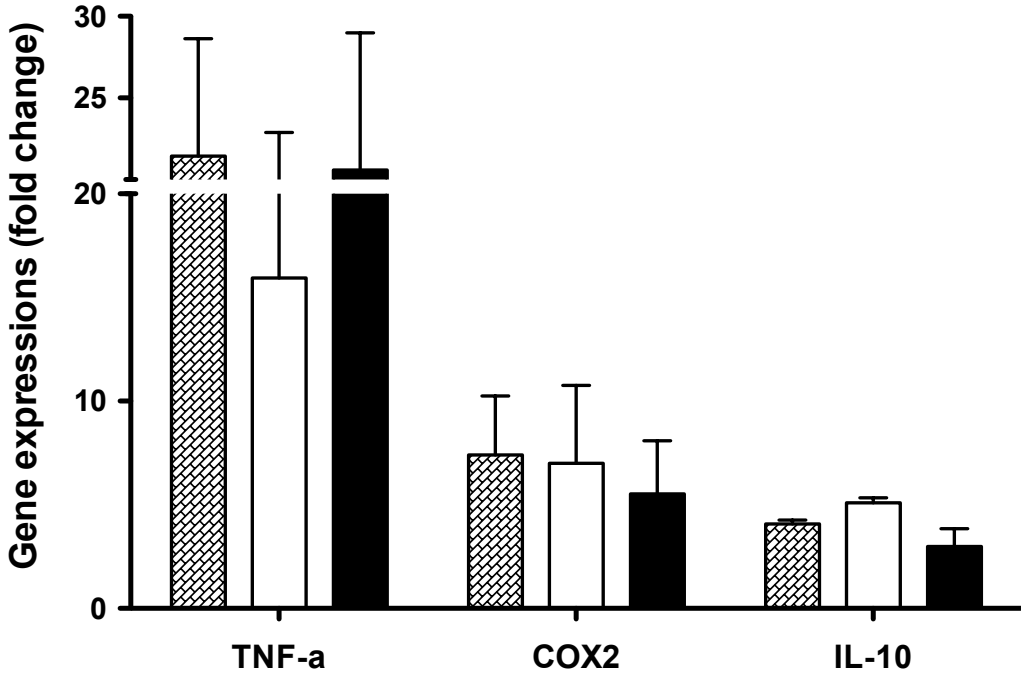

Figure 4. 7. Flagellin, LPS and Pam_3CSK_4 induce TNF-α, COX-2, and IL-10 gene expression in equine neutrophils

Expression of TNF-α, COX-2, and IL-10 mRNA in isolated equine neutrophils incubated with *E. coli* LPS (100 ng/ml; hatched bars), Pam_3CSK_4, (300ng/ml; clear bars), and flagellin (200 ng/ml; solid bars) for 1 h (TNF-α, COX-2) and 4 h (IL-10). The stimulatory concentrations of LPS and Pam_3CSK_4 were based on the results of previous studies (Sun et al.,[30] unpublished data). Values (mean ± SD) are expressed as the fold-increase over time-matched control (non-stimulated) cells.

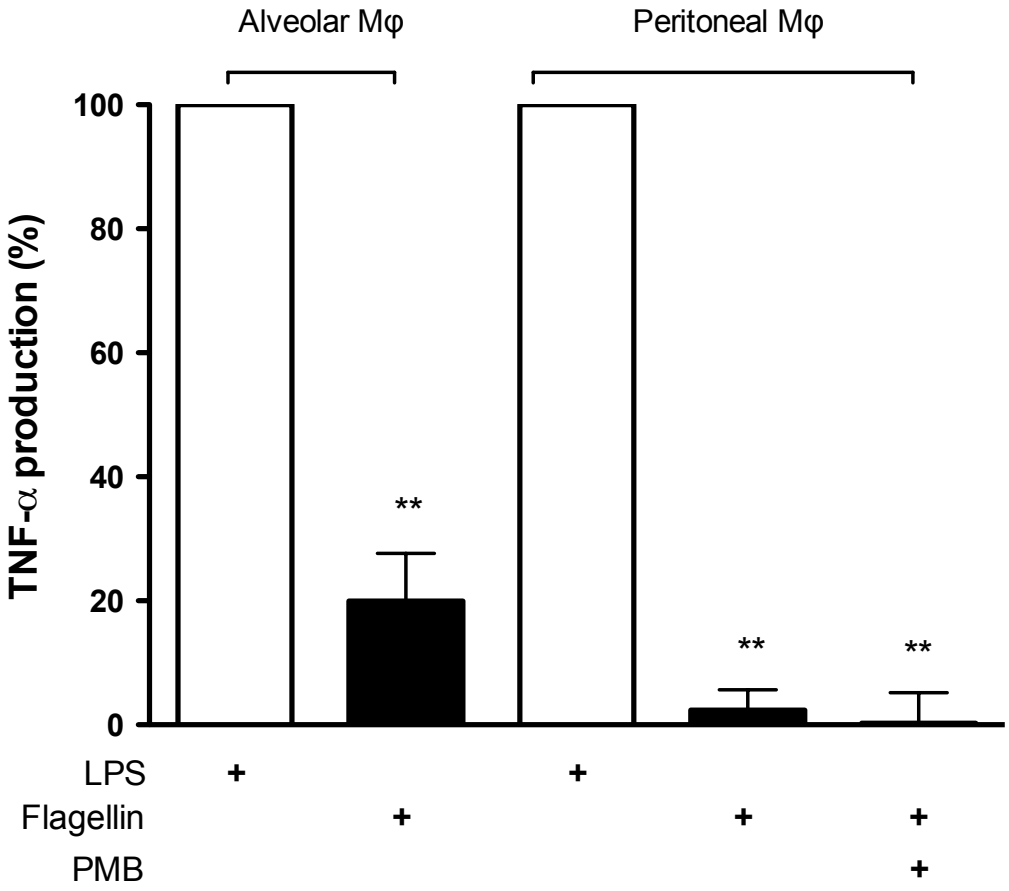

Figure 4. 8. Flagellin does not induce TNF-α production by equine alveolar (n=2 horses) or peritoneal macrophages (n=6 horses)

Relative concentrations (mean ± S.E.M.) of TNF-α in supernatants of isolated equine alveolar and peritoneal macrophages incubated with *E. coli* 0111:B4 LPS alone (1 ng/ml), flagellin (200 ng/ml) alone or flagellin in the presence of PMB (13 units/ml) for 6 hours. TNF-α concentrations for unstimulated, control cells and LPS stimulated cells were set at 0% and 100% respectively. **P<0.001 flagellin treatment versus LPS alone.

VI. References

1. Moore JN, Barton MH. Treatment of endotoxemia. *Vet Clin North Am Equine Pract.* Dec 2003;19(3):681-695.
2. Palsson-McDermott EM, O'Neill LA. Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4. *Immunology.* Oct 2004;113(2):153-162.
3. Jackson JJ, Kropp H. Antibiotic-induced endotoxin release: Important parameters dictating responses. In: Brade H, Opal SM, Vogel SN, Morrison DC, eds. *Endotoxin in Health and Disease*. New York: Marcel Dekker Inc.; 1999:67-75.
4. Dinges MM, Schlievert PM. Comparative analysis of lipopolysaccharide-induced tumor necrosis factor alpha activity in serum and lethality in mice and rabbits pretreated with the staphylococcal superantigen toxic shock syndrome toxin 1. *Infect Immun.* Nov 2001;69(11):7169-7172.
5. Steverink PJ, Salden HJ, Sturk A, Klein WR, van der Velden MA, Nemeth F. Laboratory and clinical evaluation of a chromogenic endotoxin assay for horses with acute intestinal disorders. *Vet Q.* May 1994;16 Suppl 2:S117-121.
6. Lohmann KL, Vandenplas M, Barton MH, Moore JN. Lipopolysaccharide from Rhodobacter sphaeroides is an agonist in equine cells. *J Endotoxin Res.* 2003;9(1):33-37.
7. Sun WC, Moore JN, Hurley DJ, et al. Adenosine A2A receptor agonists inhibit lipopolysaccharide-induced production of tumor necrosis factor-alpha by equine monocytes. *Vet Immunol Immunopathol.* Jan 15 2008;121(1-2):91-100.

8. Moore JN, Garner HE, Shapland JE, Schaub RG. Equine endotoxemia: an insight into cause and treatment. *J Am Vet Med Assoc.* Sep 1 1981;179(5):473-477.

9. Duncan SG, Meyers KM, Reed SM, Grant B. Alterations in coagulation and hemograms of horses given endotoxins for 24 hours via hepatic portal infusions. *Am J Vet Res.* Jun 1985;46(6):1287-1293.

10. Poltorak A, He X, Smirnova I, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science.* Dec 11 1998;282(5396):2085-2088.

11. Nagai Y, Akashi S, Nagafuku M, et al. Essential role of MD-2 in LPS responsiveness and TLR4 distribution. *Nat Immunol.* Jul 2002;3(7):667-672.

12. Van Asten FJ, Hendriks HG, Koninkx JF, Van der Zeijst BA, Gaastra W. Inactivation of the flagellin gene of Salmonella enterica serotype enteritidis strongly reduces invasion into differentiated Caco-2 cells. *FEMS Microbiol Lett.* Apr 15 2000;185(2):175-179.

13. Miao EA, Andersen-Nissen E, Warren SE, Aderem A. TLR5 and Ipaf: dual sensors of bacterial flagellin in the innate immune system. *Semin Immunopathol.* Sep 2007;29(3):275-288.

14. Samatey FA, Imada K, Nagashima S, et al. Structure of the bacterial flagellar protofilament and implications for a switch for supercoiling. *Nature.* Mar 15 2001;410(6826):331-337.

15. Liaudet L, Szabo C, Evgenov OV, et al. Flagellin from gram-negative bacteria is a potent mediator of acute pulmonary inflammation in sepsis. *Shock.* Feb 2003;19(2):131-137.

16. Eaves-Pyles T, Murthy K, Liaudet L, et al. Flagellin, a novel mediator of Salmonella-induced epithelial activation and systemic inflammation: I kappa B alpha degradation, induction of nitric oxide synthase, induction of proinflammatory mediators, and cardiovascular dysfunction. *J Immunol.* Jan 15 2001;166(2):1248-1260.

17. Sitaraman SV, Klapproth JM, Moore DA, 3rd, et al. Elevated flagellin-specific immunoglobulins in Crohn's disease. *Am J Physiol Gastrointest Liver Physiol.* Feb 2005;288(2):G403-406.

18. Gewirtz AT. Flag in the crossroads: flagellin modulates innate and adaptive immunity. *Curr Opin Gastroenterol.* Jan 2006;22(1):8-12.

19. Lunardi C, Bason C, Dolcino M, et al. Antiflagellin antibodies recognize the autoantigens Toll-Like Receptor 5 and Pals 1-associated tight junction protein and induce monocytes activation and increased intestinal permeability in Crohn's disease. *J Intern Med.* Feb 2009;265(2):250-265.

20. Raoust E, Balloy V, Garcia-Verdugo I, Touqui L, Ramphal R, Chignard M. Pseudomonas aeruginosa LPS or flagellin are sufficient to activate TLR-dependent signaling in murine alveolar macrophages and airway epithelial cells. *PLoS One.* 2009;4(10):e7259.

21. Liaudet L, Deb A, Pacher P, et al. The Flagellin-TLR5 Axis: Therapeutic Opportunities. *Drug News Perspect.* Sep 2002;15(7):397-409.

22. Lodes MJ, Cong Y, Elson CO, et al. Bacterial flagellin is a dominant antigen in Crohn disease. *J Clin Invest.* May 2004;113(9):1296-1306.

23. Feary DJ, Hassel DM. Enteritis and colitis in horses. *Vet Clin North Am Equine Pract.* Aug 2006;22(2):437-479, ix.

24. Metcalfe HJ, Best A, Kanellos T, La Ragione RM, Werling D. Flagellin expression enhances Salmonella accumulation in TLR5-positive macrophages. *Dev Comp Immunol.* Mar 3 2010.

25. Fleckenstein JM, Kopecko DJ. Breaching the mucosal barrier by stealth: an emerging pathogenic mechanism for enteroadherent bacterial pathogens. *J Clin Invest.* Jan 2001;107(1):27-30.

26. Huang GT, Eckmann L, Savidge TC, Kagnoff MF. Infection of human intestinal epithelial cells with invasive bacteria upregulates apical intercellular adhesion molecule-1 (ICAM)-1) expression and neutrophil adhesion. *J Clin Invest.* Jul 15 1996;98(2):572-583.

27. Dallap Schaer BL, Aceto H, Rankin SC. Outbreak of salmonellosis caused by Salmonella enterica serovar Newport MDR-AmpC in a large animal veterinary teaching hospital. *J Vet Intern Med.* Sep-Oct 2010;24(5):1138-1146.

28. McSorley SJ, Ehst BD, Yu Y, Gewirtz AT. Bacterial flagellin is an effective adjuvant for CD4+ T cells in vivo. *J Immunol.* Oct 1 2002;169(7):3914-3919.

29. Figueiredo MD, Vandenplas ML, Hurley DJ, Moore JN. Differential induction of MyD88- and TRIF-dependent pathways in equine monocytes by Toll-like receptor agonists. *Vet Immunol Immunopathol.* Jan 15 2009;127(1-2):125-134.

30. Sun WC, Moore JN, Hurley DJ, Vandenplas ML, Linden JM, Murray TF. Pharmacologic characterization of novel adenosine A2A receptor agonists in equine neutrophils. *Am J Vet Res.* Sep 2007;68(9):981-987.

31. Beutler BA. TLRs and innate immunity. *Blood.* Feb 12 2009;113(7):1399-1407.

32. Womble A, Giguere S, Lee EA. Pharmacokinetics of oral doxycycline and concentrations in body fluids and bronchoalveolar cells of foals. *J Vet Pharmacol Ther.* Jun 2007;30(3):187-193.

33. Barton MH, Morris DD, Norton N, Prasse KW. Hemostatic and fibrinolytic indices in neonatal foals with presumed septicemia. *J Vet Intern Med.* Jan-Feb 1998;12(1):26-35.

34. Hurley DJ, Parks RJ, Reber AJ, et al. Dynamic changes in circulating leukocytes during the induction of equine laminitis with black walnut extract. *Vet Immunol Immunopathol.* Apr 15 2006;110(3-4):195-206.

35. Figueiredo MD, Salter CE, Andrietti AL, Vandenplas ML, Hurley DJ, Moore JN. Validation of a reliable set of primer pairs for measuring gene expression by real-time quantitative RT-PCR in equine leukocytes. *Vet Immunol Immunopathol.* Sep 15 2009;131(1-2):65-72.

36. Burgener IA, Jungi TW. Antibodies specific for human or murine Toll-like receptors detect canine leukocytes by flow cytometry. *Vet Immunol Immunopathol.* Jul 15 2008;124(1-2):184-191.

37. Berghaus LJ, Moore JN, Hurley DJ, et al. Innate immune responses of primary murine macrophage-lineage cells and RAW 264.7 cells to ligands of Toll-like receptors 2, 3, and 4. *Comp Immunol Microbiol Infect Dis.* Sep 2 2009.

38. Means TK, Hayashi F, Smith KD, Aderem A, Luster AD. The Toll-like receptor 5 stimulus bacterial flagellin induces maturation and chemokine production in human dendritic cells. *J Immunol.* May 15 2003;170(10):5165-5175.

39. Akira S, Takeda K. Toll-like receptor signalling. *Nat Rev Immunol.* Jul 2004;4(7):499-511.

40. Figueiredo MD, Moore JN, Vandenplas ML, Sun WC, Murray TF. Effects of the second-generation synthetic lipid A analogue E5564 on responses to endotoxin in [corrected] equine whole blood and monocytes. *Am J Vet Res.* Jun 2008;69(6):796-803.

41. O'Mahony DS, Pham U, Iyer R, Hawn TR, Liles WC. Differential constitutive and cytokine-modulated expression of human Toll-like receptors in primary neutrophils, monocytes, and macrophages. *Int J Med Sci.* 2008;5(1):1-8.

42. Ziegler TR, Luo M, Estivariz CF, et al. Detectable serum flagellin and lipopolysaccharide and upregulated anti-flagellin and lipopolysaccharide immunoglobulins in human short bowel syndrome. *Am J Physiol Regul Integr Comp Physiol.* Feb 2008;294(2):R402-410.

43. Oliver OE, Stampfli H. Acute diarrhea in the adult horse: case example and review. *Vet Clin North Am Equine Pract.* Apr 2006;22(1):73-84.

44. Hornung V, Rothenfusser S, Britsch S, et al. Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. *J Immunol.* May 1 2002;168(9):4531-4537.

45. Lun SW, Wong CK, Ko FW, Hui DS, Lam CW. Expression and functional analysis of toll-like receptors of peripheral blood cells in asthmatic patients: implication for immunopathological mechanism in asthma. *J Clin Immunol.* May 2009;29(3):330-342.

46. Salamone GV, Petracca Y, Fuxman Bass JI, et al. Flagellin delays spontaneous human neutrophil apoptosis. *Lab Invest.* Jul 2010;90(7):1049-1059.

47. Applequist SE, Wallin RP, Ljunggren HG. Variable expression of Toll-like receptor in murine innate and adaptive immune cell lines. *Int Immunol.* Sep 2002;14(9):1065-1074.

48. Werling D, Piercy J, Coffey TJ. Expression of TOLL-like receptors (TLR) by bovine antigen-presenting cells-potential role in pathogen discrimination? *Vet Immunol Immunopathol.* Jul 15 2006;112(1-2):2-11.

49. House AK, Gregory SP, Catchpole B. Pattern-recognition receptor mRNA expression and function in canine monocyte/macrophages and relevance to canine anal furunuclosis. *Vet Immunol Immunopathol.* Aug 15 2008;124(3-4):230-240.

50. Palmer JE, Benson CE, Whitlock RH. Salmonella shed by horses with colic. *J Am Vet Med Assoc.* Aug 1 1985;187(3):256-257.

51. Alaniz RC, Cummings LA, Bergman MA, Rassoulian-Barrett SL, Cookson BT. Salmonella typhimurium coordinately regulates FliC location and reduces dendritic cell activation and antigen presentation to CD4+ T cells. *J Immunol.* Sep 15 2006;177(6):3983-3993.

52. Hornef MW, Frisan T, Vandewalle A, Normark S, Richter-Dahlfors A. Toll-like receptor 4 resides in the Golgi apparatus and colocalizes with internalized lipopolysaccharide in intestinal epithelial cells. *J Exp Med.* Mar 4 2002;195(5):559-570.

53. Guillot L, Medjane S, Le-Barillec K, et al. Response of human pulmonary epithelial cells to lipopolysaccharide involves Toll-like receptor 4 (TLR4)-

dependent signaling pathways: evidence for an intracellular compartmentalization of TLR4. *J Biol Chem.* Jan 23 2004;279(4):2712-2718.

54. Gewirtz AT, Navas TA, Lyons S, Godowski PJ, Madara JL. Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression. *J Immunol.* Aug 15 2001;167(4):1882-1885.

CHAPTER 5

BACTERIAL FLAGELLIN AND IMMUNOGLOBULINS DIRECTED AGAINST FLAGELLIN IN SERUM SAMPLES FROM HORSES WITH GASTROINTESTINAL DISEASES

Soyoung Kwon, Marco Lopes, Kelsey Hart, David J. Hurley, Thomas P. Robertson,

Andrew T. Gewirtz, James N. Moore and Michel L. Vandenplas

Provisional authors in no particular order

Results presented in this chapter was submitted to
American Quarter Horse Association research proposal, 12/01/2011

I. Introduction

Colic and sepsis are the most significant causes of mortality and morbidity in horses and foals, respectively.[1,2] The pathogenesis of many gastrointestinal (GI) diseases of horses, and sepsis in foals, has a significant inflammatory component. Of particular importance to these diseases is the translocation of structural components of enteric bacteria into the bloodstream, activation of the animal's innate immune system, and generation of inflammatory mediators that are responsible for patient morbidity and mortality. In the gut, the animal's first line of defense against enteric bacteria is an anatomically intact, mucus-coated barrier of intestinal epithelial cells. In addition, leukocytes in the underlying lamina propria contribute to the protection against bacteria. In many equine GI diseases, there is evidence that this normal intestinal mucosal barrier function is lost, allowing the translocation of enteric bacterial lipopolysaccharide (LPS) into the general circulation.[3] Similarly, LPS and Gram-negative bacteria are commonly detected in the blood of septic equine neonates.[4] After gaining access to the circulation, LPS binds to its pattern recognition receptor, Toll-like receptor 4, to initiate the signaling pathways that ultimately induce potent innate immune responses.[5] As a result, clinical signs consistent with the systemic inflammatory response syndrome develop in horses with severe GI disease and foals with sepsis. Although LPS clearly is responsible for some of the alterations that occur in affected animals, it is equally likely that other bacterial components enter the circulation.[6] However, no studies have been performed to detect bacterial components other than LPS in either horses with GI diseases or foals with sepsis.

There is recent evidence in the literature that serum concentrations of flagellin and anti-flagellin antibodies increase significantly in human patients with certain intestinal diseases[7,8] suggesting that bacterial components other than LPS may be responsible for the development of the systemic inflammatory response syndrome in these patients. The results of the study summarized in *Chapter 4* indicate that flagellin induces changes in inflammatory gene expression in equine neutrophils that are indistinguishable from those induced by LPS.[9] Moreover, we have determined that flagellin selectively activates equine neutrophils but not monocytes, whereas LPS activates both cell types. Thus, in horses flagellin elicits an innate immune response that is distinct from that induced by LPS. Flagellated bacterial species that are present in the horse's gastrointestinal tract include *Bacillus spp., Citrobacter spp. Campylobacter, Bordetella bronchiseptica, E. coli, and Salmonella spp*. Therefore, horses and foals have large reservoirs of flagellin and depend upon an effective intestinal mucosal barrier system to restrict these bacterial components to the intestinal lumen.

Immunoglobulin G (IgG) is important in the host defense against bacterial diseases because it promotes bacterial phagocytosis and reduces intracellular bacterial growth.[10] Furthermore, IgGs reduce microbial infection by interfering with microbial adherence and cell signaling, inhibiting translocation of bacteria in the gut, assisting with phagocytosis of pathogens. Therefore, transfer of colostoral IgGs within the first 24 hours of life in neonatal foals is critical to protect newborns against the development of disease.[11]

Therefore, the objective of this study is to test the hypothesis that flagellin enters the circulation of septic foals and horses with GI diseases. If our hypotheses are proven to be true, our findings may help account for the inconsistent responses obtained with the use of treatments specifically directed against LPS (i.e., hyperimmune serum and polymyxin B).[12,13] Furthermore, we will determine whether adult horses respond to the presence of flagellin in the circulation by producing IgG directed against it, and whether changes occur in serum concentrations of colostrum-derived flagellin IgGs in septic newborn foals are associated with the presence of flagellin.

II. Materials & methods

General materials:

Immulon 4 microtiter 96 well plates were purchased from Nunc (Rochester, NY), Mouse monoclonal *FliC* flagellin antibody was purchased from BioLegend (San Diego, CA). Goat polyclonal *E. coli* flagellin antibody was obtained from Dr. A. Gewirtz, Emory University. Secondary antibodies against mice and goat, or horse that are conjugated with HRP were purchased from Santa Cruz (Santa Cruz, CA) and Serotec (Raleigh, NC), respectively. All other high-grade chemicals including carbonate bicarbonate buffer were obtained from Sigma Aldrich (St. Louis, MO).

Study population and sample collection:

This study consisted of a retrospective portion involving serum samples from 38 hospitalized neonatal foals and 57 hospitalized horses with GI disease all presented to the Veterinary Teaching Hospital at the University of Georgia. The 38 hospitalized foals had previously been assigned to either septic or non-septic categories, based on clinical and clinicopathological data at the time of admission as well as blood culture results. Briefly, sepsis was determined by either a positive blood culture or a sepsis score $\geq$ 11 at admission. [14] Clinical evidence of the systemic inflammatory response syndrome (SIRS) in adult horses was defined as the presence of at least two of the following at admission: heart rate > 60 bpm, respiratory rate > 40 bpm, rectal temperature >101.5 °F, leukopenia (<4,500 cells/µl), leukocytosis (>12,500 cells/µl), or >10% band neutrophils.[15] Serum samples obtained from 18 healthy foals and 26 adult horses were included as controls.

Serum samples obtained from all horses and foals were assayed for both flagellin and anti-flagellin antibodies. All collected samples were stored frozen in aliquots at -80^{o}C, and all experimental protocols using animals were reviewed and approved by the Animal Care and Use Committee at the University of Georgia.

Serum flagellin assay:

The concentration of flagellin in each of the serum sample was determined using an ELISA assay. Multiple concentrations of antibody dilutions were tested to optimize this assay prior to the study (Appendix Figure 1 and 2). Briefly, wells of 96-well microtiter plates were coated with 100 µl of diluted mouse monoclonal *FliC* flagellin antibody (1:500) in 0.05M carbonate-bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. After blocking wells with 0.5% BSA in PBS for 1 h at room temperature, 100 µl of diluted serum samples (1:10) were added to adjacent wells in a duplicate manner. Plates were then incubated for 2h at 37°C with gentle shaking followed by the addition of goat polyclonal anti-flagellin IgG (1:500) and incubated for 1.5 h at 37°C with gentle shaking. To detect Ag-IgG complexes, the wells were treated with 100 µl of peroxidase-conjugated donkey anti-goat IgG (1:3000) for one hour at 37°C. After each step, unbound reagents in the wells were removed by repeated washing with 1% Tween-20 in PBS (PBST). Finally, 100 µl of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) substrate was added to each well and color development was measured at 405 nm absorbance on an automated microplate reader after ~ 20 min. Different amounts of purified flagellin (obtained from Dr. A. Gewirtz) in 1% equine donor serum in PBST were used as to create a standard curve.

Serum anti-flagellin antibody assay:

Concentrations of IgG directed against flagellin in serum samples from hospitalized foals and horses were measured using an ELISA assay. Purified flagellin was used to coat microtiter plates to capture and detect equine anti-flagellin IgG in the samples prepared as described above. Briefly, microtiter plates were coated with purified flagellin protein (100 ng/well) in 0.05M carbonate-bicarbonate buffer (pH 9.6) overnight at 4°C. Wells were blocked with 0.5% BSA in PBS for 1 h and 100 µl aliquots of diluted serum samples from control and hospitalized foals or horses were added to wells. After a 1-hour incubation at room temperature and washing, 100 µl of HRP-conjugated goat anti-horse IgG (1:30,000) (Serotec, Raleigh, NC) were added to the wells and allowed to incubate for 30 min at room temperature. After each step, unbound reagents in the wells were removed by repeated washing with PBST. Quantification of flagellin IgG was performed colorimetrically using ABTS substrate with plates read at 405 nm in an automated microplate reader. IgG prepared from equine donor serum by ammonium sulfate precipitation and spiked with polyclonal or monoclonal anti-flagellin antibody served as a positive control. Antigen concentration, primary and secondary antibody dilutions were determined prior to assays using ranges of dilutions for both assays (Appendix Figure 1 and 3).

Assay validation:

The precision of both flagellin and anti-flagellin antibody ELISAs were monitored for intra- and inter-assay coefficient of variation (CV) as methods of assay validation. Briefly, aliquots of 1% equine donor serum spiked with purified flagellin or anti-flagellin Ab were run in the same plate to test reproducibility of the assays. A standard was pipetted on each individual plate, and the goodness of fit (R^2) of the standard curve was assessed by using a 4-parameter logistic regression algorithm calculated with GraphPad Prism Version 5.0. Intra-assay CV (%) was calculated ((S.D/duplicate mean) x 100). Inter-assay CV also was determined to monitor plate-to-plate variation. To calculate inter-assay CV (%), a standard curve was used; two points (e.g. one high point and one low point) from standard samples were monitored in multiple, different plates, and the mean of means and S.D. of means were calculated to generate percentile CV of means. Inter-assay CV was then calculated ((average of high and low control CV) x 100).

Statistical analysis:

Serum flagellin or flagellin IgG values in sick foals and horses were compared to each control group with unpaired *t*-tests. *P*-values ≤ 0.05 were considered to be statistically significant.

III. Results

Study populations

The 57 horses admitted for GI disease represented a variety of breeds (21 Quarter horses, 10 Thoroughbreds, 4 Arabians, 4 pony, 3 Warm bloods, 5 Paint, 2 Paso Fino, 4 Tennesee Walking Horses, and 1 each of four other breeds). There were 33 geldings and 24 mares, with a mean age of 12.8 years (range 1.0 – 36.0 years). Of the 57 horses, 15 horses had GI inflammation, 8 horses had strangulation obstruction, 27 horses had non-strangulating obstruction, and 7 horses had colic with unknown origin. Of all 57 horses, 28 horses underwent celiotomy, 24 horses received medical treatment, and 5 horses were euthanized because the treatment was declined by the owners. Of the 57 horses with colic, SIRS was detected in 12 (27.3 %); 8 horses fulfilled 2 criteria, 3 horses fulfilled 3 criteria, and 1 horse fulfilled 4 criteria for SIRS as described above. The 26 healthy control horses comprised variety of breeds (18 quarter horses, 4 mixed breed, 3 thoroughbreds, and 1 warm blood), 18 mares, 5 geldings, and 3 stallions with a mean age of 10.0 years (range 2.0 – 18.0 years). The 38 hospitalized neonatal foals with a mean age of 2.5 days (range 1 – 13 days) met criteria for sepsis; 15 foals had positive blood culture and 13 foals had sepsis score ≥ 11 at admission. 18 clinically healthy Quarter Horse foals from the equine breeding facility of University of Georgia were included as a control group. Serum samples from 18 mares of healthy foals were obtained to monitor passive immunoglobulin transfer. Also, two mare and foal pairs were obtained to compare immunoglobulin levels in the mother and young before and after ingestion of colostrum.

Assay validation

Serial dilution of flagellin and mouse anti-FliC Ab in the range of 0 to 300 ng/ml and 0 to 1.67 µg, respectively, were used to generate the standard curve. The linearity of both flagellin and anti-flagellin antibody assays were evaluated by analysis of five calibration standards prepared above using a second order polynomial curve fit to measure the response [optical density (OD)] versus log transformed concentration of either flagellin or mouse anti-FliC Ab, respectively (Fig. 5. 1). 10 % serum dilution was optimized for both assays (Appendix Fig. 1). The precision and accuracy of assays were determined by intra- and inter-assay CV (%) of two concentrations of the calibration (Table 5. 1).

Detection of flagellin in serum of horses

Flagellin was detected in 28 of 57 (49 %) serum samples obtained from adult horses admitted with a variety of GI diseases and 12 of 26 (46 %) clinically healthy adult horses (Figure 5. 2). In horses with GI disease, the mean flagellin concentration (163.7 ± 50.8 ng/ml) was higher than that in the healthy horses (33.8 ± 17.9 ng/ml). Consequently, 21 of 57 (37.8 %) horses with GI disease had increased levels of flagellin in their circulation when compared to the healthy control horses. When compared as a group, the mean flagellin concentration in horses diagnosed with strangulation such as large colon volvulus and small intestinal strangulating obstruction was significantly higher than that of healthy control group (Table 5. 2). However, there was no significant difference between values for medical and surgical colic cases, and survivors and non-survivors (Table 5. 2). Similarly, flagellin was detected in serum of 17 of 28 (68 %) septic foals, 2

of 10 (20 %) non-septic foals, and 4 of 18 (22 %) healthy foals. The mean flagellin concentration of hospitalized foals (139.9 ± 45.9) was significantly higher than that in healthy foals (13.3 ± 6.1). When compared as a group, the mean flagellin concentrations in septic foals were significantly higher than that of healthy foals. Similarly, there was a significant difference between survivors and non-survivors (Table 5. 3).

Detection of serum flagellin-specific immunoglobulin in horses

Serum samples obtained from two healthy mare and foal pairs were used to evaluate anti-flagellin IgG levels in the mother and young (Fig. 5. 3A). In these two foals, there was a sharp increase in the level of anti-flagellin IgG after colostrum ingestion that was comparable to that of the mare. Serum samples obtained from healthy newborn foals at various times after ingestion of colostrum allowed identification of anti-flagellin IgG concentrations that were maintained for 30 days (Fig. 5. 3B). In contrast, anti-flagellin IgG concentrations were significantly lower in samples obtained from septic foals (mean age of 2 days) hospitalized at the UGA Teaching Hospital when compared to samples collected from clinically healthy foals (mean age of 1.25 day; Fig. 5. 4). Adult horses admitted to the UGA Teaching Hospital for various GI diseases had serum flagellin IgG levels similar to those of clinically healthy horses (Fig. 5. 4). However, flagellin IgG levels were increased in samples obtained after 10 to 14 days of hospitalization in horses treated for GI diseases (Fig. 5. 5).

IV. Discussion

It is generally accepted that LPS released from Gram-negative bacteria is an important trigger of the inflammatory response that characterizes patients with sepsis. This conclusion is based on substantial evidence that parenteral administration of LPS produces a sepsis-like syndrome in animals and humans,[16-18] that LPS can be measured in the circulation of patients with many diseases including Gram-negative infection,[19] and that LPS induces pro-inflammatory responses in cultured cells in vitro.[20] However, LPS is not always detected in the circulation of many ill equine patients, and intervention strategies specifically directed to prevent biological effects of LPS, such as hyperimmune anti-LPS sera and polymyxin B, do not always yield profound positive effects. The results of recent experimental and clinical studies indicate that PAMPs other than LPS, such as flagellin, peptidoglycan, and CpG nucleotides, may be important in many species including horses.[21] Furthermore, the ability of these PAMPs to induce pro-inflammatory responses mediated by activation of their corresponding TLRs is well documented in both in vivo and in vitro studies. In addition, the shedding of soluble PAMPs including lipopeptide and flagellin have been detected in culture supernatants from both Gram-negative and Gram-positive bacteria.[22] Therefore, bacterial components other than LPS may be important for the development of SIRS and consequently responsible for the lack of clinical efficacy of treatments specifically directed against LPS.

For the first time, we report that flagellin is detectable in the blood of horses with GI disease and septic foals. Our results provide additional evidence for the loss of normal intestinal mucosal barrier function in equine GI diseases, allowing the translocation of enteric bacterial components other than LPS into the general circulation.[3,23] However, the

detection of low concentrations of flagellin in serum samples obtained from clinically healthy horses in our current study may reflect the fact that small amounts of bacterial translocation occurs in healthy animals. It is feasible that this small exposure to bacterial components may contribute to the development of protective mechanisms by allowing the immune system constant contact with external antigens.[24]

Of many bacterial components, flagellin might have the unique potential to contribute to the development of SIRS in many conditions including equine GI diseases. For example, TLR5, a pattern recognition receptor for flagellin, has limited expression on the basolateral surface of gut epithelium.[25] This polarization is unique as it allows only invading or translocating microbes, but not commensal bacteria that are contained to the intestinal lumen, to induce inflammatory responses. Further, the importance of flagellin is also highlighted by the presence of its cytosolic receptor, Ipaf. When flagellin enters the host cell via the type-3 secretion (T3SS) system that transports bacterial products into host cells, it is recognized by Ipaf. As a result of its recognition, Ipaf signaling activates caspase-1 that contributes to the maturation of pro-inflammatory cytokines. Therefore, the combination of TLR5 and Ipaf activation results in a more robust response to invading pathogens expressing virulent factors that might permit evasion of basic immune mechanisms.

In the final component of the current study, we detected an enhanced adaptive immune response to flagellin in horses during hospitalization for treatment of GI diseases. We hypothesize that the translocation of flagellin stimulates adaptive immunity that is reflected by increased IgG levels in those horses. Increased adaptive immune response to PAMPs is also reported in human patients with Crohn's disease and short

bowel syndrome that are characterized by loss of intestinal barrier function.[7,8] The results of the current study indicate that healthy adult horses have substantial levels of anti-flagellin IgG. The latter finding may reflect the translocation of small amounts of PAMPs, such as flagellin, and/or bacteria and their detection by the gut-associated immune system. The healthy neonatal foals in the current study that received colostrum had substantial levels of anti-flagellin IgG in their circulation, which was maintained for a month. Although foals have the ability to produce IgG as early as 16 days of age in response to appropriate antigenic stimulation, protective humoral immunity is not reached until they are approximately 2 months old.[26] Even though the phagocytic ability of neutrophils in newborn foals is comparable to that of adult horses, opsonization does not reach adult levels until 3-4 weeks of age.[27] Therefore, transfer of colostoral IgG within the first 24 hours of life is critical for newborn foals, as documented in independent studies.[10,11] For example, IgG and IgA were detected in sera and nasopharyngeal mucosa of foals after ingestion of colostrum.[28] Similarily, IgG directed against various parts of *Actinobacillus equuli* were detected in mares and their newborn foals, and they persisted approximately 30 days in the foals.[29] Consequently, the anti-flagellin IgG detected in the serum samples from the healthy foals in the current study most likely represents colostral transference from their mares. Also, in human infants, breast-feeding reduces the incidence of severe diarrhea caused by various enteric infectious agents including *E. coli*.[30] In contrast, the septic foals in the current study had markedly lower levels of anti-flagellin IgG, and 10 of the 30 septic foals had low amounts of total IgG (< 200) at admission. These findings can be explained by either the

lack of absorption of anti-flagellin antibody from the mare's colostrum or consumption of antibody as a result of interaction with circulating flagellin.

In summary, we report that horses with GI disease and septic foals can be exposed to increased systemic levels of bacterial flagellin similar to what occurs with LPS, probably due to intestinal barrier dysfunction. Furthermore, adaptive immune response to flagellin was detected in horses with GI disease that was reflected by increased level of antibody directed to flagellin during hospitalization. Given the robust proinflammatory response of equine neutrophils to flagellin, translocation of this PAMP may contribute to the pathogenesis of SIRS in horses and neonatal foals.

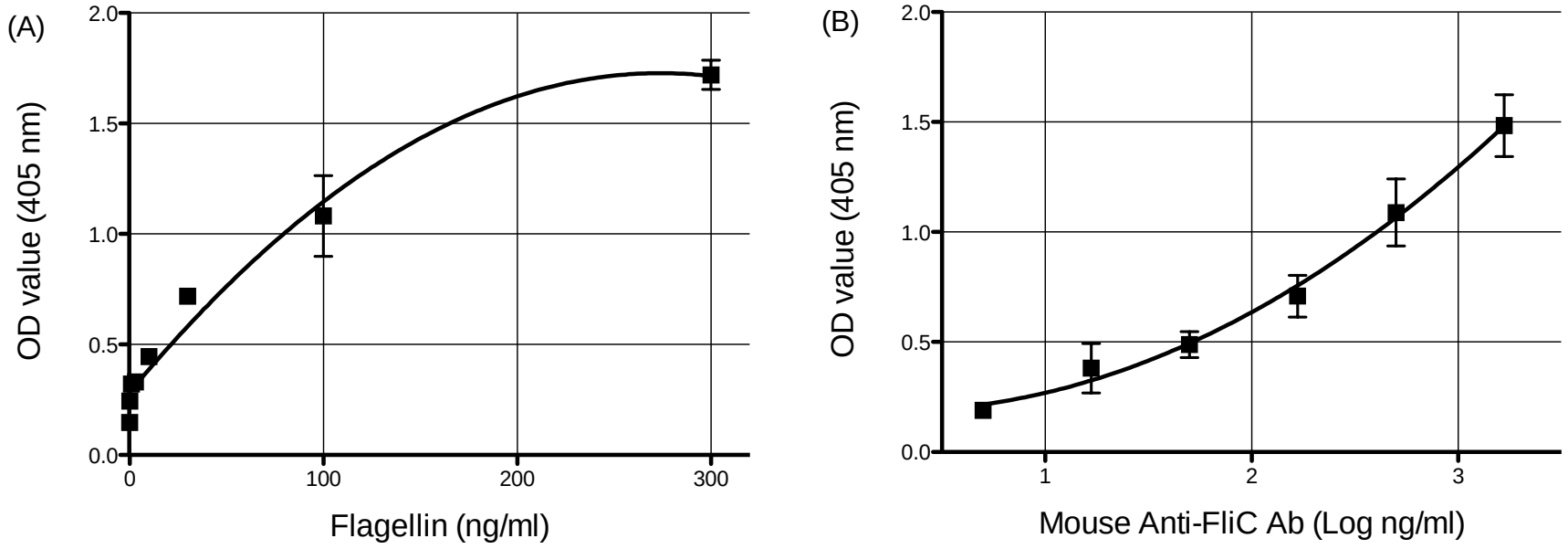

Figure 5. 1. Representative standard curves from enzyme-linked immunosorbent assay (ELISA) for flagellin (A) and flagellin antibody (B) calculated with a second order polynomial curve fit: Y = Y=A+ B*X + C*X^2 where A=0.2838, B=0.01055, C=0.1927 (R^2=0.90) and A=01955, B=-0.07331, C=0.1466 (R^2=0.93) respectively. X axis shows a standard concentration ranging from 0 to 300 ng/ml and 0 to 1.67 µg/ml for flagellin and anti-flagellin Ab assays respectively. Y axis shows the optical density (OD) measured at 450 nm. The Y axis displays the absorbance at a wavelength of 405 nm.

Table 5. 1. Intra- and inter-assay precision for flagellin and anti-flagellin Ab assays

	Statistics	**Flagellin ELISA** Flagellin (ng/ml)		**Anti-flagellin Ab ELISA** Mouse anti-FliC Ab (ng/ml)	
		30	300	167	1,670
Intra-assay	Mean	36.36	252.20	71.21	2,233.07
	S.D.	4.74	28.17	21.25	106.05
	CV (%)	13.03	11.17	29.84	4.75
	Recovery (%)	121.19	84.06	42.74	133.72
Inter-assay	Mean	35.61	278.97	111.67	1,549.91
	S.D.	5.97	24.47	16.56	25.72
	CV (%)	16.74	8.77	14.82	1.66
	Recovery (%)	118.73	93.00	66.87	92.81

Intra-assay variability is shown with five replicates of test samples containing low (30 ng/ml) and high (300 ng/ml) concentrations of flagellin on a single plate. Inter-assay variability is shown with test samples containing low (30 ng/ml) and high (300 ng/ml) concentrations of flagellin on five different plates. Average calculated concentrations and percentage of coefficient of variation (CV) are shown for each concentration. CV values of < 15% were accepted for intra- and inter-assays. The goodness of fit (R^2) of the standard curve was assessed as described iin the manuscript. Recovery (%) represents the difference between detected and theoretical concentration.

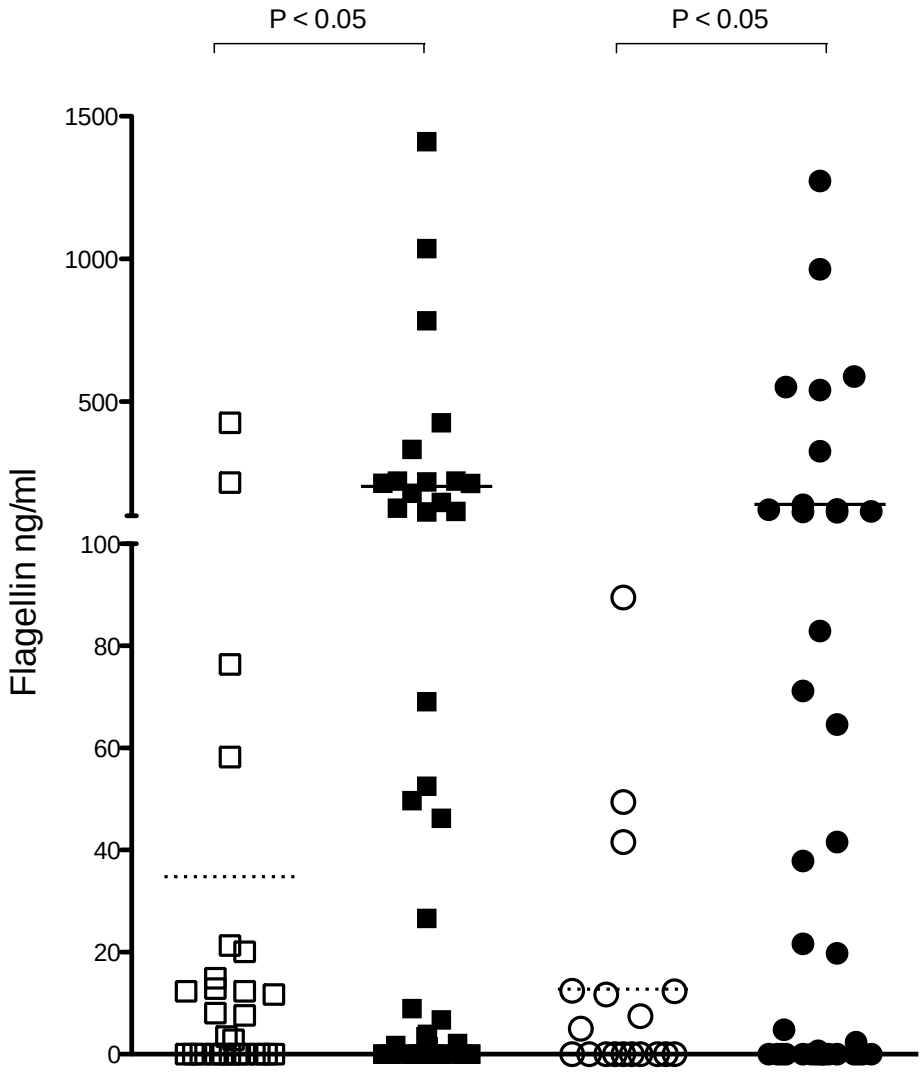

Figure 5. 2. Flagellin concentration from serum samples of horses and foals determined by an ELISA

Serum flagellin concentrations in healthy (control) horses (n = 24, ⊟), horses with GI disease (n = 57 ■), healthy (control) foals (n = 18, ⊖), and hospitalized foals (n = 38, ●). The horizontal lines represent the mean concentration for each group. Samples with undetectable level of flagellin in our assay were assigned to 0 for statistical analysis.

Table 5. 2. Association of disease types and clinical outcomes to serum flagellin concentrations in hospitalized horses.

Variables		N	Mean ± SEM	*P* value
Disease category	Inflammation	15	199.2 ± 115.9	0.077
	Obstruction	27	145.6 ± 71.1	0.143
	Strangulation	8	259.2 ± 170.3	0.027
	Non-identified colic	7	48.5 ± 32.7	0.723
Correction	Medical	32	162.6 ± 73.9	0.934
	Surgical	21	153.2 ± 80.5	
Survival	Survivor	42	129.3 ± 48.9	0.260
	Non-survivor	15	260.0 ± 136.6	

Table 5. 3. Association of disease types and clinical outcome to serum flagellin concentrations in hospitalized foals.

Variables		N	Mean ± SEM	*P* value
Disease category	Positive blood culture	15	263.3 ± 92.8	0.012
	Sepsis score ≥ 11	13	104.9 ± 47.8	0.037
	Non-septic	10	0.3 ± 0.2	0.116
Survival	Survivor	28	76.9 ± 28.1	0.046
	Non-survivor	10	344.4 ± 141.4	

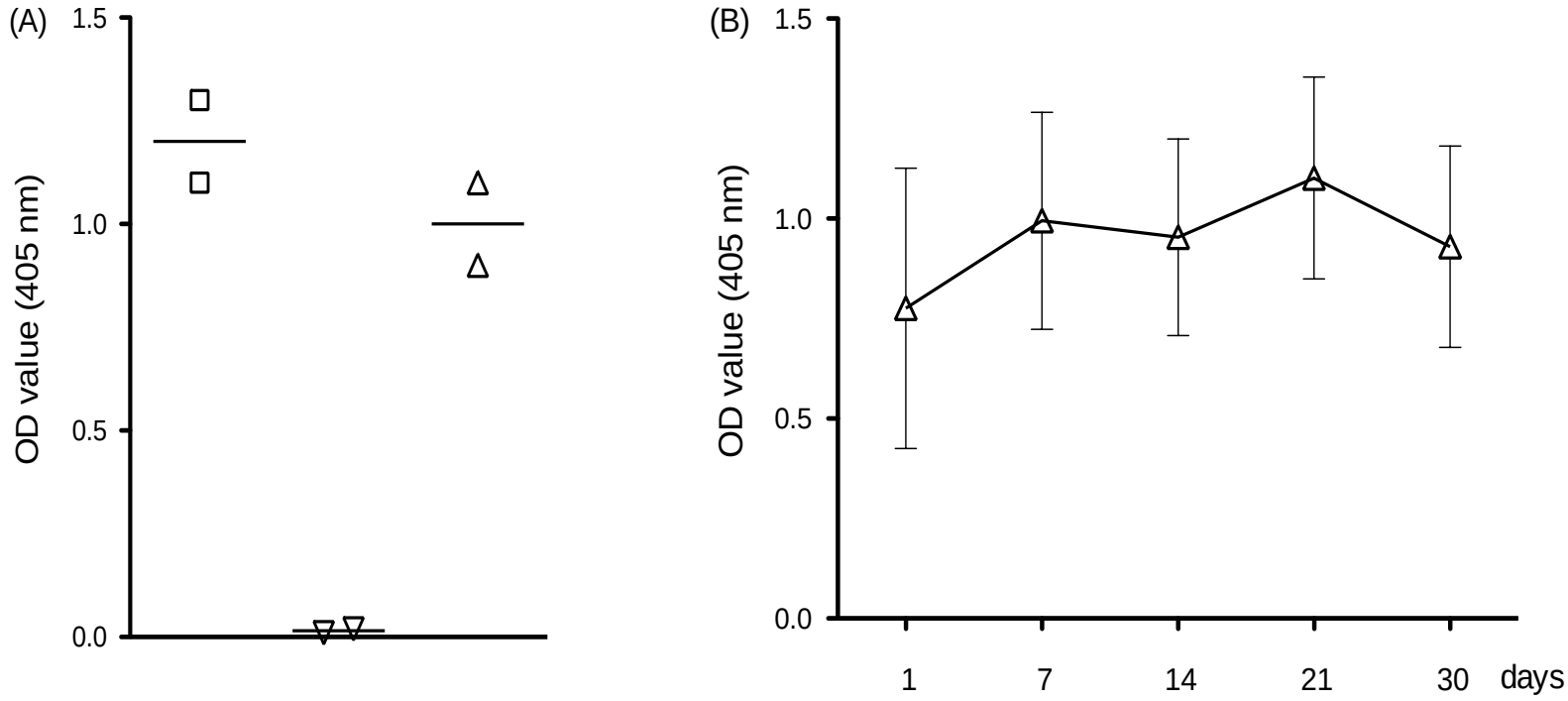

Figure 5.3. Anti-flagellin immunoglobulin levels in serum of foals determined by an ELISA

(A) A sharp increase in anti-flagellin IgG concentrations were detected in serum samples obtained from a small number (n = 2) of healthy newborn foals immediately before suckling (▽) and after ingestion of colostrum (day 1, △). Anti-flagellin IgG was also detected in samples obtained from the mares of these foals (□). **(B)** Anti-flagellin IgG concentrations in 13 healthy neonatal foals in serum samples from 1-2 day old to 30 days.

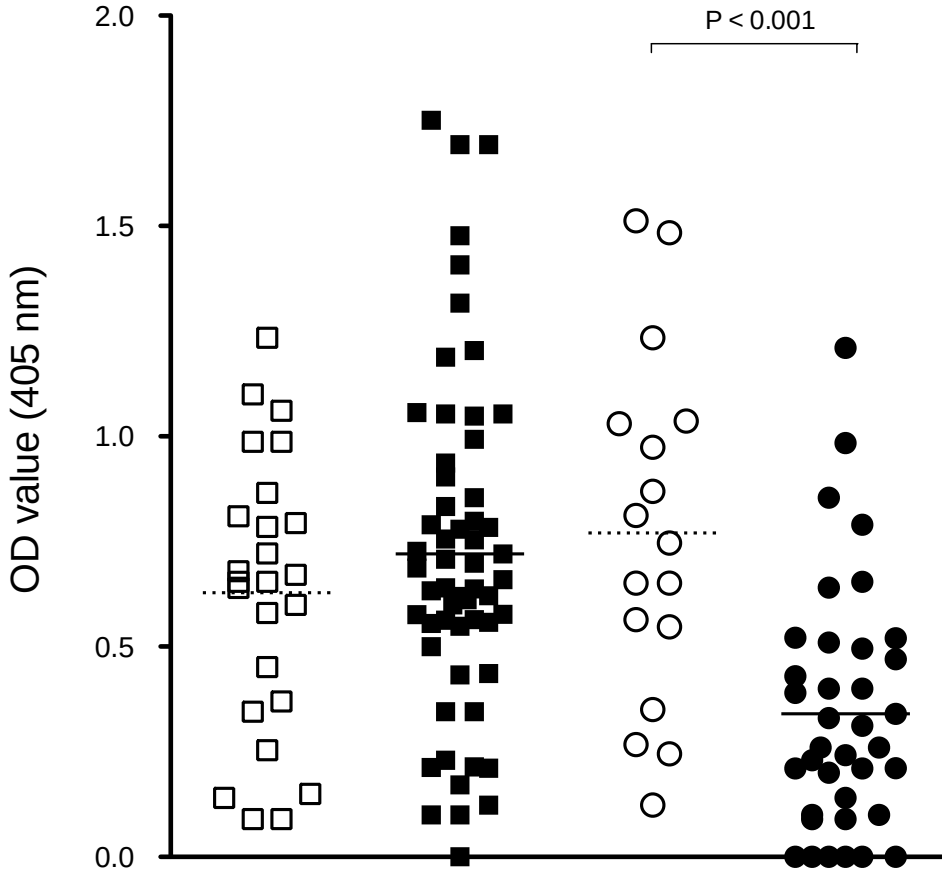

Figure 5. 4. Detection of flagellin antibody in horses and foals by an ELISA

Serum anti-flagellin immunoglobulin concentrations in healthy (control) horses (n = 24, ⊟), horses with GI disease (n = 57, ■), hospitalized foals (n = 38, ●), and healthy (control) foals (n = 18, ⊖). The horizontal lines represent the mean concentration for each group. Samples with undetectable level of flagellin in our assay were assigned to 0 for statistical analysis.

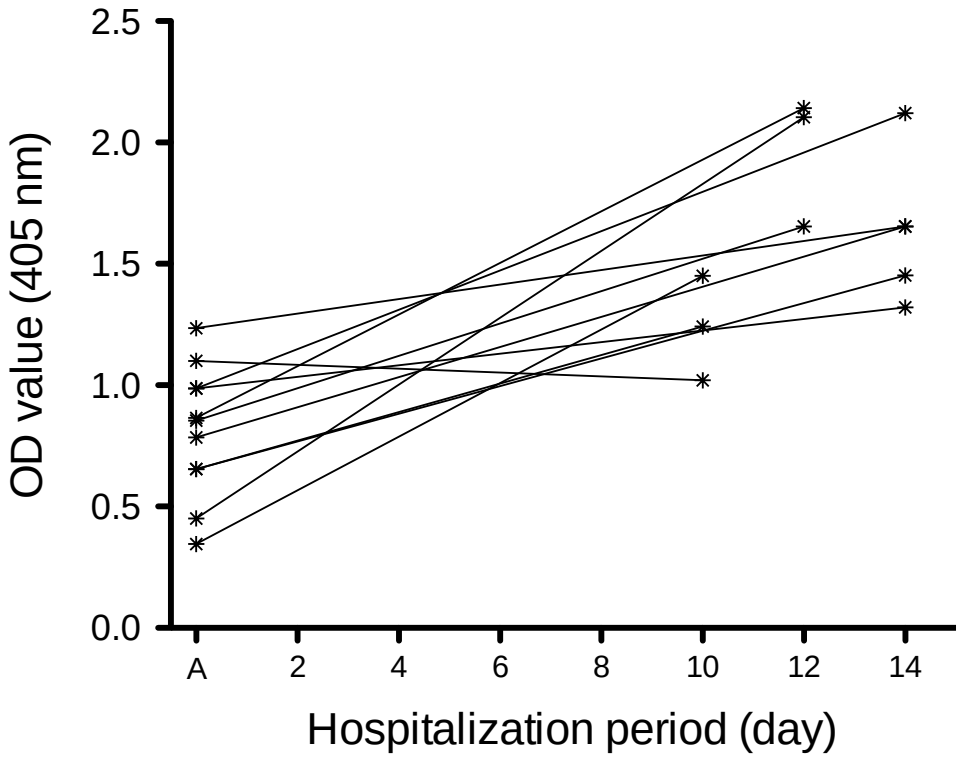

Figure 5. 5. Change of flagellin antibody in horses during hospitalization

Change in serum anti-flagellin immunoglobulin concentrations in horses with GI disease during the hospitalization (n = 10).

V. References

1. Morris DD. Endotoxemia in horses. A review of cellular and humoral mediators involved in its pathogenesis. *J Vet Intern Med.* May-Jun 1991;5(3):167-181.
2. Barton MH, Morris DD, Norton N, Prasse KW. Hemostatic and fibrinolytic indices in neonatal foals with presumed septicemia. *J Vet Intern Med.* Jan-Feb 1998;12(1):26-35.
3. King JN, Gerring EL. Detection of endotoxin in cases of equine colic. *Vet Rec.* Sep 3 1988;123(10):269-271.
4. Moore JN, Morris DD. Endotoxemia and septicemia in horses: experimental and clinical correlates. *J Am Vet Med Assoc.* Jun 15 1992;200(12):1903-1914.
5. Akira S, Takeda K. Toll-like receptor signalling. *Nat Rev Immunol.* Jul 2004;4(7):499-511.
6. Sparwasser T, Miethke T, Lipford G, et al. Bacterial DNA causes septic shock. *Nature.* Mar 27 1997;386(6623):336-337.
7. Ziegler TR, Luo M, Estivariz CF, et al. Detectable serum flagellin and lipopolysaccharide and upregulated anti-flagellin and lipopolysaccharide immunoglobulins in human short bowel syndrome. *Am J Physiol Regul Integr Comp Physiol.* Feb 2008;294(2):R402-410.
8. Sitaraman SV, Klapproth JM, Moore DA, 3rd, et al. Elevated flagellin-specific immunoglobulins in Crohn's disease. *Am J Physiol Gastrointest Liver Physiol.* Feb 2005;288(2):G403-406.
9. Kwon S, Gewirtz AT, Hurley DJ, Robertson TP, Moore JN, Vandenplas ML. Disparities in TLR5 expression and responsiveness to flagellin in equine

neutrophils and mononuclear phagocytes. *Journal of immunology.* Jun 1 2011;186(11):6263-6270.

10. Dawson TR, Horohov DW, Meijer WG, Muscatello G. Current understanding of the equine immune response to Rhodococcus equi. An immunological review of R. equi pneumonia. *Vet Immunol Immunopathol.* May 15 2010;135(1-2):1-11.
11. Feist N, Berger D, Speer CP. Anti-endotoxin antibodies in human milk: correlation with infection of the newborn. *Acta Paediatr.* Sep 2000;89(9):1087-1092.
12. Sykes BW, Furr MO. Equine endotoxaemia--a state-of-the-art review of therapy. *Aust Vet J.* Jan-Feb 2005;83(1-2):45-50.
13. Kelmer G. Update on treatments for endotoxemia. *Vet Clin North Am Equine Pract.* Aug 2009;25(2):259-270.
14. Brewer BD, Koterba AM. Development of a scoring system for the early diagnosis of equine neonatal sepsis. *Equine Vet J.* Jan 1988;20(1):18-22.
15. Epstein KL, Brainard BM, Gomez-Ibanez SE, Lopes MA, Barton MH, Moore JN. Thrombelastography in horses with acute gastrointestinal disease. *Journal of veterinary internal medicine / American College of Veterinary Internal Medicine.* Mar-Apr 2011;25(2):307-314.
16. Martich GD, Boujoukos AJ, Suffredini AF. Response of man to endotoxin. *Immunobiology.* Apr 1993;187(3-5):403-416.
17. Taveira da Silva AM, Kaulbach HC, Chuidian FS, Lambert DR, Suffredini AF, Danner RL. Brief report: shock and multiple-organ dysfunction after self-

administration of Salmonella endotoxin. *The New England journal of medicine.* May 20 1993;328(20):1457-1460.

18. Fessler JF, Bottoms GD, Coppoc GL, Gimarc S, Latshaw HS, Noble JK. Plasma endotoxin concentrations in experimental and clinical equine subjects. *Equine Vet J Suppl.* Jun 1989(7):24-28.

19. Barton MH, Collatos C. Tumor necrosis factor and interleukin-6 activity and endotoxin concentration in peritoneal fluid and blood of horses with acute abdominal disease. *J Vet Intern Med.* Sep-Oct 1999;13(5):457-464.

20. Figueiredo MD, Moore JN, Vandenplas ML, Sun WC, Murray TF. Effects of the second-generation synthetic lipid A analogue E5564 on responses to endotoxin in [corrected] equine whole blood and monocytes. *Am J Vet Res.* Jun 2008;69(6):796-803.

21. Tabata T, Tani T, Endo Y, Hanasawa K. Bacterial translocation and peptidoglycan translocation by acute ethanol administration. *J Gastroenterol.* 2002;37(9):726-731.

22. Erridge C, Duncan SH, Bereswill S, Heimesaat MM. The induction of colitis and ileitis in mice is associated with marked increases in intestinal concentrations of stimulants of TLRs 2, 4, and 5. *PloS one.* 2010;5(2):e9125.

23. Sagar PM, MacFie J, Sedman P, May J, Mancey-Jones B, Johnstone D. Intestinal obstruction promotes gut translocation of bacteria. *Dis Colon Rectum.* Jun 1995;38(6):640-644.

24. Tsujimoto H, Ono S, Mochizuki H. Role of translocation of pathogen-associated molecular patterns in sepsis. *Digestive surgery.* 2009;26(2):100-109.

25. Gewirtz AT, Navas TA, Lyons S, Godowski PJ, Madara JL. Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression. *J Immunol.* Aug 15 2001;167(4):1882-1885.

26. Metzger N, Hinchcliff KW, Hardy J, Schwarzwald CC, Wittum T. Usefulness of a commercial equine IgG test and serum protein concentration as indicators of failure of transfer of passive immunity in hospitalized foals. *J Vet Intern Med.* Mar-Apr 2006;20(2):382-387.

27. Demmers S, Johannisson A, Grondahl G, Jensen-Waern M. Neutrophil functions and serum IgG in growing foals. *Equine Vet J.* Nov 2001;33(7):676-680.

28. Galan JE, Timoney JF, Lengemann FW. Passive transfer of mucosal antibody to Streptococcus equi in the foal. *Infect Immun.* Oct 1986;54(1):202-206.

29. Holyoak GR, Smith CM, Boyette R, et al. Serum antibodies in mares and foals to Actinobacillus equuli whole cells, outer membrane proteins, and Aqx toxin. *Vet Immunol Immunopathol.* Aug 15 2007;118(3-4):310-316.

30. Long K, Vasquez-Garibay E, Mathewson J, de la Cabada J, DuPont H. The impact of infant feeding patterns on infection and diarrheal disease due to enterotoxigenic Escherichia coli. *Salud Publica Mex.* Jul-Aug 1999;41(4):263-270.

CHAPTER VI

CONCLUSION

The main objectives of the studies comprising this dissertation were: (1) to determine the effects of stimulation of equine monocytes with microbial ligands or TNF-α on the regulation of TLR 2, TLR 3, and TLR 4 by monitoring the expression of genes for these TLRs; (2) to characterize the responses of equine leukocytes to bacterial flagellin and expression patterns of TLR5 on different leukocyte populations; (3) to determine circulating concentrations of flagellin in equine colic patients and septic foals, and (4) to investigate adaptive immune responses directed against flagellin in hospitalized horses.

Infectious and inflammatory diseases are major equine health problems in which systemic inflammatory responses to microbial molecules contribute to disease pathogenesis and the development of life-threatening complications such as sepsis. TLRs are germ-line encoded pathogen recognition receptors (PRR) expressed by various cells, including leukocytes, that recognize pathogen-associated molecular patterns (PAMPs). Recognition of PAMPs by TLRs leads to stimulation of intracellular signaling pathways that involve adaptor proteins such as MyD88 and TRIF. Recruitment of adaptor proteins

triggers distinct cascade of events in the signaling pathways that ultimately results in activation of transcription factors such as NFκB and IRF-3. Genes that encode various inflammatory cytokines, type I IFN, and chemokines are the targets of these transcription factors, and these products are key molecules that initiate immediate immune responses and ultimately shape adaptive immunity. In naturally occurring infections, multiple PRRs are likely to be activated during the initiation of the host response to infection. Recently, it has become apparent that PRR signaling is regulated in a complex manner as a result of cross-talk among intracellular signaling cascades. The results of the first study in this dissertation indicate that activation of equine monocytes by TLR 2 and TLR 4 ligands increases the expression of genes for these two TLRs, but not for TLR 3. Conversely, cellular activation via TLR 3 selectively causes induction of the TLR 3 gene. These differences in receptor gene profile expression segregate with the different adaptor proteins utilized by TLR 2 and TLR 4 (MyD88) and TLR 3 (TRIF). This pattern of TLR expression may represent an energetically economical strategy for the cells, as TLR 2 and TLR 4 recognize bacterial components while TLR 3 senses viral double-stranded RNA.

In the second study in this dissertation, we investigated the effect of bacterial flagellin on equine monocytes and neutrophils in vitro. In many equine gastrointestinal diseases, there is evidence that the normal intestinal mucosal barrier function is lost, allowing the translocation of bacterial PAMPs from the intestinal lumen into the general circulation, where they activate the innate immune system to generate pro-inflammatory mediators. Although LPS clearly is responsible for some of the alterations that occur in affected animals, it is equally likely that other bacterial components enter the circulation. Flagellin is a motor unit of bacteria, which is also important in bacterial adherence to

epithelial cells and invasion to the host tissue. Flagellin has a highly conserved structure among different bacterial species and it activates the immune/inflammatory axis via TLR5 on the cell surface. Because TLR5 recognizes a highly conserved region of flagellin, this receptor detects a wide variety of microbes, including both Gram-negative and Gram-positive flagellated bacteria. The molecular mechanisms by which TLR 5 signals are similar to that used by other TLRs, as ligation with flagellin forms a TLR 5 homodimer or heterodimer which then activates the MyD88/IRAKs/TRAF6/NF-κB and MAPK intracellular signaling pathways to transcriptionally regulate pro-inflammatory genes. There is considerable evidence for the clinical importance of flagellin in the pathogenesis of various diseases, including Crohn's disease and local infectious disease of the eyes and lungs. Therefore, a more complete understanding of flagellin-induced stimulation of the TLR 5 pathway in equine leukocytes was undertaken. The results obtained in this study revealed that even high concentrations of flagellin (~3 μg/ml) failed to induce equine monocytes to produce TNF-α production, while the same cell type from the same horses responded robustly to LPS. Similarly, flagellin had no stimulatory effect on equine peritoneal and alveolar macrophages. In contrast, flagellin stimulated equine neutrophils to produce reactive oxygen species with an EC_{50} of 20 ng/ml. This response was absent when flagellin was pre-treated with pronase, but not altered by co-incubation with polymyxin B. Neutrophils had a strong signal for surface expression of TLR 5 by flow cytometry, whereas no signal was detected for monocytes. Both cell types expressed TLR 4 and TLR 5 mRNA, but in different abundancies, and TLR5 mRNA from both cells was translated into protein in cell-free translation assays. Additionally, flagellin and LPS induced similar levels of expression of TNF-α, COX-2

and IL-10 mRNA in neutrophils, but not in monocytes. Finally, incubation with neither LPS nor IFN-γ altered TLR5 expression by the monocytes. The pronounced differences in the responses of monocytes and neutrophils to flagellin identified in this study appears to be unique to the horse, as mononuclear phagocytes and neutrophils from other species both respond strongly to flagellin. The apparent lack of surface expression of TLR 5 on equine monocytes explains such hypo-responsiveness. This may be due to a block in TLR 5 mRNA translation or to rapid turnover of the small amount of TLR 5 protein synthesized by these cells. Results from this study provide additional evidence that innate immune responses can be species-specific and that it is difficult to extrapolate data obtained in one species to another.

To substantiate a role for flagellin in the pathogenesis of equine inflammatory diseases, we further investigated the concentrations of flagellin in serum samples from adult horses with GI diseases and septic foals to test the hypothesis that flagellin enters the circulation of sick foals and horses. In addition, we determined whether adult horses responded to the presence of flagellin in the circulation by producing IgG directed against it, and whether changes occur in serum concentrations of colostrum-derived flagellin IgGs in septic newborn foals were associated with the presence of flagellin. In the first part of this study, we validated both flagellin and flagellin antibody ELISA assays by using antibodies against flagellin tested for specific recognition of flagellin by dot blot assay using highly purified flagellin. Standard curves and confirmation of the linear relationship between absorbance and concentrations of either flagellin or anti-flagellin antibody were made by adding known amounts of flagellin or anti-flagellin antibody to 1% equine donor serum. Reproducible standard curves were obtained for both flagellin and

anti-flagellin antibody. Serum samples obtained from adult horses admitted to the UGA Teaching Hospital with a variety of GI diseases had increased concentrations of flagellin when compared to samples obtained from clinically healthy horses. Similarly, higher concentrations of flagellin were detected in serum samples obtained from septic foals when compared to values for samples from healthy foals. Furthermore, serum concentrations of anti-flagellin antibodies increased over the period of hospitalization in some adult horses being treated for GI diseases in the UGA Teaching Hospital, providing evidence that flagellin enters the circulation during such diseases and initiates an immune response. While serum samples obtained from healthy newborn foals after ingestion of colostrum allowed identification of a sharp increase in anti-flagellin IgG concentrations that were maintained for 30 days, anti-flagellin IgG concentrations were significantly lower in samples obtained from septic foals hospitalized at the UGA Teaching Hospital when compared to samples from clinically healthy foals. The latter findings are consistent with either the lack of absorption of anti-flagellin antibody from the mare's colostrum or absorption of antibody to circulating flagellin. Taken together, increased circulating concentrations of flagellin in horses with intestinal diseases and septic foals suggest that bacterial components other than LPS may be responsible for the development of the systemic inflammatory response syndrome in sick horses with GI diseases and in septic foals.

In conclusion, the studies conducted for this dissertation project have demonstrated that the horse is in possession of a unique pattern of inflammatory regulation. First, the horse utilizes an essentially monomorphic MyD88 based response to TLR 4 in contrast with the MyD88 and TRIF bifurcated pattern observed for mice,

human and bovine responses. Other classical responses to microbial cell wall products and double stranded RNA are similar to those seen in other mammals. Second, the response to flagellin in horses is strong and robust in neutrophils, but lacking in cells of monocyte origin. This is in stark contrast with the pattern of flagellin response in other mammals. While both neutrophils and monocyte derived cells express TLR 5 mRNA, only neutrophils produce surface receptor and show dose dependent flagellin responses.

I believe our findings from the studies described in this dissertation improve our understanding of how different types of leukocytes are involved in immune responses to diseases that interfere with intestinal barrier function and in bacterial infections. Furthermore, these findings suggest that the responses of horses to bacteria are different from those of other species and that treatment of diseases involving bacteria and/or their components should be approached in a species-specific fashion. Finally, the findings from these studies shed new light on the responses of horses to bacterial components other than LPS that may enter the circulation during gastrointestinal diseases, and provide the basis for the development of compounds other than hyperimmune anti-LPS sera and polymyxin B for use in affected horses.

APPENDIX

1. Assay validation of flagellin and anti-flagellin antibody ELISA

1) Optimal antibody dilutions for flagellin and anti-flagellin Ab assays

Optimal antigen/antibody coating dilutions were determined using serial dilutions of flagellin or mouse anti-FliC Ab for anti-flagellin Ab and flagellin ELISA assays, respectively. For example, wells of 96-well microtiter plate were coated with serially diluted mouse monoclonal anti-FliC Ab (1:500, 1:1,000, 1:2,000) in 0.05 M carbonate-bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. After blocking wells with 0.5% BSA in PBS for 1 h at room temperature, 100 µl of spiked flagellin samples in PBST and 10% EDS ranging from 0 to 300 ng were added to wells in a duplicate manner. The plate was incubated for 2h at 37°C followed by the addition of serially diluted goat polyclonal anti-flagellin IgG (1:500, 1:1,000, 1:3,000) and incubated for 1.5 h at 37°C (Fig. 1). To detect Ag-IgG complexes, the wells were treated with 100 µl of peroxidase-conjugated donkey anti-goat IgG (1:3000). After each step, unbound reagents in the wells were removed by repeated washing with 1% Tween-20 in PBS (PBST). Finally, 100 µl of substrate were added to each well and color development was measured at 405 nm absorbance on an automated microplate reader after ~ 20 min.

This protocol was adopted from *Criss-Cross serial dilution analysis* from the *Current Protocols in Molecular Biology, 11.2.16*. We have determined monoclonal FliC Ab at 1:500 and goat polyclonal anti-flagellin IgG at 1:500 are optimal for our in-house flagellin assay (Fig. 2). For flagellin Ab ELISA, wells of 96-well microtiter plate were coated with 100 ng flagellin / well based on personal communication with Mr. Moore from Dr. Gewirtz laboratory. Similar to flagellin assay, serial dilutions of HRP-conjugated goat anti-horse IgG were tested and 1:30,000 dilution was determined to be optimal for our in-house flagellin Ab assay (data not shown).

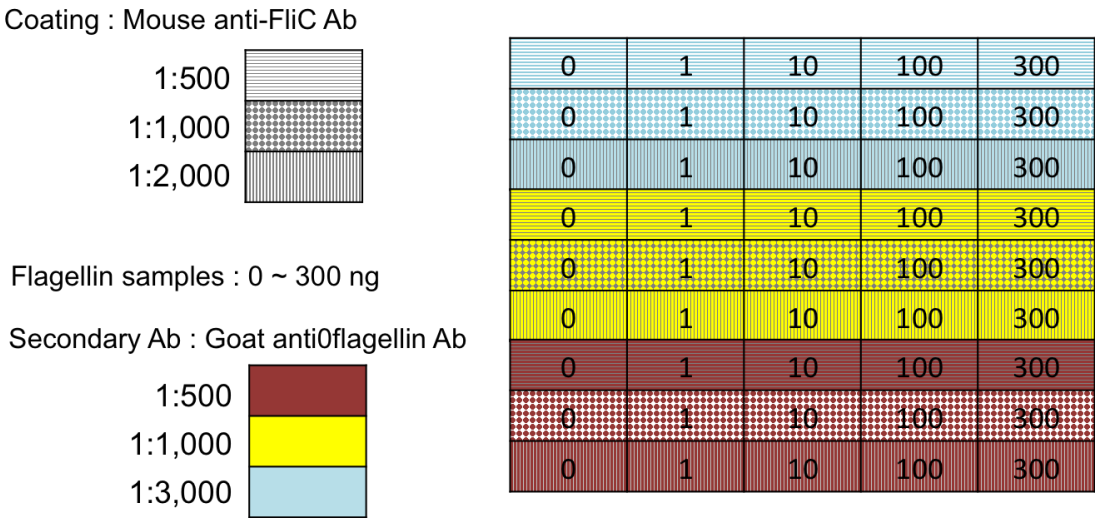

Figure 1. Criss-cross serial dilution analysis for optimal antibody dilution determination

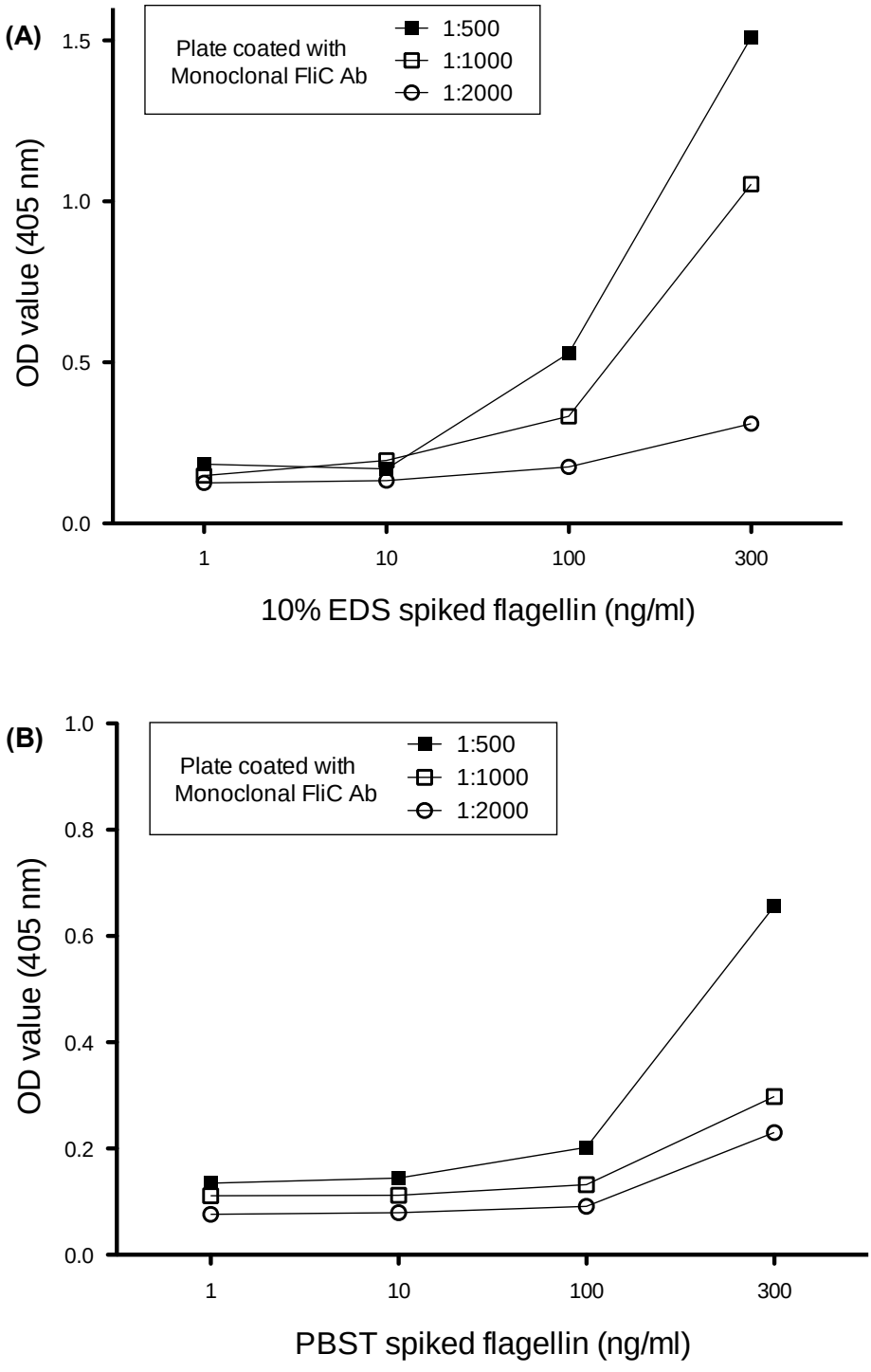

Figure 2. Serial dilution analysis of optimal antibody dilutions for a flagellin ELISA assay.

2) Optimal antibody dilutions for flagellin and anti-flagellin Ab assays

Optimal sample dilution was determined by different dilutions of EDS spiked with known concentrations of flagellin or anti-FliC Ab. For example, a range (1:100 ~ 10,000) of mouse anti-FliC Ab was spiked in PBST, EDS, or EDS diluted with PBST at 1% and 10%. A flagellin Ab ELISA was run using these samples. Anti-FliC Abs spiked in either 1% or 10% EDS showed linear relationship ($r^2 \geq 0.9$) (Fig. 2). Similar experiment was done for flagellin ELISA assay using flagellin spiked EDS (Fig. 3). Later, 10% serum sample dilution was used for both flagellin and flagellin Ab assays.

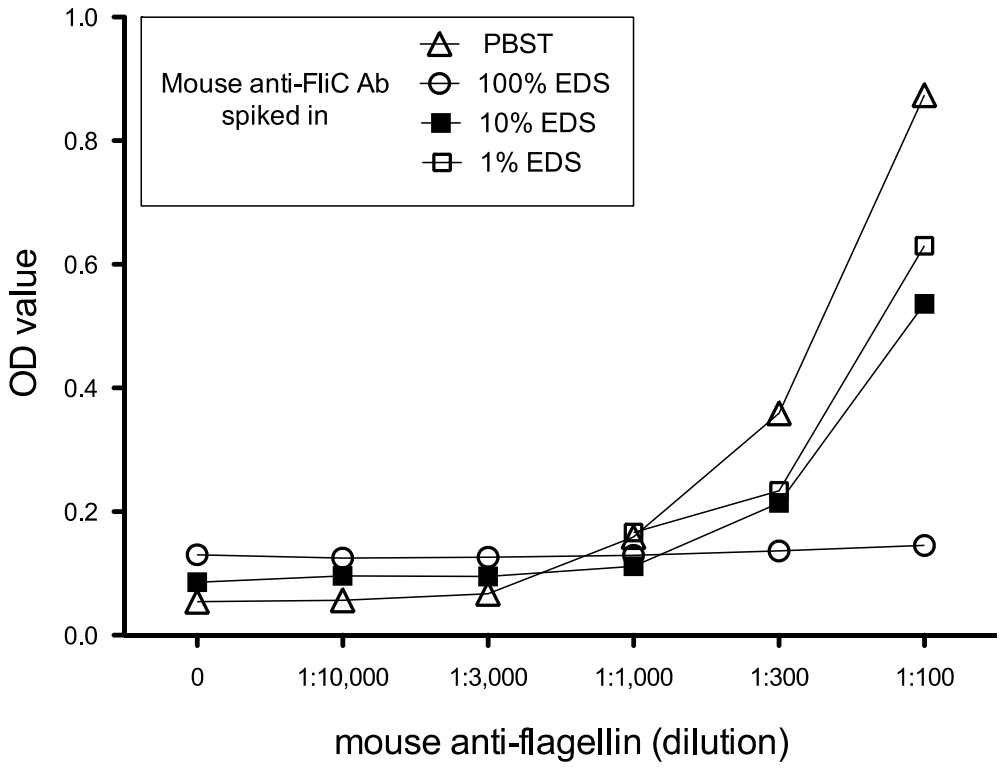

Figure 3. Optimal sample dilution validation for flagellin Ab assay.

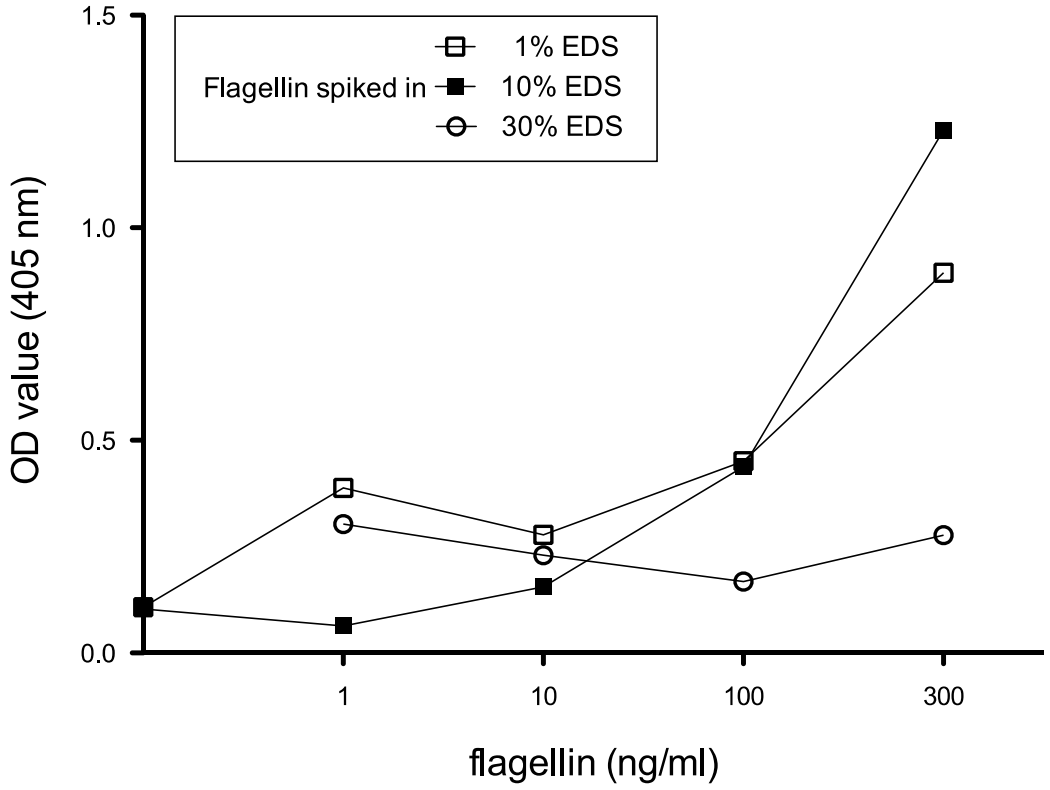

Figure 4. Optimal sample dilution validation for flagellin assay.

Initially, we also isolated IgGs from each serum sample to minimize non-specific binding to other serum proteins. Briefly, 200 µl of serum samples were mixed with an equal volume of saturated ammonium sulfate and incubated for 10 min at 4°C. Precipitated IgGs were collected by centrifugation at 20,000 x *g* for 10 min at 4°C. The antibody pellets were dissolved in 400 µl PBS and concentrated through Amicon PM-30 centrifugal concentrators to remove the ammonium sulfate. The IgGs were re-constituted with PBS to the original volume of serum used, and stored frozen at -80^{o}C. However, there was no difference between using diluted serum or isolated IgG samples (data not shown). Therefore, we used intact serum samples for flagellin Ab assays.