

**TLR4 expression on equine B lymphocytes: a clue to LPS
sensitivity?**

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Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of

Master of Science
In
Animal and Poultry Sciences

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(9:00 AM August 7th, 2017)

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Abstract for General Public

Horses are prone to potentially lethal infections due to their surrounding fecal containing environment and their predisposition to colic. Their gastrointestinal tract and environment naturally contain gram-negative bacteria such as *E.coli*. The gram-negative bacterial cell membrane expresses a molecule called lipopolysaccharide (LPS) which can cause inflammation by binding to an immune cell receptor called toll-like receptor 4 (TLR4). In cases where epithelial barriers are compromised or breached, LPS has the potential to enter the circulation and cause a severe inflammatory response capable of progressing to shock and death. Humans are also highly sensitive to LPS yet their B lymphocytes express non-functional TLR4. Mice, in contrast, are highly tolerant of LPS but their B lymphocytes express functional TLR4. The objective of this study was to determine TLR4 presence and functionality on equine B lymphocytes. Studies in horse have perhaps been limited by the small number of antibody markers available for use in horse. Two B lymphocyte markers not previously assessed in horse tissue were used in this experiment to enrich for B lymphocytes. A TLR4 marker, also not previously described in the horse, was used in conjunction with the two B lymphocyte markers determined TLR4 is present on equine B lymphocytes. However, equine B lymphocytes failed to proliferate in the presence of LPS similar to human B cells. Transcriptional changes were observed in the equine cells in the TLR4 pathway upon treatment with LPS.

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Scholarly Abstract

Horses are prone to potentially lethal endotoxemia due to their surrounding fecal containing environment and their predisposition to colic. Their gastrointestinal tract and feces naturally contain gram-negative bacteria. These bacteria express lipopolysaccharide (LPS) on their cell membranes, which is recognized by Toll-like receptor 4 (TLR4). In cases where epithelial barriers are compromised or breached LPS has the potential to enter circulation and cause the inflammatory symptoms seen with endotoxemia. The objective of this study was to determine TLR4 presence and functionality on equine B cells. TLR4 expression on B lymphocytes has been studied in mouse, human and many other mammals, but has not been well characterized in the horse. Humans are highly sensitive to LPS but their B cells express non-functional TLR4. Mice in contrast are highly tolerant of LPS yet their B cells express functional TLR4. Studies in horse have perhaps been limited by the limited array of antibody markers available for use in horse. Anti-human CD21 has previously been shown to mark equine B lymphocytes. We show rat anti-mouse CD45R(B220) mAbs also accurately labels equine B lymphocytes. To investigate TLR4 expression in horses 12 Thoroughbred geldings, ages 5-10, were used for blood collection. By using the density gradient, Lympholyte, lymphocytes were separated from peripheral blood and incubated with or without LPS. B lymphocyte proliferation, TLR4 expression and mRNA changes were examined before or after culture in the presence or absence of LPS. We demonstrate TLR4 is expressed on equine B lymphocytes through the use of a mouse anti-human TLR4 antibody, clone 76B357.1, not previously used in horse. We demonstrated equine B cells fail to proliferate under LPS challenge as opposed to highly proliferative mouse B lymphocytes. However, transcriptional changes were observed in the equine cells within the TLR4 pathway upon treatment with LPS.

Acknowledgements

This thesis was accomplished with Dr. Caroline Leeth, who gave me the opportunity to work with her and guided me throughout the entire study. I would also like to thank my committee members Dr. Sally Johnson and Dr. Sharon Witonsky for contributing their knowledge and expertise to the study. I am thankful for the graduate students in the Johnson and El-Kadi labs, especially Amanda Reeg and Sydney McCauley, who graciously taught me lab techniques and gave their assistance wherever it was needed. I also thank my parents who have supported, pushed and always encouraged me to be the best I could be.

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List of Definitions

1. CD14-Cluster of differentiation 14
2. CD21-Cluster of differentiation 21
3. CD45R/B220-Cluster of differentiation 45 Receptor
4. CpG-Cytosine triphosphate deoxynucleotide-Phosphodiester link-Guanine triphosphate deoxynucleotide
5. C_T -Threshold cycle
6. dsDNA-Double stranded deoxyribose nucleic acid
7. GAPDH-Glyceraldehyde 3-phosphate dehydrogenase
8. IL-1R-Interleukin 1 receptor
9. IRF3-Interferon regulatory factor 3
10. IRF7-Interferon regulatory factor 7
11. IKK ϵ -I-kappa-B kinase epsilon
12. IRAK-Interleukin 1 receptor associated kinase
13. IL-1 β -Interleukin 1 beta
14. IL-6-Interleukin 6
15. IL-10-Interleukin 10
16. IFN β - Interferon beta
17. IP-10-Interferon gamma-induced protein 10
18. LBP-Lipopolysaccharide binding protein
19. LPS-Lipopolysaccharide
20. MAPK-Mitogen activated protein kinase
21. mCD14-Membrane CD14

22. MD-2-Lymphocyte antigen 96
23. mRNA-Messenger ribonucleic acid
24. MyD88-Myloid differentiation 88
25. NF- κ B-Nuclear factor-kappa-B
26. PAMP-Pathogen associated molecular pattern
27. PRR-Pattern recognition receptor
28. RANTES-Regulated on Activation, Normal T Cell Expressed and Secreted
29. RIP1-Receptor-interacting serine/threonine-protein kinase 1
30. TAK1-Transforming growth factor beta-activated kinase 1
31. TBK1-TANK-binding kinase 1
32. TLR-Toll-like receptor
33. TIR-Toll/IL-1 Receptor
34. TNF α -Tumor necrosis factor alpha
35. TRAF-TNF receptor associated factor
36. TRAM-Translocation associated membrane protein 1
37. TRIF-TIR domain-containing adaptor protein inducing interferon beta
38. ssDNA-Single stranded deoxyribose nucleic acid

CHAPTER I

Introduction

Endotoxemia is a prominent concern in human and equine medicine. In comparison to other species, humans and horses experience an extreme inflammatory response to lipopolysaccharide (LPS), the causative molecule in endotoxemia (Brade, 1999). This syndrome can lead to lethal conditions such as septic shock, a sequela accounting for the majority of human deaths seen in U.S. hospitals (Liu, 2014). Found on all gram-negative bacteria, LPS can enter the body when any sort of breach in the epithelial tissue occurs (Moore, 1988). Since gram-negative bacteria can be readily found in the environment and within the gastrointestinal tract of humans and equines the potential for endotoxemia to occur is high.

To be able to mount an immune reaction resulting in an inflammatory state, the LPS molecule must be recognized by the body. The recognition of LPS is achieved through Toll-like receptor 4 (TLR4)(Hoshino, 1999). TLR4 is located principally on immune cells but can be found on other cell types such as liver and skeletal muscle (Frost, 2002; Takeda, 2004). TLR4 belongs to a family of receptors that function within the innate immune system (O'Neill, 2013). In most cases, when the body is exposed to a foreign antigen it will take up to 14 days to initiate a specific adaptive immune response (Werling, 2003; Ademokun, 2010). With receptors like TLR4, the body recognizes certain pathogens very quickly and mounts a response in a matter of hours to protect the body (Janeway, 2005).

The mechanism of recognition for LPS has been well documented in mouse and humans, but is less characterized in horse (Vaure, 2014). Even though humans and equines are considered to be the most sensitive species to LPS, not many studies have been conducted to understand if this similarity can be seen at a molecular level. Understanding the differences and similarities between humans and equines could allow for a more applicable animal model in human septic shock studies. This could open a line of communication between human and veterinary medicine to search for further treatment options for both species. The objective of this study was to examine TLR4 expression on equine B cells and their response to LPS treatment in vitro.

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CHAPTER II

Review of Literature

Toll-like Receptors

The surge in discovery of Toll-like receptors (TLRs) can be traced back to the Cold Spring Harbor Symposia on Quantitative Biology in 1989, where Charles Janeway, Jr. proposed that there were nonclonal origins to the immune system yet to be characterized. He hypothesized that innately conserved receptors existed and could recognize pathogen-associated molecular patterns (PAMPs) of non-host origin and termed these receptors pattern recognition receptors (PPRs) (Janeway, 1989). Although there are a many types of PPRs, TLRs are one of the most studied of the group. The first

Table 1: Presence of homologous TLR gene sequences.

	Human	Mouse	Horse
TLR1	Yes	Yes	Yes
TLR2	Yes	Yes	Yes
TLR3	Yes	Yes	Yes
TLR4	Yes	Yes	Yes
TLR5	Yes	Yes	No
TLR6	Yes	Yes	Yes
TLR7	Yes	Yes	Yes
TLR8	Yes	Non-functional	Yes
TLR9	Yes	Yes	Yes
TLR10	Yes	No	Yes
TLR11	No	Yes	No
TLR12	No	Yes	No
TLR13	No	Yes	No

of the Toll family was cloned in mice and called the Interleukin-1 Receptor (IL-1R), but at that time the mechanism of the receptor was unknown (Sims, 1988). In 1989, the IL-1R was cloned in human T lymphocytes with still no understanding of its mechanism (Sims, 1989). During the same period, the *Toll* gene was characterized in *Drosophila melanogaster* and found to be involved in the dorso-ventral axis formation in the fly's embryo (Hashimoto, 1988). A few years later the IL-1R and *Toll* genes were found to have homologous domains, leading to the hypothesis of a shared signal transduction (Gay, 1991). In 1998, 4 more members of the Toll family (TLR1-5) were identified in human and the group was renamed Toll-like receptors (Rock, 1998).

To date, 13 TLRs have been identified. Of these TLR genes 10 of the TLR genes are expressed in humans and 12 are expressed in

mice. Some species differences exist for TLR expression (Table 1). For example TLR1-TLR9 are conserved between human and mice, however TLR10 is not expressed in mice. In addition TLR11-TLR13 are not found in humans, but are present in mice (Kawai, 2009). Each identified TLR has a unique structure which results in the recognition of unique ligands. Akira and colleagues (1999) created several *Tlr* and adapter molecule-knockout mice that elucidated many TLR ligands. The human homologue of *Drosophila* Toll, *hToll* also known as *TLR4*, was the first of these *Tlr* knockout mice created and confirmed Lipopolysaccharide (LPS) as the TLR4 ligand (Hoshino, 1999).

The following is a summary of TLRs and their known ligands. TLR2, which is able to heterodimerize with TLR1 or TLR6, can recognize triacetylated lipoproteins and diacetylated lipoproteins respectively (Takeuchi, 2001; Takeuchi, 2002). TLR2^{-/-} and TLR1^{-/-} murine cells were needed to recognize and respond to triacetylated lipoproteins. (Takeuchi, 2002). It was shown that murine TLR6^{-/-} cells were unresponsive to diacetylated lipoproteins, but were responsive to other lipoproteins. In conjunction, TLR2^{-/-} TLR6^{-/-} embryonic fibroblasts were unresponsive to diacetylated lipoproteins demonstrating TLR2 and TLR6 are imparative for diacetylated lipoprotein recognition (Takeuchi, 2001). TLR3 is able to aid in the defense against viral infections by sensing double stranded DNA (dsDNA). A TLR3^{-/-} mouse was observed to have reduced responses and cytokine production to polyinosine-polycytidylic acid, a synthetic analog of dsDNA (Alexopoulou, 2001). Flagellin, an inherent component of bacterial flagella, can be recognized by TLR5. This was confirmed using tandem mass spectrometry, analyzing *Listeria monocytogenes* culture supernatants, which identified the bacterial flagella as having the highest level of TLR5-stimulating activity (Hayashi, 2001). A synthetic group of molecules called imidazoquinolines activate immune cells and are recognized by TLR7. Although, it was known that imidazoquinolines had strong anti-viral and anti-bacterial properties, it was not understood how until Hemmi *et al.* demonstrated that TLR7^{-/-} mouse cells produced no protective responses after imidazoquinolines treatment (Hemmi, 2002). TLR7, along with TLR8, also recognizes single stranded DNA (ssDNA), which was observed using TLR7- and TLR8- deficient murine cells (Heil, 2004). Although, TLR8 is non-functional in mice, transgenic mice were developed to express human TLR8 in order to uncover its ligand (Kugelberg, 2014).

TLR9 provides anti-viral immunity. It was demonstrated that unmethylated CpG dinucleotides, common in bacterial DNA, activated TLR9 when TLR9 deficient murine cells were unresponsive to CpG DNA (Hemmi, 2000). TLR10 is the only TLR with an unidentified ligand, but demonstrated anti-inflammatory effects. This was observed in the increase of proinflammatory cytokine release after blocking TLR10 during stimulation (Oosting, 2014). TLR11, which is highly expressed in kidney and bladder tissue, is as a key defense against uropathogenic bacteria. TLR11-deficient mice failed to recognize and respond to uropathogenic *E. coli* (Zhang, 2004). This TLR also oligomerizes with TLR12 to recognize and respond against *Toxoplasma gondii* infection, which was observed when transfection of both TLR11 and TLR12 was needed for murine macrophages to produce high levels of IL-12 (Andrade, 2013). Finally, TLR13 was shown to recognize ribosomal RNA from bacteria (Oldenburg, 2012).

Overview of Toll-Like Receptor 4

TLR4 is among one of the most studied TLRs and is known to play an intricate role in eliciting the symptoms seen in endotoxemia and sepsis in many species. TLR4 was originally identified as hToll as it resembled *Drosophila melanogaster's Toll* (Medzhitov, 1997). A year later, a group of 4 related receptors were found in humans and the group was renamed Toll-like receptors (Rock, 1998). At TLR4's discovery no function was known, but it was hypothesized this TLR would function with the innate immune system similar to *Toll* in *D. melanogaster* (Lemaitre, 1996). It was discovered that two mouse strains, C3H/HeJ and C57BL/10ScCr, had defective lipopolysaccharide (LPS) signaling that corresponded with mutations in the TLR4 gene (Poltorak, 1998). Akira *et al.* (1999), produced a *TLR4* knockout mouse that failed to respond to LPS, verifying LPS as the TLR4 ligand (Hoshino, 1999). It was observed that after TLR4 cDNA transfection, LPS responsiveness was undetected thus, it was hypothesized that TLR4 did not directly bind LPS (Kirschning, 1998; Wright, 1999). Myeloid differentiation factor 2 (MD-2) was discovered as the key link between LPS and TLR4 to allow recognition (Shimazu, 1999).

In addition to MD-2, TLR4 uses several other molecules to recognize LPS. Before LPS can be bound by the TLR4 complex, Lipopolysaccharide binding protein (LBP) binds

LPS in the serum (Tobias, 1989). LBP then transfers LPS to another accessory protein, membrane CD14 (Park, 2009). Membrane CD14 (mCD14) is a glycoprotein that is anchored in the cellular membrane and transfers LPS to the TLR4-MD-2 complex for recognition and signal transduction (Wright, 1990).

After TLR4 binds to LPS on the cell surface through MD-2 interaction, the intracellular domain of two TLR4 proteins called the Toll/IL-1R (TIR) domain homodimerize (Kawai, 2009; Vaure, 2014). TLR4 is able to signal through two adapter molecules, Myloid differentiation factor 88 (Myd88) and TIR-domain-containing adapter-inducing interferon- β (TRIF), resulting in two distinct signal cascades (Takeda, 2004; Figueiredo, 2009; Kawai, 2009). The recruitment of MyD88 results in the activation of MAP kinases (MAPKs) and Nuclear Factor Kappa B (NF- κ B) that controls inflammatory cytokine gene expression. TRIF recruitment activates MAPKs and NF- κ B as well, along with interferon regulatory factor 3 (IRF3), which leads to the production of type I interferons (Kawai, 2007).

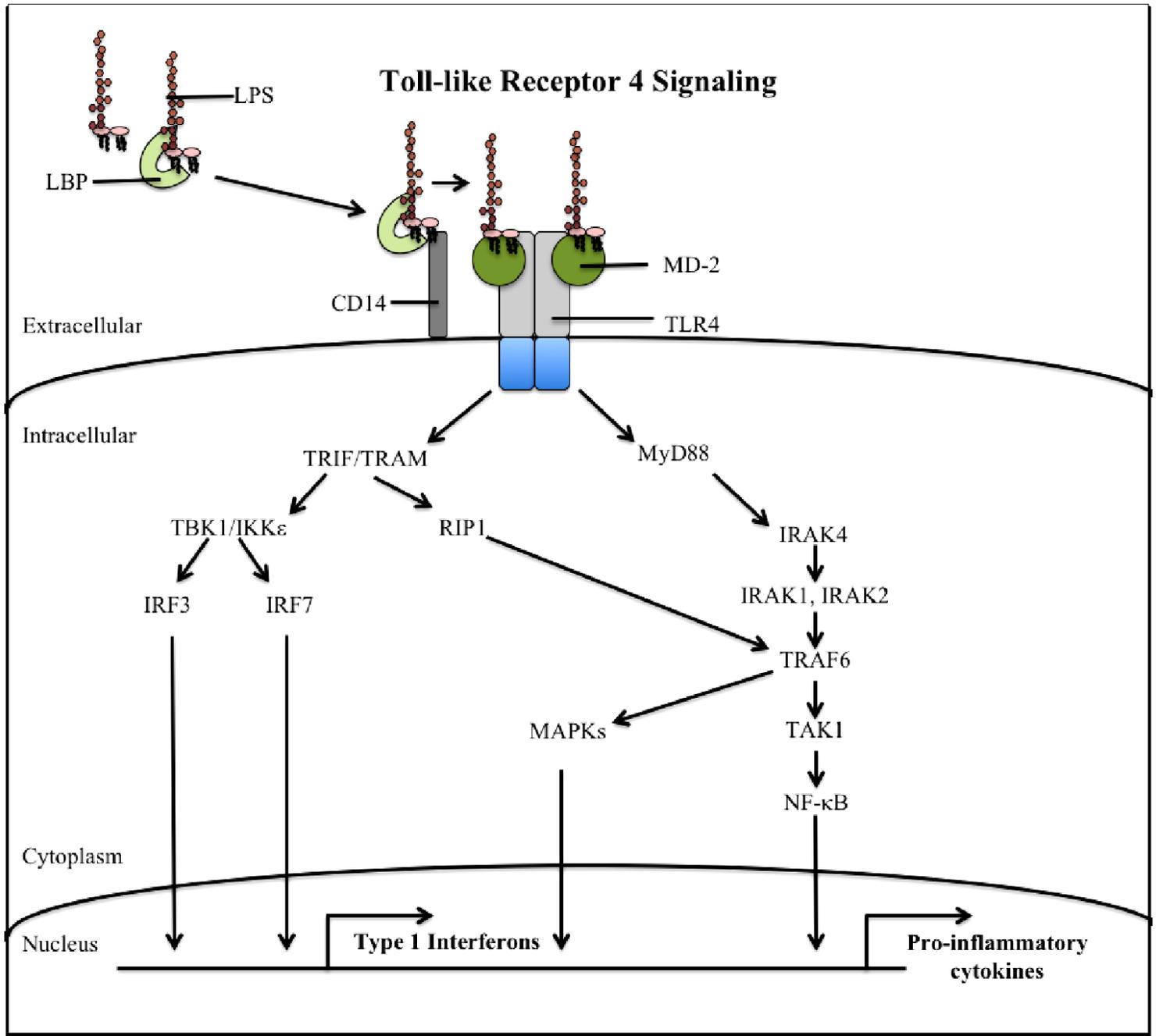


Figure 1: TLR4 signaling pathways.

Human TLR4 expression on immune cells

TLR4 expression varies between cell types and between species. TLR4 expression is higher in myeloid derived cells compared to lymphoid derived cells. In human samples, monocytes were found to produce high levels of TLR4 mRNA, in contrast to T cells, natural killer cells, and plasmacytoid dendritic cells that show little to

no TLR4 mRNA (Hornung, 2002; Ketloy, 2008). Human B cells express low, but definite levels of TLR4 mRNA. Human B cells are non-responsive to LPS (Hornung, 2002; Peng, 2005; Ketloy, 2008). B cell responsiveness to LPS can be induced through *in vitro* co-treatment with IL-4 in humans (Mita, 2002).

Mouse TLR4 expression on immune cells

Similar to humans, murine TLR4 expression is higher in myeloid derived cells compared to lymphoid derived cells. In contrast to human, murine plasmacytoid dendritic cells express high levels of TLR4 (Ketloy, 2008). In addition, TLR4 mRNA was present in higher quantities in murine B cells and T cells than seen in humans (Iwami, 2000; Gururajan, 2007). TLR4 on murine B cells acts contrary to the subfunctional TLR4 found on human B cells. Murine B cell TLR4 is able to recognize LPS to initiate signal transduction that leads to B cell proliferation, cytokine production, and class-switch recombination (Xu, 2008; Stavnezer, 2014). Differences in TLR4 expression and functionality become of interest when studying disease states related to LPS such as endotoxemia and septic shock. When picking a model to perform studies, understanding species differences will lead to accurate and applicable data (Vaure, 2014).

Endotoxemia and Sepsis

Endotoxemia and the further development of sepsis is a leading cause of death in both humans and equines. Sepsis is estimated to cause 250,000 human deaths annually in the U.S. (Tidswell, 2011). Septic shock patients positive for endotoxemia experience a higher mortality rate than those who did not have endotoxemia (Danner, 1991). The death toll of endotoxemia is hard to quantify in horses, but since it plays a role in the pathogenesis of many gastrointestinal diseases such as colic and neonatal septicemia, it is considered a principal killer in equines (Morris, 1991).

Endotoxin, also known as LPS, is expressed in all Gram negative bacteria. LPS, which is structurally necessary for the bacterial outer membrane, is composed of a lipid A region, core section, and an O-polysaccharide. Positioned to the inside of the membrane, the hydrophobic lipid A region is the endotoxically active portion of the molecule (Erridge, 2002). As Gram-negative bacteria proliferate or die, LPS is released from the

membrane into the surrounding microenvironment (Morris, 1991). This becomes a concern in gastrointestinal and inflammatory diseases that compromise the epithelial barrier and allow LPS to enter bodily tissues and circulation from the gastrointestinal tract (Moore, 1979). In a healthy individual, LPS can be cleared from the circulation by the liver, however in compromised or persistent cases, LPS may reach damaging levels (Herring, 1963; Satoh, 2008). Once in the circulation the pro-inflammatory mediators released in response to LPS can cause systemic inflammation, which can lead to organ failure and death (Brandtzaeg, 1989).

Conclusion

During the last 20 years there has been a burgeoning interest in toll-like receptors among different species and their function in the immune system. Research has included many species, however most attention has been paid to mouse and human TLRs. For medical reasons, human TLRs are of higher relevance, although mouse TLRs are often used and studied to model human function.

TLRs are a group of highly conserved receptors that function as a facet of our innate defense against pathogens. In many species, these TLRs are located on and within many different cell types where they are able to recognize common patterns presented by pathogens. Recognition of a specific PAMP will cause a conformation change in the TLR. This change induces the recruitment of adapter molecules, which signal to intermediate proteins and finally leads to the production of cytokine NF- κ B and type-I interferons. These cytokines result in the attraction of immune cells to the threatened area to neutralize and remove pathogens. Understanding the activity of TLRs can possibly indicate ways to manipulate disease states. Currently, TLR ligands are used as adjuvants with some vaccines to increase the bodies immune response to subsequently increase the effectiveness of the vaccine (Steinhagen, 2011).

TLR4 was one of the first TLRs discovered and fit the PRR hypothesis. TLR4 recognizes LPS, a potent TLR ligand, that is presented on gram-negative bacteria. When LPS is present in the circulation it is considered endotoxemia, which corresponds to an increase in lethality among sepsis patients. Humans have a high systemic inflammatory response to LPS as do equines, mice have a lower inflammatory response. Therefore, the

equine TLR4 may be more comparable to human TLR4. Studies investigating the species differences and similarities between human, horse and mouse in regards to TLR4 expression may give rise to a more applicable human model to study diseases involving LPS. Hopefully, research in this area will lead to the development of new and effective treatment options for humans and equines.

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CHAPTER III

TLR4 expression on equine B lymphocytes: a clue to LPS sensitivity?

Abstract

TLR4 recognizes LPS as its ligand and elicits an immune response leading to the production of pro-inflammatory cytokines. TLR4 is located extracellularly and intracellularly on many immune cells, including B lymphocytes. TLR4 expression on B lymphocytes has been studied in mouse, human and many other mammals, but has not been well characterized in horse. Equine cellular assessment is hampered by the lack of validated antibody reagents capable of binding horse proteins. Anti-human CD21 marks equine B cells. We show rat anti-mouse CD45R(B220) mAbs also accurately labels equine B lymphocytes. To investigate TLR4 expression in horses, 12 Thoroughbred geldings, ages 5-10 years, were used for blood collection. By using the density gradient, Lympholyte, lymphocytes were separated from peripheral blood and incubated with or without LPS. B lymphocyte proliferation, TLR4 expression and mRNA changes were examined before or after culture in the presence or absence of LPS. We demonstrated equine B lymphocytes failed to proliferate under LPS challenge as opposed to highly proliferative mouse B lymphocytes. We demonstrated TLR4 was expressed on equine B lymphocytes through the use of a mouse anti-human TLR4 antibody, clone 76B357.1, not previously used in horse. In contrast to observations made from the proliferation assays, transcriptional changes were observed in the equine cells within the TLR4 pathway upon treatment with LPS.

Introduction

Endotoxemia develops as a system wide inflammatory response to LPS. It is the leading cause of death in horses and is also a contributor to other serious equine diseases such as laminitis (Moore, 1979; Moore, 1988; Morris, 1991). LPS is a molecule expressed on the cell membrane of gram-negative bacteria. The gram-negative bacteria make up a portion of the gut microbiome and during a highly proliferative period or death they can release LPS from their membranes (White, 1998). Usually LPS released in the gut does not negatively effect the health of the horse (Moore, 1979), but if the integrity of the intestinal epithelium is compromised by inflammation or other damage, the LPS molecules can move out of the intestinal tract and into the tissues (Moore, 1981; Meyers, 1982; King, 1988). Once in the blood or tissue LPS can be recognized by circulating immune cells through TLR4 interaction (Lohmann, 2007).

TLR4 activation and its resulting signal cascade have been well studied in murine and human B lymphocytes. TLR4 responds through two separate pathways with adapter molecules Myd88 and TRIF (Kawai, 2001; Zughaier, 2005). Myd88 recruitment is needed to produce the cytokines TNF- α , IL-1 β , IL-6 and IL-10 (Kawai, 2001). TRIF activation leads to the production of IFN- β , RANTES, IP-10 and TRAF1 (Kawai, 2001; Zughaier, 2005). Humans express TLR4 on B lymphocytes, although seen at drastically lower levels than found on monocytes (Hornung, 2002). No activation signals are observed when stimulating human B lymphocytes with LPS, demonstrating a non-functional TLR4 (Bekeredjian-Ding, 2005). Mice however show moderate expression levels of TLR4 on B lymphocytes and a functional TLR4 that results in B lymphocyte proliferation, class switch recombination and cytokine production upon stimulation (Coutinho, 1974; Gronowicz, 1979; Genestier, 2007). Interestingly, horses and humans share a unique systemic sensitivity to LPS where mice show low sensitivity to LPS (Brade, 1999). In horses, no studies have investigated the role of equine B lymphocytes in the recognition and response to LPS. The purpose of this study was to quantify TLR4 expression on equine B lymphocytes and understand their response to LPS stimulation. Unlike in mice and humans, equine B lymphocyte markers are very limited and their efficacy can be questionable. In this study we show CD45R (B220), clone RA-6B2, accurately and consistently labels equine B lymphocytes. With the use of the CD45R

(B220) antibody and a TLR4 antibody, clone 76B357.1 as well as quantitative PCR, we characterized TLR4 expression on equine B lymphocytes.

Methods and Materials

Materials

Lympholyte Mammal was purchased from Cedarlane (Burlington, NC). Ficoll-Paque Premium was purchased from GE Healthcare (Pittsburg, PA). Kwik-Diff staining kit was purchased through Thermo Fisher (Waltham, MA). X-Vivo, serum free hematopoietic cell media, was purchased from Lonza (Basel, Switzerland). 2-Mercaptoethanol and Lipopolysaccharides from E.coli O55:B5 were purchased from Sigma-Aldrich (Saint Louis, MO). ODN D-SL01 type B CpG synthetic oligonucleotides were purchased from InvivoGen (San Diego, CA). Propidium Iodide solution was purchased from Biolegend (San Diego, CA). PureLink RNA mini kits, High-capacity cDNA reverse transcription kits, *Power* SYBR green PCR master mixes, DNase I (RNase-free) kits, and CellTrace Violet cell proliferation kits were purchased through Thermo Fisher (Waltham, MA).

Horses

All experimental procedures were approved by the Institution of Animal Care and Use Committee at Virginia Tech. Twelve thoroughbred geldings ages 5-10 years were used. The geldings were housed at Virginia Tech's horse breeding farm, Smithfield Horse Center. The horses were turned out 24 hours a day and separated into groups of three. All horses were up to date on vaccinations prior to one month before the beginning of the study. Horses were also negative for equine infectious anemia. Blood samples were collected from the jugular vein into 4 ml BD Vacutainers containing EDTA as an anticoagulant.

Mice

All experimental protocols were approved by the Institution of Animal Care and Use Committee at Virginia Tech. C57BL/6J male mice (Jackson Laboratory, ME), 8-10 weeks of age were used. Blood samples were collected via cardiac perfusion into a syringe and deposited into 4 ml BD Vacutainers containing EDTA as an anticoagulant.

Isolation of equine peripheral lymphocytes

Lymphocytes were isolated by density-gradient centrifugation over lympholyte or ficoll-paque premium. For Lympholyte separation 2 mls of blood was mixed with 2 mls of PBS and layered over 3 mls of room temperature Lympholyte. Samples were centrifuged at 800 xg for 20 min at 18° C. After centrifugation the serum layer was aspirated and discarded and the lymphocyte layer was aspirated and placed in a new tube. 3x the volume of the lymphocyte layer was added of PBS. The samples were centrifuged at 800 xg for 10 min at 4° C. The supernatant was decanted the pelleted lymphocytes and samples were washed with 10 mls PBS at 270 xg for 6 min at 4° C. For Ficoll separation, 2 mls of blood was mixed with 2 mls of PBS and layered over 3 mls of room temperature Ficoll. Samples were then spun at 400 xg for 35 min at 18° C. After centrifugation the serum layer was aspirated and discarded and the lymphocyte layer was aspirated and placed in a new tube. 3x the volume of the lymphocyte layer was added of PBS. The samples were centrifuged at 800 xg for 10 min at 4° C. The supernatant was decanted the pelleted lymphocytes and samples were washed with 10 mls PBS once more at 270 xg for 6 min at 4° C. Lymphocytes were resuspended in X-Vivo RPMI at a final concentration of $2 \times 10^6 \text{ ml}^{-1}$; lymphocytes were added to each well of a sterile 96-well or 6-well polystyrene plate and incubated for up to 72 h at 37° C, in a 5% CO₂ atmosphere.

Proliferation Assay

Lymphocytes were isolated by density-gradient centrifugation over lymphocyte separation media. Lymphocytes were suspended in X-Vivo RPMI at a final concentration of $2 \times 10^6 \text{ ml}^{-1}$; 2×10^5 lymphocytes were added to each well of a sterile 96-well polystyrene plate and incubated for 72 h at 37° C, in a 5% CO₂ atmosphere. After lymphocytes were isolated, cells were resuspended in 1 ml of PBS. Cell suspension was transferred to a new, dry, 15 ml conical and 2.5×10^{-12} mols of CellTrace Violet dye was added to the side of the tube. The tube was then vortexed to diffuse the dye equally among cells. The cells were incubated with the dye at room temperature for 20 minutes and then 14 mls of X-Vivo RPMI was added to the sample and the sample was spun at 270xg for 6 min at 4° C. The supernatant was poured off of the samples and the cells

were resuspended at 2×10^6 cells/ml. Cells were then plated into a 96-well polyethylene plate with 2×10^5 cells/well in the presence or absence of either 5 μ g/ml LPS or 5 μ M CpG and incubated for 72 h at 37° C in 5% CO₂ atmosphere.

TLR4, CD45R, and CD21 antibodies

B lymphocyte staining of the primary mouse anti-human TLR4 mAb (Clone 76B357.1)(LSBio, Seattle, WA) in equine lymphocytes was confirmed using flow cytometry. Equine expression in lymphocytes was investigated with flow cytometric staining with 0.0625 μ g mAb/ 10^6 cells (Figure 2a). Secondary rat anti-mouse IgG2b Abs, PE-conjugated, were from Biolegend. The mAb (Clone 27-35) used as an IgG2b isotype control was purchased through BD Biosciences.

Cross reactivity of the rat anti-mouse/human CD45R/B220 Abs (Clone RA3-6B2, Biolegend, San Diego, CA), BV605-conjugated, in equine lymphocytes was confirmed using flow cytometry. Equine expression in lymphocytes was investigated with flow cytometric staining with 0.025 μ g/ 10^6 cells (Figure 2b). The mAb (Clone RA3-6B2) used as an IgG2a isotype control was purchased through Biolegend.

The mouse anti-human CD21 mAb (Clone Bu33), APC-conjugated, was purchased from Novus Biologicals (Littleton, CO). No concentration was given, so equine expression in lymphocytes was investigated with flow cytometric staining with 1:160 of antibody to cells (Figure 2c).

Cells were collected using an Attune NxT cytometer running Attune NxT Software from Thermo Fisher (Waltham, MA). Histograms and dot plots were created and analyzed using FlowJo v10.1r7 software (Ashland, Oregon).

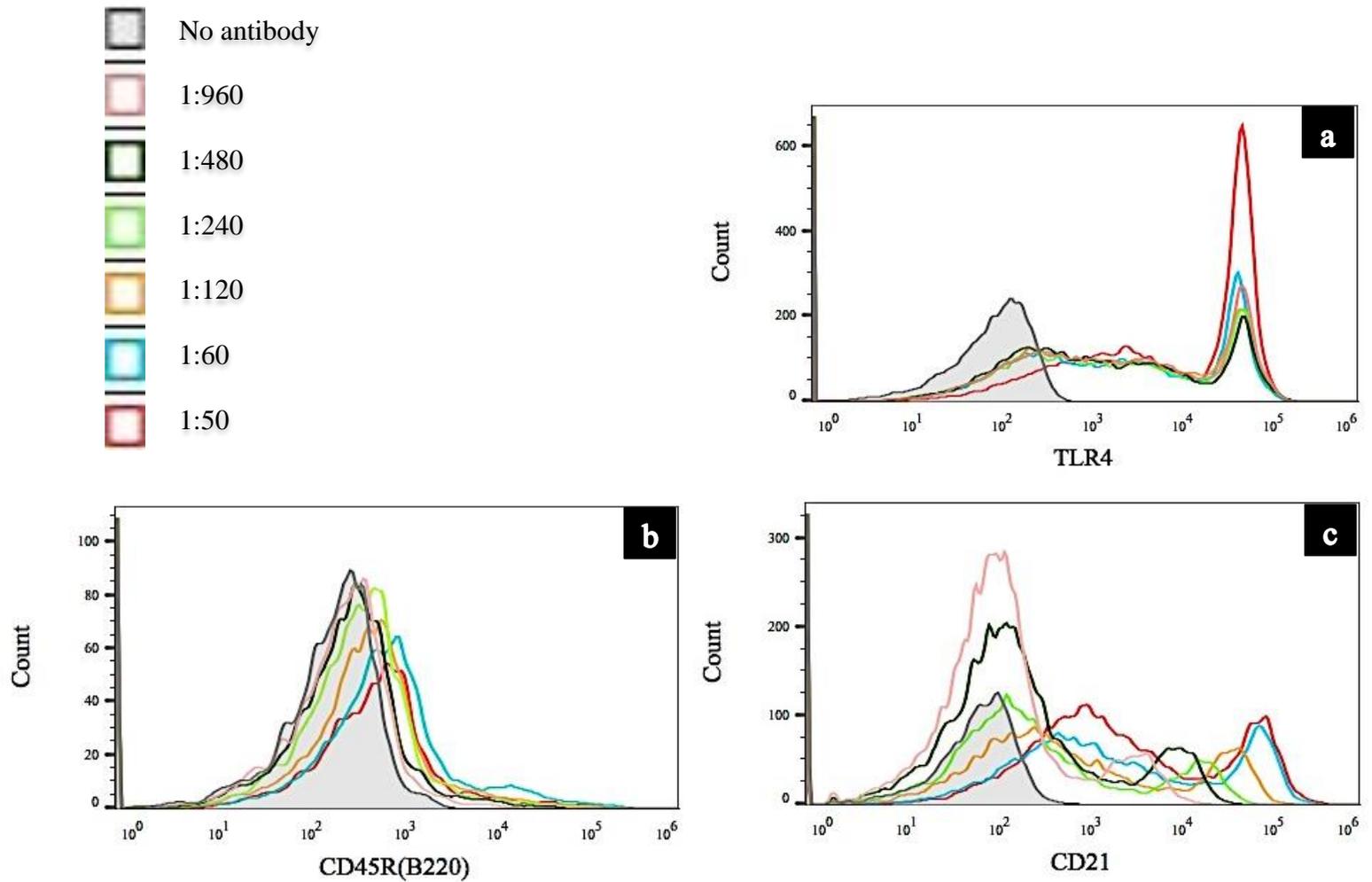


Figure 2. TLR4, CD45R, and CD21 titrations. Peripheral blood was collected via the jugular vein and lymphocytes were separated using Lymphocyte. Freshly isolated lymphocyte samples were resuspended at 2×10^7 cells/ml. Samples were incubated with titrated volumes of TLR4(a), CD45R(b), or CD21(c) and analyzed with flow cytometry.

RNA extraction and cDNA synthesis for mRNA analysis

1.5 mls of isolated equine lymphocytes at a concentration of 2×10^6 cells/ml were added into each well of a sterile polyethylene 6-well plate and incubated for 24 h at 37°C in 5% CO_2 atmosphere in the presence or absence of either $5 \mu\text{g/ml}$ LPS or $5 \mu\text{M}$ CpG. After incubation, cells were transferred to sterile 15 ml polyethylene conical tubes and spun down at 270 xg for 6 min at 4°C . Supernatant was discarded and pelleted cells were transferred into 1.5 ml eppendorf tubes. Trizol was used to lyse the lymphocytes and total RNA was extracted using the PureLink RNA mini kit according to the manufacturer's

protocol. Only samples having 260:280 nm absorbance ratios between 1.8 and 2.2 as measured on a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) were processed for cDNA synthesis. After full RNA was extracted DNase I was used as described by manufacturer to destroy remaining DNA and the High Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, Wilmington, DE) was used for cDNA synthesis with 100 ng of RNA as template.

Real-time quantitative PCR

Real-time qPCR assays using SYBR Green as detector were performed in a Mastercycler Realplex (Eppendorf, Hauppauge, NY), with GAPDH serving as endogenous controls. Conditions for amplification were 10 min at 95° C, followed by 40 cycles of 15s at 95° C, and 1 min at 60° C. This was followed by a melt curve for 20 min to reach 95° C and finally 15s at 95° C to assure only one amplicon was produced. The target equine genes were *TLR4*, *TLR9*, *TNF- α* , *IL-1 β* , *IL-6*, *IL-10*, *IFN- β* , *IP-10*, *RANTES* and *TRAF1* (Table 2). Oligonucleotide primers used for the detection of cDNA specific equine TLRs and cytokines were obtained from Integrated DNA Technologies (Coralville, IA). Dissociation curve analysis and end-point PCR revealed single products for all primers, the PCR amplification efficiency ranged between 85-110%. The PCR assays contained 10 nM of each primer, 10 μ l SYBR green, 2 μ l cDNA sample and 6 μ l RNase-free water with a final volume of 20 μ l. Changes in gene expression were calculated by relative quantification against GAPDH using the $\Delta\Delta C_T$ method; where $\Delta\Delta C_T = [(gene\ of\ interest\ C_T - GAPDH\ C_T)_{sample} - (gene\ of\ interest\ C_T - GAPDH\ C_T)_{calibrator}]$. C_T is defined as the amplification cycle at which amplification reached a comparable level of template concentration. Fold changes in gene expression were calculated using the $2^{-\Delta\Delta C_T}$ method. Results were expressed as the mean fold change in gene expression. The C_T of the non-stimulated control sample was used as the calibrator and assigned a fold change of 1. Experiments were run with technical duplicates nested in biological replications.

Table 2. Real-time quantitative RT-PCR primer pairs

Gene	GenBank Accession No.	Forward Sequence (5'-3')	Reverse Sequence (5'-3')
TLR4¹	AY005808	ATGCCCGTGCTGGGTTTTA	ACTTTTTGCAGCCAGCAAGAA
TLR9²	DQ390541	GTGACTGGCTACCTGGCAAGAC	TGGTTATAGAAGTGGCGGTTGTCC
TNF-α³	AB035735	AAGGACATCATGAGCACTGAAAG	GGGCCCCCTGCCTTCT
IL-1β³	ECU192481	ATGACTTACTGCAGCGGCAA	GTCTTGGAAGCTGCCCTTCA
IL-6³	ECU64794	TGCTGGCTAAGCTGCATTCA	GGAAATCCTCAAGGCTTCGAA
IL-10³	U38200	GCCTTGTCGGAGATGATCCA	TTTTCCCCCAGGGAGTTCAC
IFN-β³	AF134227	CCCCGAGGACACAATGAACT	ACCAATGCAGCATCCTCCTT
RANTES³	CD470151	CACTGCCACCTTCTGCACTC	CGGGAGATGTAGGCAAAGCA
IP-10³	AF506970	CCTCCAGTTGCAGCACCAT	TTCTTGAGTTCCACTCAGAGTCA
TRAF1³	BI961513	GTCCCGTCCCCATTCAGATA	CAGCCTCTTCTCCCCAGCTT
GAPDH⁴	CX593628.1	AGAAGGAGAAAGGCCCTCAG	GGAAACTGTGGAGGTCAGGA

¹Figueirido et al., 2009; ²Figueirido et al., 2009; ³Zhang et al., 2008; ⁴da Silveira et al., 2015.

Statistical Analysis

Data was analyzed using GraphPad Prism (San Diego, CA). Differences in treatments were analyzed using the Mann-Whitney U test. All data reported as mean \pm S.E.M. Significance was set at $P < 0.05$.

Results and Discussion

Lympholyte versus Ficoll-Paque Premium as an equine lymphocyte separation media

To determine what lymphocyte separation media would be most ideal to use with horse peripheral blood, the conventionally used Ficoll (GE Healthcare) was compared against Lympholyte mammal (Cedarlane). Protocols from the manufacturers of Lympholyte and Ficoll-Paque premium were used to isolate lymphocytes. Equal volumes

of equine peripheral blood were used for each protocol. Cell isolates were examined for total cell count, lymphocyte cell count and cell viability (Table 3). No significant difference was found between the lympholyte and ficoll-paque premium groups for any evaluated parameter.

After cells were counted, each sample was resuspended at 2×10^7 cells/ml. Images of Diff-Quik prepared slides show a greater number lymphocytes in the ficoll-paque premium prepared cells compared to lympholyte (Figure 3). Although the ficoll was able to provide more lymphocytes in some samples the cell counts were more variable between samples compared to the lympholyte prepared samples. These results could be due to the separation of the blood cell, lymphocyte, and serum layers after the first centrifugation step in both protocols. In lympholyte prepared samples the layers appeared more distinct and because the lymphocyte layer was more visible, aspiration led to more consistent cell counts between samples.

Table 3. *Lympholyte and Ficoll-Paque Premium media differences*

Media	Density	Total cell count per 2 ml blood (number of cells)	Lymphocyte count per 2 ml blood (number of cells±S.E.M)	Lymphocyte viability (number of cells±S.E.M)
Lympholyte mammal	1.086±0.0001 g/ml	1.92×10^7	$6.48 \times 10^3 \pm 46.3$	$3.3 \times 10^3 \pm 44.5$
Ficoll-Paque Premium	1.077±0.0001 g/ml	3.67×10^6	$1.27 \times 10^4 \pm 1,670$	$6.49 \times 10^3 \pm 806$

Peripheral blood was collected via the jugular vein into EDTA treated tubes from thoroughbred geldings. Lympholyte or ficoll-paque premium media was used to isolate lymphocytes from equal volumes of blood for analysis. n=3 horses

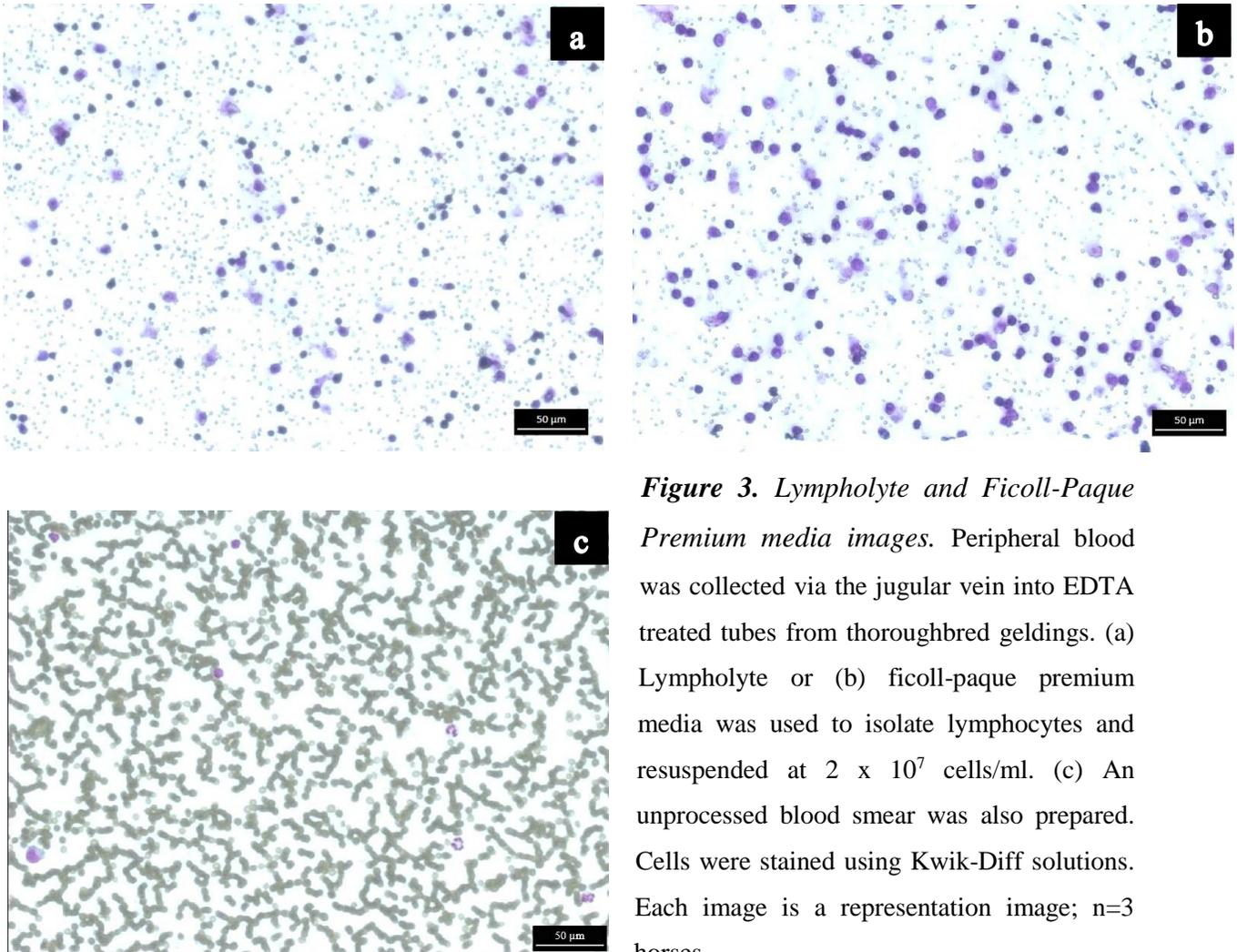


Figure 3. *Lympholyte and Ficoll-Paque Premium media images.* Peripheral blood was collected via the jugular vein into EDTA treated tubes from thoroughbred geldings. (a) Lympholyte or (b) ficoll-paque premium media was used to isolate lymphocytes and resuspended at 2×10^7 cells/ml. (c) An unprocessed blood smear was also prepared. Cells were stained using Kwik-Diff solutions. Each image is a representation image; n=3 horses

Equine lymphocyte viability during culture

Equine lymphocytes were incubated with LPS, CpG or in the absence of a stimulant for up to 72 hours. Samples were taken to be analyzed by flow cytometry for viability at predetermined time points (Table 4)(Figure 4).

Table 4. Equine lymphocyte viability *in vitro*

Treatment	Timepoint					
	0 hours	4 hours	8 hours	12 hours	24 hours	48 hours
Unstimulated	48.7±0.96	46.7±1.5	47.0±1.8	47.1±1.5	46.4±1.9	42.1±2.8
LPS	47.8±2.0	46.9±1.5	46.0±2.3	46.5±2.2	44.5±2.5	38.9±3.5
CpG	49.9±0.52	50.1±1.7	49.1±0.59	47.6±1.1	40.9±4.8	33.0±7.9

Peripheral blood was collected via the jugular vein into EDTA treated tubes from thoroughbred geldings. Lympholyte was used to isolate lymphocytes and samples were resuspended at 2×10^6 cells/ml. The cells were then placed in culture with LPS, CpG, or no stimulant. Viability is shown in percent of viable cells. Viability is shown in percent of single cells that are viable. n=6 horses.

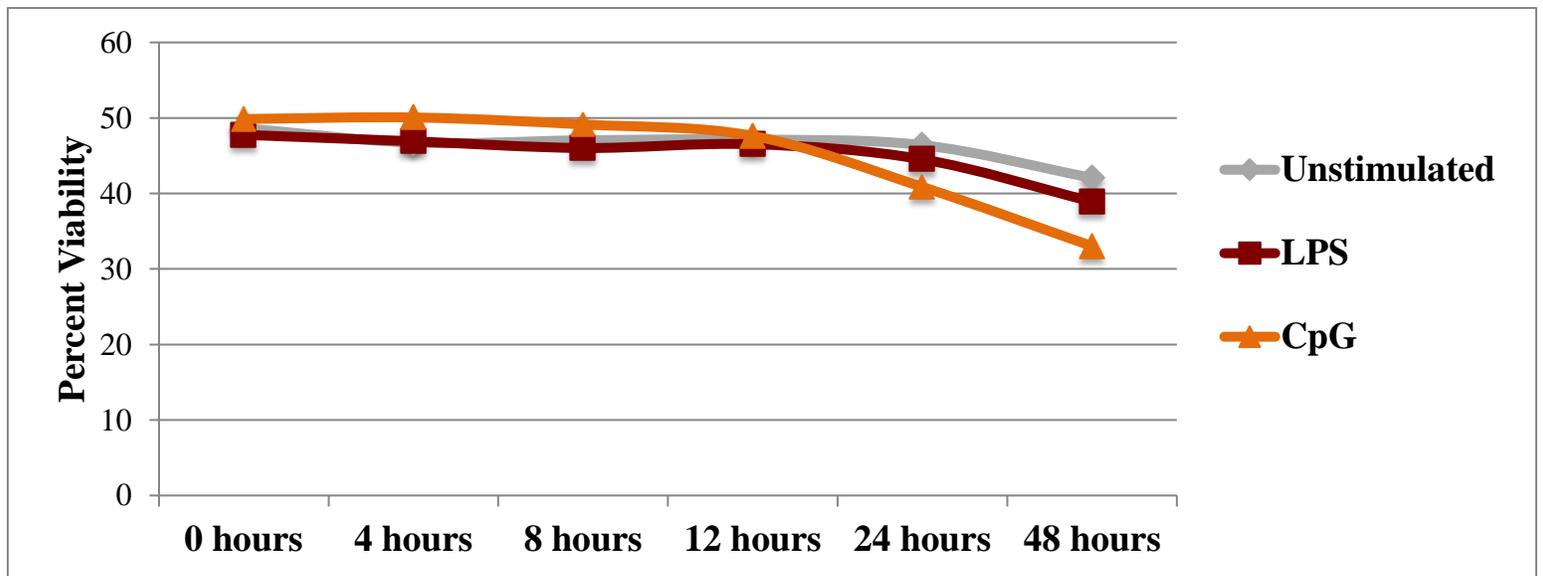


Figure 4. Lymphocyte viability curve during *in vitro* culture. Peripheral blood was collected via the jugular vein into EDTA treated tubes from thoroughbred geldings. Lympholyte media was used to isolate lymphocytes and samples were resuspended at 2×10^6 cells/ml. The cells were then placed in culture with LPS, CpG, or no stimulant. Viability is shown in percent of single cells that are viable. n=6 horses

B lymphocyte proliferation after LPS stimulation

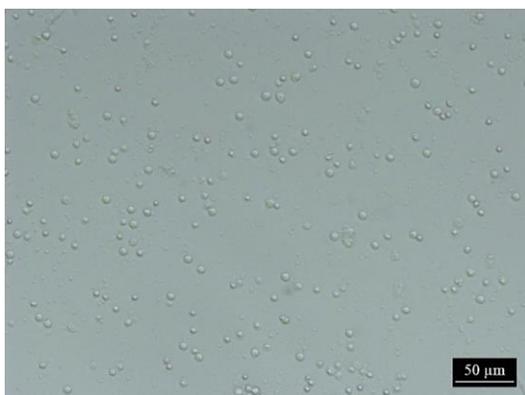
Murine peripheral lymphocytes showed proliferating or blasting cell populations when incubated with LPS in contrast to the non-proliferating unstimulated murine lymphocytes when evaluated by microscope images (Figure 5a). However, there were no proliferating cells in the equine peripheral lymphocytes incubated in the absence or presence of LPS (Figure 5b).

When evaluating CellTrace Violet histograms, gated on CD45R(B220)⁺ lymphocytes, equine lymphocytes incubated in the absence or presence showed a similar generation trend with one primary generation. In agreement with the Zeiss light microscope images, murine CD45R(B220)⁺ lymphocytes showed a primary generation, a secondary generation and a third generation for the LPS stimulated lymphocytes where the unstimulated lymphocytes showed one primary generation. Here we show CD45R(B220) as a B lymphocyte marker (Figure 5b). By gating on the CD45R(B220)⁺ lymphocytes we were able to gate on the B lymphocytes and show their activity.

Mouse

Horse

Unstimulated



LPS (5 μg/ml)



CpG (10 μg/ml)

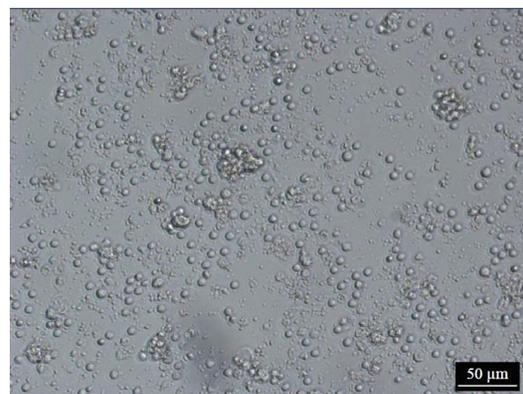
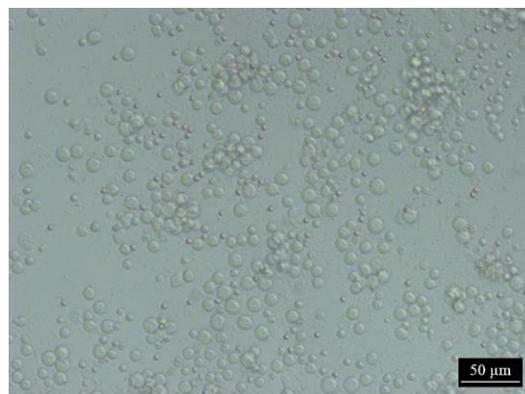


Figure 5a. Cell proliferation images of murine and equine lymphocytes with or without LPS. Images of murine and equine lymphocytes with or without LPS (5 μg/ml) or CpG (10 μg/ml) treatment. Peripheral blood was collected via the jugular vein into EDTA treated tubes from thoroughbred geldings and cardiac blood was collected via cardiac puncture from male C57BL/6J mice. Lympholyte media was used to isolate lymphocytes and then 4×10^5 cells were incubated with or without LPS for 48 hours for each species. Each image is a representative sample; n=3 horses.

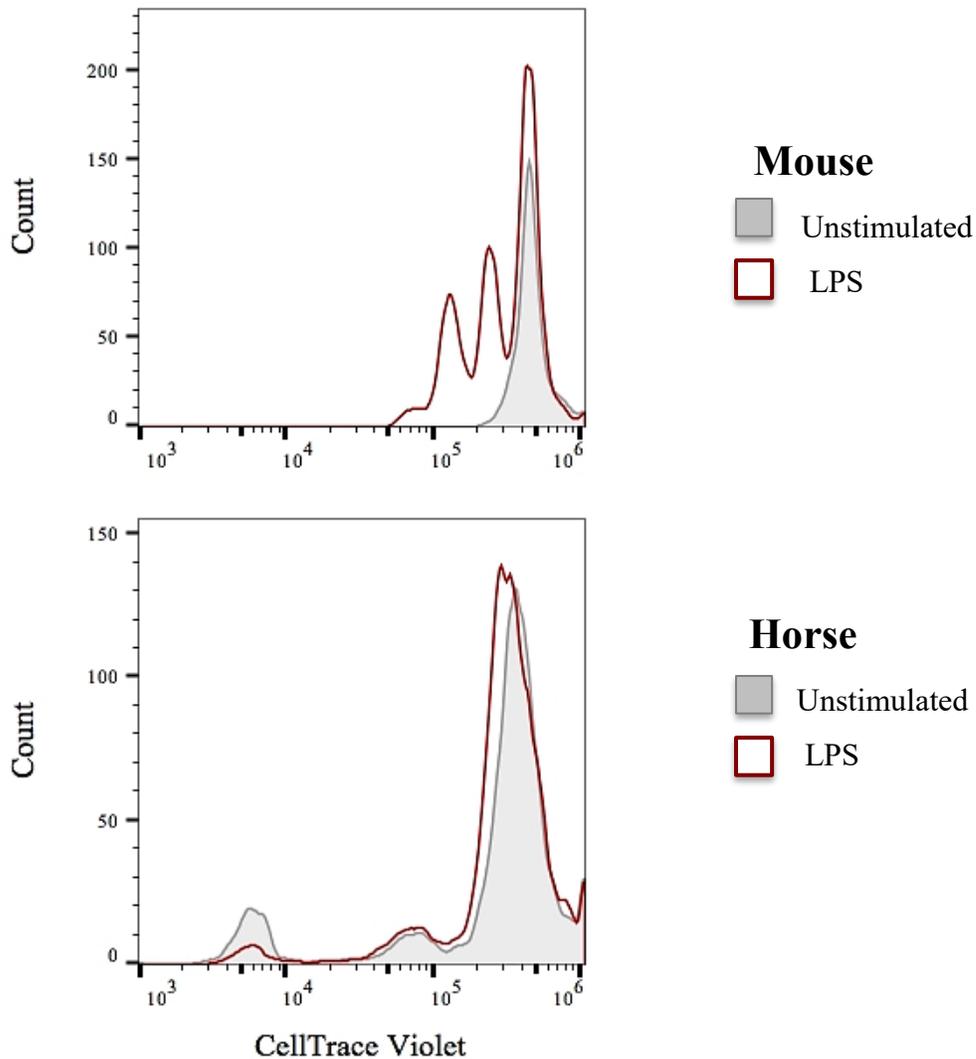
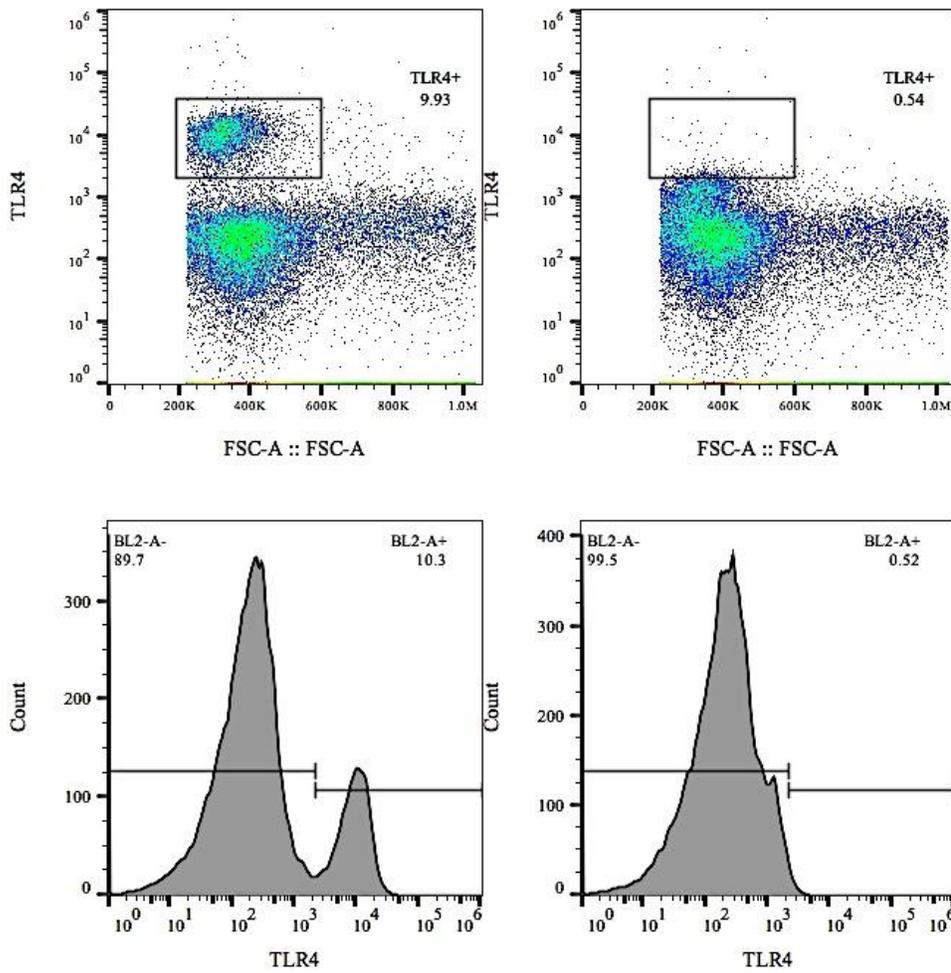


Figure 5b. CellTrace Violet proliferation assay of murine and equine lymphocytes. CellTrace Proliferation dye histogram of murine and equine lymphocytes incubated with LPS (5 $\mu\text{g}/\text{ml}$) or no stimulant for 72 hours. Equine peripheral blood was collected via jugular vein into EDTA treated tubes from thoroughbred geldings. Mouse blood was collected via cardiac puncture from C57BL/6J males and placed into EDTA treated tubes. Lymphocytes were separated using lympholyte media and then stained with CellTrace Proliferation dye. 4×10^5 cells were incubated with LPS or no stimulant for both species and analyzed through flow cytometry. Figure is a representation sample; $n=3$ horses.

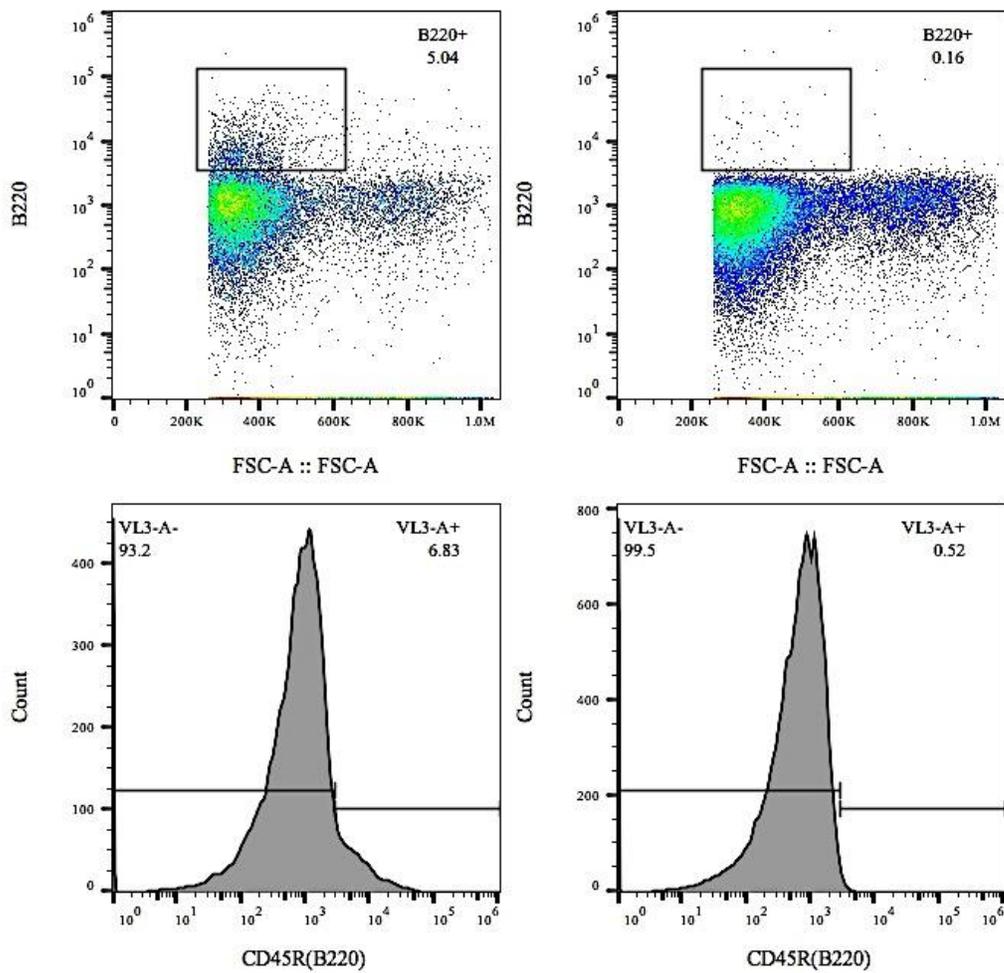
TLR4 expression on Equine B lymphocytes

To understand whether equine B lymphocytes expressed TLR4 an anti-human TLR4 mAb, not previously tried in horse, was used to detect TLR4 positive equine lymphocytes. Flow cytometric data showed a distinct TLR4 positive population in freshly isolated equine lymphocytes. Used in conjunction with anti-human CD21 or a novel anti-mouse CD45R(B220), coexpression with TLR4 was examined. Both experiments showed coexpression with TLR4 to define TLR4⁺,CD21⁺ and TLR4⁺,CD45R(B220)⁺ positive lymphocyte populations (Figure 6a,b,c,d).



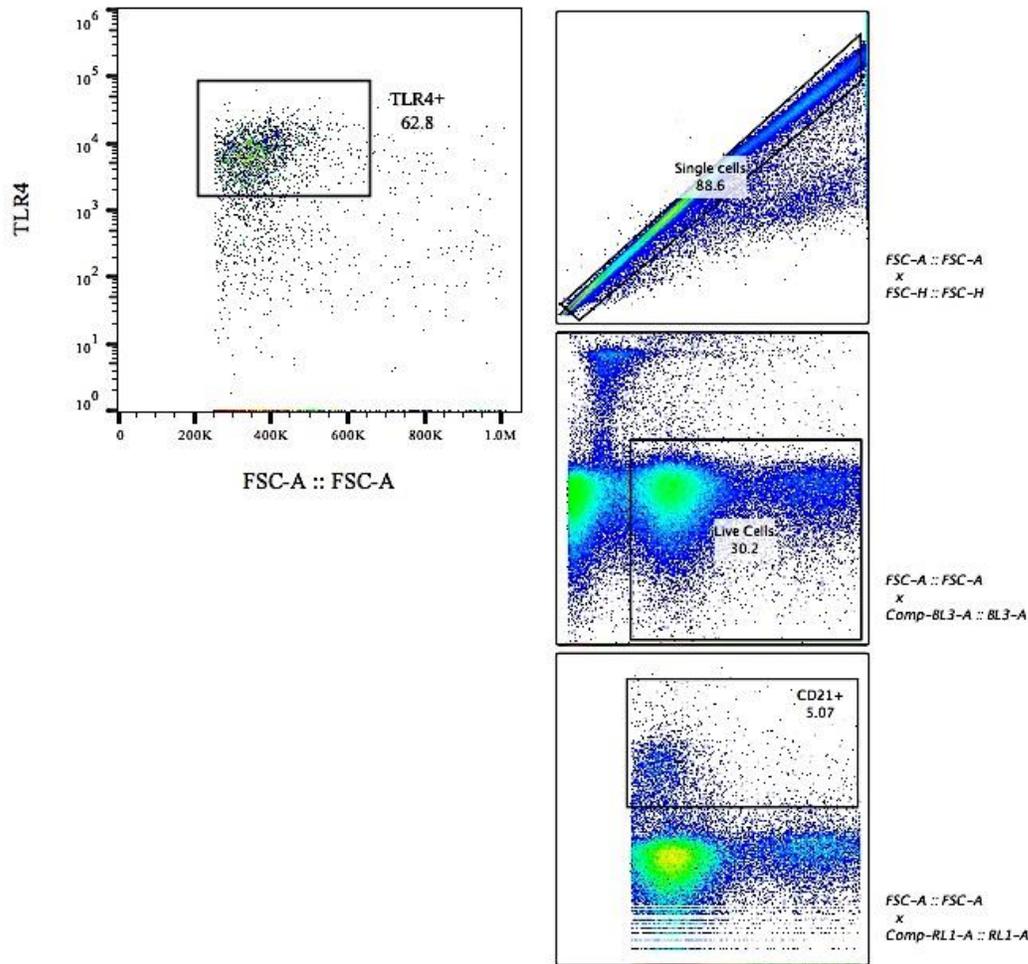
	Horse 1	Horse 2	Average
Percent of cells TLR4 positive			
TLR4	9.93	9.56	9.75
Isotype Control	0.50	0.54	0.52

Figure 6a. TLR4, clone 76B357.1, expression on equine lymphocytes. TLR4 expression on equine lymphocytes. Equine blood was collected via the jugular vein into EDTA treated tubes from thoroughbred geldings and lympholyte media was used to isolate lymphocytes. Lymphocytes were then incubated with a TLR4 antibody (left), clone 76B357.1 or its isotype control IgG2b (right), clone 27-35, and analyzed with flow cytometry. Image shown is a representative sample, but the table includes data from all samples; n=2 horses.



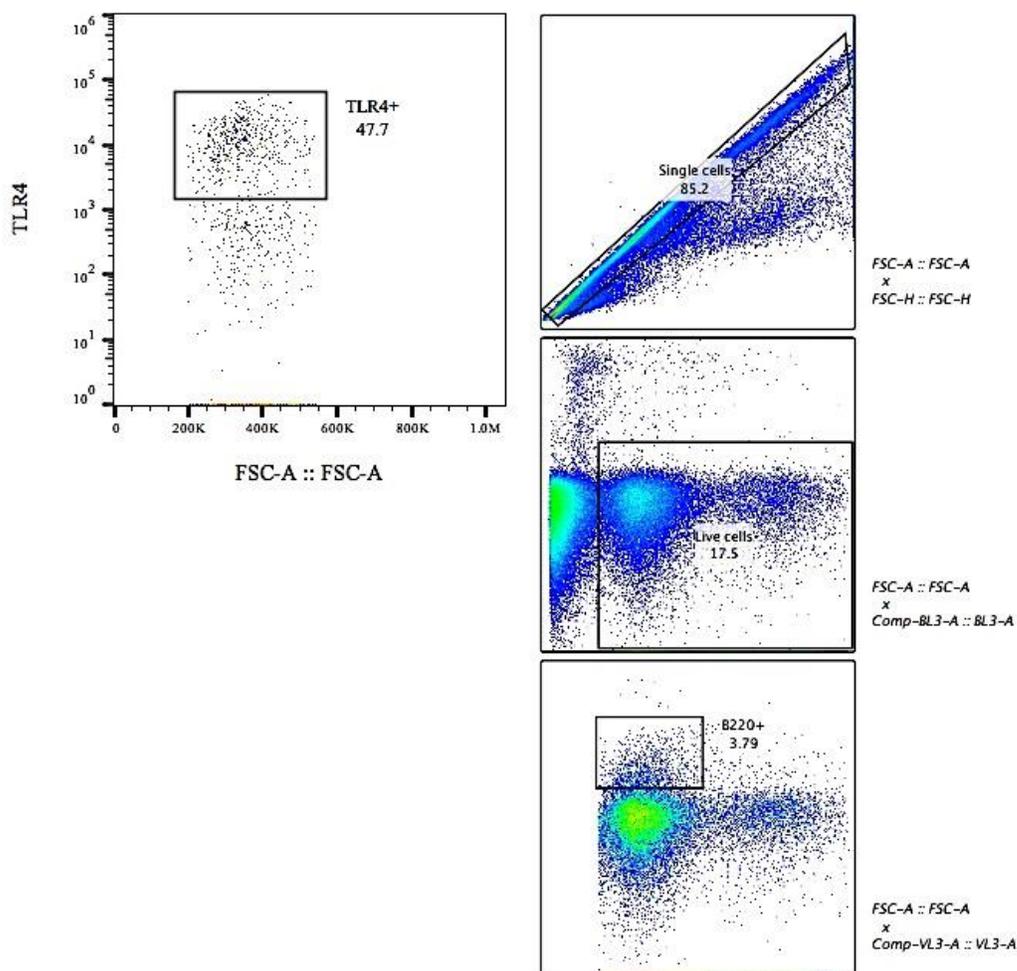
	Horse 1	Horse 2	Average
Percent of cells CD45R positive			
CD45R	5.04	4.36	4.70
Isotype Control	0.2	0.16	0.18

Figure 6b. CD45R (B220), clone RA-6B2, expression on equine lymphocytes. CD45R (B220) expression on equine lymphocytes. Equine blood was collected via the jugular vein into EDTA treated tubes from thoroughbred geldings and lympholyte media was used to isolate lymphocytes. Lymphocytes were then incubated with a CD45R (B220) antibody (left), clone RA-6B2, or its isotype control Rat IgG2a (right), clone RTK2758, and analyzed with flow cytometry. Image shown is a representative sample, but the table includes data from all samples; n= 2 horses



	Horse 1	Horse 2	Average
Percent of cells TLR4 positive			
TLR4	61.6	66	63.8

Figure 6c. CD21, clone Bu33, and TLR4, clone 76B357.1, coexpression on equine B lymphocytes. Coexpression of CD21, clone Bu33, and TLR4, clone 76B357.1, on equine B lymphocytes. Equine blood was collected via the jugular vein into EDTA treated tubes from thoroughbred geldings and lymphocyte was used to isolate lymphocytes. Lymphocytes were then incubated with with a TLR4 antibody, clone 76B357.1, and a CD21 antibody. Results were analyzed by flow cytometry. Gating strategy: Lymphocytes were first gated on single cells, then live lymphocytes, then CD21⁺ B cells and finally TLR4⁺ cells. Image shown is a representative sample, but the table includes data from all samples; n=2 horses.

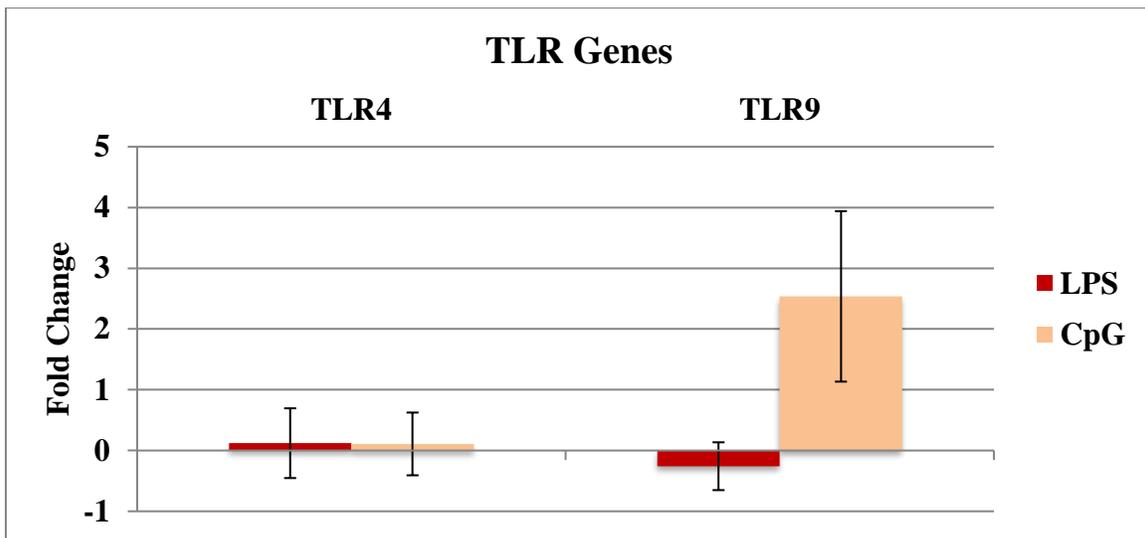


	Horse 1	Horse 2	Average
Percent of cells TLR4 positive			
TLR4	47.7	46.2	47.0

Figure 6d. CD45R(B220), clone RA-6B2, and TLR4, clone 76B357.1, coexpression on equine B lymphocytes. CD45R (B220), TLR4 coexpression on equine lymphocytes. Equine blood was collected via the jugular vein into EDTA treated tubes from thoroughbred geldings and lympholyte media was used to isolate lymphocytes. Lymphocytes were then incubated with a CD45R (B220) antibody, clone RA-6B2, and with a TLR4 antibody, clone 76B357.1, and analyzed with flow cytometry. Gating strategy: Lymphocytes were first gated on single cells, then live lymphocytes, then CD45R⁺ B cells and finally TLR4⁺ cells were gated. Image shown is a representative sample, but the table includes data from all samples; n=2 horses.

mRNA changes in Equine lymphocytes for molecules in the TLR4 pathway

Since TLR4 in other mammalian species recruits both MyD88 and TRIF upon stimulation to examine TLR4 pathway activation primers for genes in the MyD88 and TRIF dependent pathways were selected and tested, along with a TLR4 primer pair (Figure 7). For the Myd88 pathway the cytokines TNF- α , IL-1 β , IL-6 and IL-10 were examined and for the TRIF pathway the genes IFN- β , IP-10, RANTES and TRAF1 were examined. We used CpG stimulated equine lymphocytes as a positive control because we were able to see proliferation after stimulation and because it also signals through the adapter molecule TRIF. Since CpG is an agonist for TLR9 we also used a TLR9 primer pair. 5 horses were used for qPCR analysis.



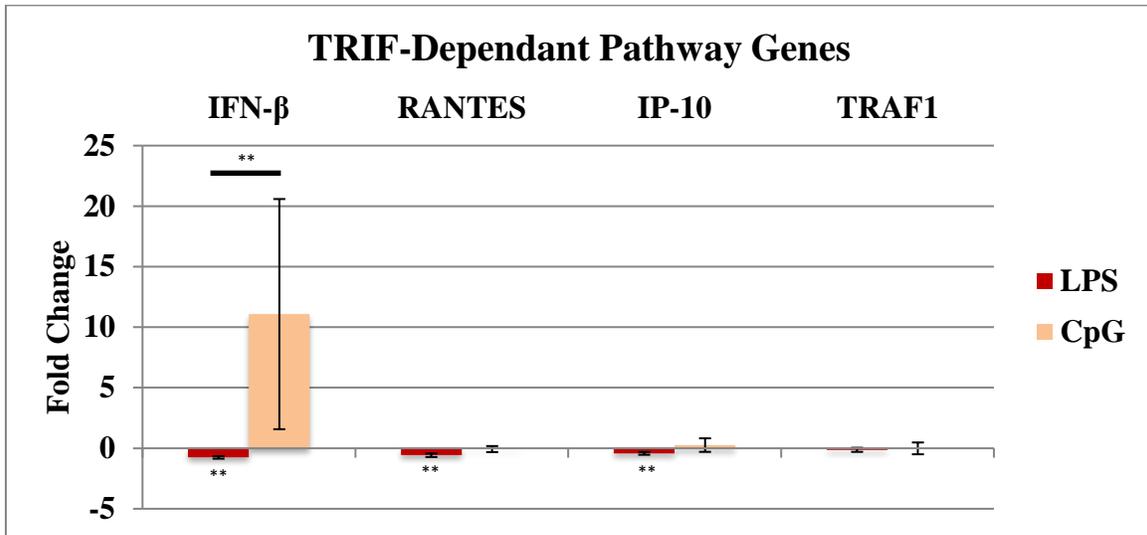
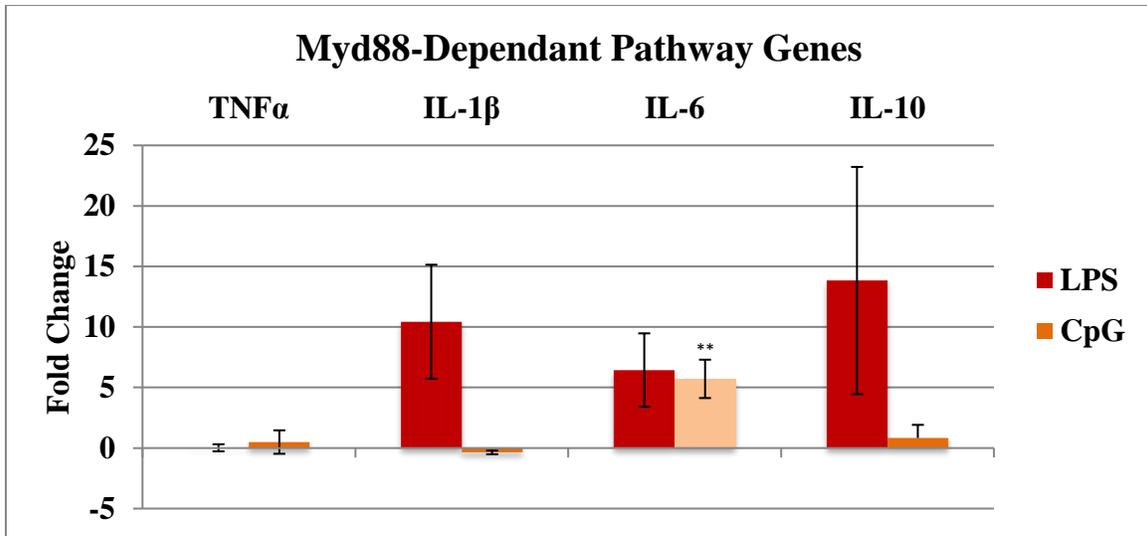


Figure 7: Quantitative analysis of mRNA expression in equine lymphocytes with LPS stimulation and CpG stimulation. Quantitative analysis of mRNA expression in equine lymphocytes (n=5). Peripheral blood was collected via the jugular vein into EDTA treated tubes from thoroughbred geldings. Isolated peripheral lymphocytes (3×10^6 cells per well) were incubated with no stimulant, LPS (5 $\mu\text{g/ml}$), or CpG (5 μM) for 24 hours. Expression of TLR4, TLR9, IL-6, IFN- β , RANTES, IP-10, TRAF1, TNF- α , IL-1 β , and IL-10 mRNA was determined by RT-qPCR and is depicted as the mean fold change of the treatment (LPS or CpG) minus the control (unstimulated) \pm SEM. Results are normalized to GAPDH. * P<0.05, ** P<0.01 is used to indicate significant differences between treatment samples and unstimulated samples. A bar with * P<0.05, ** P<0.01 is used to indicate significant differences between treatment groups; n=5 horses.

Summary and Conclusion

In conclusion, we show that equine B lymphocytes failed to proliferate in the presence of *Escherichia coli* 055:B5 LPS, compared with control mouse B lymphocytes. These results were consistent with B lymphocyte responses seen in human (Bekeredjian-Ding, 2005). We confirmed the cross-species reactivity to horse of a rat anti-mouse CD45R (B220) using flow cytometry, demonstrating a useful B lymphocyte marker not yet used in horse. We also confirmed the cross-species reactivity to horse of a mouse anti-human TLR4 using flow cytometry. By gating on the CD45R(B220)⁺ or CD21⁺ lymphocytes we were able to show B lymphocyte expression of TLR4. These results combined with the failure of equine B lymphocytes to proliferate in the presence of LPS suggests that TLR4 is present, but subfunctional in horse, which is similar to human (Bekeredjian-Ding, 2005). Since both humans and horses experience severe inflammatory responses to LPS on a full body scale and this study demonstrates similarities between the two species on a cellular level, these observations could indicate a possible mode of manipulation to control the inflammatory response and prevent the development of shock and death in both species. These results also provide a useful human model for LPS related diseases such as septic shock. Since septic shock is still a top killer in the U.S. for humans, more should be learned about the complicated mechanisms involved with the progression of this disease to prevent the loss of life in the future (Tidswell, 2011). Horses could be a useful avenue for these further investigations.

In contrast to results seen in the proliferation assays, quantitative real-time PCR data indicates mRNA changes from LPS stimulated equine lymphocytes compared to the control. Although, no changes are seen in equine B lymphocyte proliferation, transcriptional changes occur. It is possible contaminants other than B lymphocytes that could influence the results. Cell death within the samples could also influence results. EDTA was used as an anticoagulant and could be affecting results as well since EDTA is harsher on lymphocytes leading to decreased cell viability compared to other anticoagulants (Carter, 1992). A future goal could be to eliminate the other cell contaminants and dead cells, but B cell sorting can often be difficult and lead to the majority of B cells dying. In addition, repeating assays using heparin or citrate as an anticoagulant is warranted.

Future Directions

The potentially lethal effects of LPS have been well studied for decades. Researchers have worked to understand the mechanism of recognition and the resulting effects of LPS on the body. We know that *in vivo* LPS causes an inflammatory response initiated by the immune system, but there are still many unanswered questions, such as what causes such extreme reactions in some species, but not others and how can we use that knowledge to alter treatment options in a clinical setting. We have seen some similarities in B lymphocyte TLR4 expression and activity between human and horse samples, possibly indicating a source of a shared sensitivity to LPS. Next steps should work to assess this connection further by looking into other aspects of TLR activation not examined in this study. In murine studies, TLR4 activation is characterized by cytokine production, class switch recombination, and plasma cell differentiation (Bekeredjian-Ding, 2005). We would expect to see little to no increase in these measurements to confirm a sub-functional TLR4 on equine B lymphocytes. ELISAs, along with FACs data, investigating these aspects should be used with horse samples to better understand TLR4 activation.

This study suggests B lymphocytes could indicate systemic sensitivity to LPS. Several mechanisms within the TLR4 pathway exist to regulate and control the extent of pro-inflammatory mediators. Having low to non-functional TLR4 on human and equine B lymphocytes could alter this communication within the body and between immune cells and cause a less controlled inflammatory reaction to occur. To test this hypothesis a B cell specific TLR4 knockout or B cell specific non-functional TLR4 mouse strain could be used to analyze altered sensitivity to LPS.

Another aspect of this study that should be expanded on is confirming the identified equine B cell markers. Using biotinylated CD21 and CD45R(B220) antibody with anti-biotin magnetic beads in a B cell isolation protocol could create a B cell pure sample. Since FCS sorting with the use of flow cytometry staining is not an option in horse because of the lack of B cell markers, RT-qPCR could be used to analyze purity by designing primers to B cell specific genes, along with other genes of possible cell contaminants. These experiments could confirm two accurate B cell markers for equine research.

In conjunction with previous research performed in humans, studies delving into TLR4 activation in horse may uncover a key link between B cells and LPS sensitivity, as well as developing a useful human model for lethal LPS-related diseases such as septic shock.

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