RAPID COMMUNICATION: TLR4 expressed but with reduced functionality on equine B lymphocytes

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ABSTRACT: Varying susceptibility exists among mammalian species to the development of potentially fatal endotoxemia due to gram-negative bacteria molecular component, lipopolysaccharide (LPS). Toll-like receptor 4 (TLR4) is responsible for LPS-associated immune response and is expressed on numerous immune cells including B lymphocytes. TLR4 is expressed in a functional form on mouse B lymphocytes, a species much less susceptible to endotoxemia compared with humans who are highly sensitive to endotoxin. Humans possess B lymphocytes that are not responsive to LPS. Likewise, horses are highly susceptible to endotoxemia but the expression and function of TLR4 on horse B lymphocytes is not known. Colic, the major cause of mortality in horses, is often complicated by resultant endotoxemia. The objective of this study was to determine the expression and function of TLR4 on equine B lymphocytes. Lymphocytes were isolated from peripheral blood mononuclear cells that were collected from six horses, and the expression and function of TLR4 was analyzed for

each horse. Flow cytometry results indicate TLR4 is expressed on horse B lymphocytes but stimulation with LPS did not alter this expression ($P = 0.99$) compared with unstimulated B lymphocytes after 24 h. After 72 h of in vitro LPS stimulation, analysis of cell proliferation dye by flow cytometry demonstrated that equine B lymphocytes did not proliferate, while mouse B lymphocytes predictably did. Furthermore, the total number of LPS stimulated equine B lymphocytes did not significantly differ from unstimulated cells after 72 h of culture ($P = 0.92$). Horse lymphocytes exhibited no significant differences in the measured TLR4 signaling pathway genes (TLR4, IL-10, IL-6, IFN β , and TNF α) when expression was compared with LPS stimulated vs. unstimulated cells. In conclusion, while TLR4 is expressed on horse B lymphocytes, it appears minimally responsive to LPS in vitro, similar to results seen in human B lymphocytes. While further studies are still needed, our work reveals a potential link between B lymphocyte TLR4 expression and endotoxin sensitivity.

Key words: B lymphocytes, endotoxemia, equine, lipopolysaccharide, Toll-like receptor 4

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J. Anim. Sci. 2019.97:2175–2180

doi: 10.1093/jas/skz074

INTRODUCTION

The family of Toll-like receptor (TLR) proteins recognize and respond to microbial pathogens and initiate an inflammatory response important for eliminating invading pathogens. One of the most

extensively investigated TLRs is Toll-like receptor 4 (TLR4) and its ligand lipopolysaccharide (LPS) due to its role in endotoxemia and associated detrimental health complications. Endotoxin response and sensitivity varies greatly amongst mammalian species such as that observed in the mouse vs. human and of interest here, the horse. The horse's environment frequently exposes it to gram-negative bacteria and molecular component LPS without consequence. However, endotoxin is associated

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Received January 16, 2019.

Accepted February 18, 2019.

with the pathogenesis of several severe equine ailments, most commonly, colic (Tinker, 1997). During episodes of gastrointestinal ailments (colic), the protective mucosal intestinal epithelial barrier can become compromised, allowing LPS to enter systemic circulation and leading to endotoxemia and subsequent hyperinflammatory response (Moore, 1981, 1992, 2014). Why the proinflammatory response associated with systemic LPS infection varies so greatly amongst species is not fully understood, but insights may lie in the variation of cellular TLR4 expression amongst different species. Murine B lymphocytes express functional TLR4 (Nagai, 2002) and B lymphocyte TLR4–LPS interaction results in cellular activation with cytokine and antibody production and proliferation (Xu et al., 2008; Stavnezer and Schrader, 2014; Lu, 2016). Humans are highly sensitive to endotoxin, yet human B lymphocytes express subfunctional TLR4 that does not respond when exposed to LPS (Hornung et al., 2002; Wagner, 2004). We hypothesize that TLR4 expression in horses will more closely resemble that seen in humans as opposed to that observed in mice. The objectives of this study are to determine the expression of TLR4 on equine B lymphocytes and assess the functionality of this receptor.

MATERIALS AND METHODS

Horses

Blood was collected via jugular venipuncture from healthy thoroughbred and warmblood geldings between the ages of 7 to 15 years old in sodium citrate vacutainers. A total of six individuals were sampled. The experimental protocols were approved by the Virginia Tech University Animal Care and Use Committee.

Equine Peripheral Blood Mononuclear Cell (PBMC) Isolation

PBMCs were isolated from whole blood with the density gradient cell separation reagent Lympholyte-M (Cedarlane Laboratories, Burlington, Canada). A total of 20 mL of blood was collected from each horse ($n = 6$) and the collected sample was divided into five 4 mL aliquots. For each aliquot, 4 mL of whole blood was diluted 1:1 with $1\times$ PBS and layered on top of 3 mL of Lympholyte-M and centrifuged at $800 \times g$, for 20 min, at room temperature. For each horse, lymphocytes were collected from the Lympholyte/serum interface from each of the five aliquots and

combined back into a single sample, and then washed and suspended in $1\times$ PBS.

Cell Culture and Proliferation Assay

Lympholyte isolated cells were stained with the cell proliferation dye 5(6)-carboxyfluorescein diacetate *N*-succinimidyl ester (Thermo Fisher Scientific, Waltham, MA) as described (Driver, 2017) and resuspended in the X-vivo 20 media (Lonza, Allendale, NJ) supplemented with 50 μ M 2-mercaptoethanol (2-ME, Millipore Sigma, St Louis, MO). A total of 2×10^5 cells were plated in 96-well flat bottom polystyrene plate and stimulated with either *Escherichia coli* LPS 055:B5 (Millipore Sigma) at 5 μ g/mL, pokeweed mitogen (PWM; Ref# L8777, Millipore Sigma) at 2 μ g/mL for positive control or with no stimulant for negative control. All proliferation assay cell cultures were performed in triplicates for each horse ($n = 6$). Cells isolated from each horse ($n = 6$) were incubated at 37 °C, in 5% CO₂ for 72 h and then harvested for flow cytometry analysis.

Cultured lymphocytes intended for the use of RNA extraction were suspended in X-vivo 20 + 2 ME at a concentration of 2×10^6 cells/mL, after Lympholyte isolation. A total of 1×10^6 cells, from each horse ($n = 5$), were stimulated with either *E. coli* LPS (5 μ g/mL), PWM (2 μ g/mL) or with no stimulant and incubated at 37 °C, in 5% CO₂ for 24 h. Cell cultures were performed in duplicates for each horse ($n = 5$).

Mice

C57Bl6/J male mice (8 to 10 wk of age) ($n = 3$) were obtained from the Jackson Laboratory (Maine). Mice were housed with oversight and approval from the Virginia Tech University Animal Care and Use Committee. Blood was collected via cardiac puncture, lymphocytes were isolated with Lympholyte-M and analyzed for in vitro proliferation as above (see Section “Cell Culture and Proliferation Assay”).

Flow Cytometry

For each horse, lymphocytes which were harvested after 24 h ($n = 5$) or 72 h ($n = 6$) of culture, were pelleted by centrifugation. Antibodies used were anti-CD21 (clone BU33, Novus Technologies) (Mayall, 2001) and anti-TLR4 (clone 76B357.1, LS Bio, Seattle, WA) with PE-conjugated anti-IgG2b secondary (clone RMG2b-1, Biolegend, San Diego,

CA). Anti-IgG2b (clone 27 to 35, BD Biosciences, San Jose, CA) was used for the TLR4 isotype control. Cells were incubated with antibodies for 30 min, at 4 °C and analyzed on an Attune NxT Flow Cytometer with Attune NxT software v 2.4 (Life Technologies, Thermo Fisher, Waltham, MA). Cells were gated for single cell populations and then for lymphocytes using FSC vs. SSC. Propidium iodine was used for viability in assessing TLR4 expression on cells cultured for 24 h (Figure 1A). For mouse cells, anti-CD45R/B220 (clone RA3-6B2, Biolegend) was used instead of anti-CD21. Data were analyzed utilizing the software Flow Jo version 10 (FlowJo, LLC, Ashland, OR).

RNA Extraction and mRNA Expression Analysis

For each horse ($n = 5$), RNA was extracted from 2×10^6 lymphocytes cultured for 24 h utilizing Quick-RNA microprep kit including DNase I (Zymo Research, Irvine, CA). RNA samples were analyzed on NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). RNA samples with a 260:280 absorbance ratio between 1.8 and 2 were processed for cDNA synthesis with High-Capacity Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to manufacturer's protocol.

Real-time qPCR assays used Power-UP SYBR green master mix (Applied Biosystems) and were performed in the 7500 HT Fast RT-qPCR system (Applied Biosystems). Conditions for amplification were 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 60 s at 60 °C. Melt curve assessment ensured the presence of single amplicon for each primer used. The genes of interest included:

TLR4, TNF α , IL-10, IL-6, and IFN β . Samples from each horse ($n = 5$) were run in triplicate. Primers were purchased from Integrated DNA Technologies (Coralville, IA), using previously validated sequences (Figueiredo, 2009a). The amplification efficiencies for all primers were ~100% with equivalent target and reference gene efficiencies. The qPCR assay contained 5 ng of cDNA, 300 nm of primer, and with a final reaction volume of 10 μ L. Changes in cytokine expression were calculated using the $\Delta\Delta$ CT method relative to the unstimulated samples.

Statistical Analysis

Culture results after 72 h and qPCR comparisons were analyzed using one- or two-way ANOVA post hoc testing Tukey *T*-test. 24-h cell culture data comparisons were assessed using Kruskal–Wallis test post hoc testing Dunn's multiple comparisons. Normal distribution was determined using the D'Agostino-Pearson normality test. Significance was set at $P = <0.05$ and all analysis was done using GraphPad Prism (San Diego, CA).

RESULTS

TLR4 Expression on B Lymphocytes

The antibody clone 76B357.1 for TLR4 is useful for analysis of equine cells by flow cytometry as a well-defined-positive population is seen compared with isotype control (Figure 1A). Co-expression of previously reported B-lymphocyte marker anti-CD21 (clone BU33, Mayall, 2001) and TLR4 demonstrated the presence of TLR4+ B lymphocytes (Figure 1A). The

Figure 1. (A) Representative flow cytometry dot plots demonstrate the specificity of Toll-like receptor 4 (TLR4) antibody (plot 1) compared with isotype control (plot 2). The expression of TLR4 on B lymphocytes (CD21) prior to 24-h cell culture (plot 3). (B) A bar graph depicting the median fluorescence intensity (MFI) of TLR4 on CD21 positive B lymphocytes preculture or after 24 h culture with no stimulation (unstimulated), lipopolysaccharide or pokeweed mitogen stimulated ($n = 6$).

remaining population of TLR4+ lymphocytes is presumed to be T lymphocytes, a population previously reported to not proliferate or produce cytokines in response to LPS stimulus alone in neither humans nor mice (Hornung et al., 2002; Gelman, 2004). The median fluorescence intensity (MFI) of TLR4 in B lymphocytes ($n = 6$) stimulated with LPS did not differ from unstimulated or PWM stimulated B lymphocytes ($n = 6$) (Figure 1B). In conclusion, the results from these in vitro experiments demonstrate that equine B lymphocytes do express TLR4 but cell culture and antigenic stimulation do not increase or otherwise alter cell surface receptor expression.

Functionality of LPS-Stimulated Equine B Cells

Following Lymphocyte isolation, equine lymphocytes cultured for 72 h with no stimulation (unstimulated), LPS or PWM (positive control), showed no proliferation in response to LPS stimulation ($n = 6$) (Figure 2A). For each horse ($n = 6$), lymphocytes stimulated with the positive control, PWM, demonstrated proliferation (Figure 2A), and a significant increase in total number of B lymphocytes compared with unstimulated (P

$= 0.004$) and LPS stimulated ($P = 0.002$) samples (Figure 2B). There was no significant difference between the total number of unstimulated and LPS stimulated B lymphocytes ($P = 0.924$) (Figure 2B). Mouse B lymphocytes stimulated with LPS demonstrated proliferation after 72 h of culture, serving as a positive LPS control (Figure 2A).

An abbreviated overview of the TLR4 signaling pathway shows the two primary signaling pathways with resultant cytokine production (Figure 2C). For each horse ($n = 5$), gene expression analyses results for cytokines; TNF α , IL-10, IL-6, and IFN β further support the sub-functionality of TLR4 on equine B lymphocytes. LPS stimulation resulted in downregulation of TLR4 with no significant differences in measured cytokines between unstimulated and LPS stimulated cells. In comparison of LPS to PWM stimulated cells, TLR4 mRNA levels were not significantly increased, TNF α and IL-10 levels were similar, IL-6 and IFN β levels were increased but not significantly (Figure 2D). Based on the horses sampled for this study, these results indicate that in vitro stimulation of equine B lymphocytes with LPS did not result in proliferation or cytokine upregulation.

Figure 2. (A) Representative flow cytometry histogram plots depicting proliferation of CD21 positive horse B-lymphocytes and B220 positive mouse B lymphocytes measured by attenuating carboxyfluorescein diacetate *N*-succinimidyl ester fluorescence intensity signal (log scale). Increase number of peaks correlates with increased proliferation. (B) Bar graph depicting the total number of horse B lymphocytes after 72 h of culture time. (C) Abbreviated Toll-like receptor 4 signaling pathways diagram. (D) Bar graph of qPCR data displaying the fold change of analyzed genes relative to unstimulated cells ($n = 5$). ** $P = <0.005$.

DISCUSSION

TLR4 expression varies amongst mammalian species affecting responses to LPS, the causative protein in endotoxemia. Horses, like humans, are sensitive to LPS and develop severe endotoxemia, a primary cause of death in cases of equine colic. In this study, we sought to determine whether the expression of TLR4 on equine B lymphocytes was like that seen in mouse or human. We found that equine B lymphocytes express TLR4 on their cell surface but these cells are not responsive to LPS in culture, similar to results seen in humans (Hornung et al., 2002; Wagner, 2004). PWM was chosen as a positive control for this study as it acts independently of TLR4 in humans (Bekeredjian-Ding et al., 2005) and has previously been shown to activate equine B lymphocytes (Lewis, 2014). For each horse sampled in this study, culture for 24 h resulted in no significant TLR4 expression change between unstimulated samples and those stimulated with LPS or PWM. While TLR4 is present on equine B lymphocytes, LPS fails to induce these cells to proliferate or upregulate cytokines. This is contrary to mouse studies where TLR4 expression, cytokine gene expression, and proliferation increases with LPS stimulation (Gururajan et al., 2007; Xu et al., 2008; Stavnezer and Schrader 2014; Lu, 2016) and similar to human B lymphocytes which express low levels of TLR4 but are nonresponsive when exposed to LPS (Hornung et al., 2002; Wagner, 2004). The qPCR results of this study indicated that LPS stimulation did not result in upregulation of any analyzed cytokine genes or TLR4 itself in any of the individual horses sampled for this study. In fact, TLR4 mRNA levels were downregulated compared with unstimulated samples, further supporting sub-functional TLR4 on equine B lymphocytes.

The cytokine genes evaluated in this study have previously been studied and associated with TLR4 signaling pathways. When LPS binds to carrier protein LPS-binding protein (LBP) in circulation, it is then transferred to receptor molecule CD14. This ligation leads to the interaction of LPS with TLR4 and co-receptor MD-2, initiating pro-inflammatory NF κ B signaling pathway. The transcription factor NF κ B is activated through the MyD88 signaling pathways, and the TRIF signaling pathways leading to the production of cytokines; TNF α , IL-6, IL-1 β (MyD88 pathway) and interferons and IL-10 (TRIF pathway) (Björkbacka, 2004) (Figure 2C). Previous in vitro equine immune cell studies investigating the response of monocytes to LPS have demonstrated that TLR4 signaling in this cell population primarily

occurs through the MyD88 pathway (Figueiredo, 2009b). Since little is known about TLR4 signaling in equine B lymphocytes, the current study analyzed gene expression of cytokines produced by both potential TLR4 signaling pathways.

The lymphocyte population used in the gene expression assays for this study contained both T and B lymphocytes, suggesting possibility that gene expression results are not a direct reflection of B lymphocyte activity. However, previous mouse studies have demonstrated that TLR activation on T lymphocytes requires TCR activation and LPS itself is not a known TCR agonist (Gelman, 2004). Additionally, human T lymphocytes are known to express low levels of TLR4 (Hornung et al., 2002). Therefore, any changes in cytokine expression can be attributed to B lymphocyte activity in this study.

These results suggest similarities in human and horse B lymphocytes in response to LPS. The susceptibility of these species to endotoxin may lie in the hypo-responsiveness of B lymphocytes to this antigen. This lack of direct response could result in delayed antibody responses, allowing for significant immune activation prior to antibody neutralization of LPS. This delayed immune response would be detrimental to the patient, allowing for endotoxemia to develop. Successful supportive treatments for equine endotoxemia include the use of hyperimmune plasma which contains antibodies against endotoxin inner core molecule Lipid A (Spier et al., 1989; Forbes et al., 2012). This treatment strategy further supports a potential essential and crucial role of B lymphocyte antibody production in the prevention of equine endotoxemia. Further studies are warranted to fully understand the role of B lymphocytes in equine endotoxemic disease.

Conflict of interest statement. None declared.

LITERATURE CITED

- Bekeredjian-Ding, I. B., M. Wagner, V. Hornung, T. Giese, M. Schnurr, S. Endres, and G. Hartmann. 2005. Plasmacytoid dendritic cells control TLR7 sensitivity of naive B cells via type I IFN. *J. Immunol.* 174: 4043–4050.
- Björkbacka, H., K. A. Fitzgerald, F. Huet, X. Li, J. A. Gregory, M. A. Lee, C. M. Ordija, N. E. Dowley, D. T. Golenbock, and M. W. Freeman. 2004. The induction of macrophage gene expression by LPS predominantly utilizes myd88-independent signaling cascades. *Physiol. Genomics* 19:319–330. doi:10.1152/physiolgenomics.00128.2004.
- Driver, J. P., J. J. Racine, C. Ye, D. J. Lamont, B. N. Newby, C. M. Leeth, H. D. Chapman, T. M. Brusko, Y. G. Chen, C. E. Mathews, et al. 2017. Interferon- γ limits diabetogenic CD8⁺ T-cell effector responses in type 1 diabetes. *Diabetes* 66:710–721. doi:10.2337/db16-0846.

- Figueiredo, M. D., Salter, C. E., Andrietti, A. L. P., Vandenplas, M. L., Hurley, D. J., and Moore, J. N. 2009a. Validation of a reliable set of primer pairs for measuring gene expression by real-time quantitative RT-PCR in equine leukocytes. *Vet. Immunol. Immunopathol.* 131, 65–72. doi:10.1016/J.VETIMM.2009.03.013
- Figueiredo, M. D., M. L. Vandenplas, D. J. Hurley, and J. N. Moore. 2009b. Differential induction of MyD88- and TRIF-dependent pathways in equine monocytes by Toll-like receptor agonists. *Vet. Immunol. Immunopathol.* 127, 125–134. doi:10.1016/J.VETIMM.2008.09.02
- Forbes, G., S. Church, C. J. Savage, and S. R. Bailey. 2012. Effects of hyperimmune equine plasma on clinical and cellular responses in a low-dose endotoxaemia model in horses. *Res. Vet. Sci.* 92:40–44. doi:10.1016/j.rvsc.2010.10.020
- Gelman, A. E., J. Zhang, Y. Choi, and L. A. Turka. 2004. Toll-like receptor ligands directly promote activated CD4+ T cell survival. *J. Immunol.* 172:6065–6073.
- Gururajan, M., J. Jacob, and B. Pulendran. 2007. Toll-like receptor expression and responsiveness of distinct murine splenic and mucosal B-cell subsets. *PloS one.* 2:e863.
- Hornung, V., S. Rothenfusser, S. Britsch, A. Krug, B. Jahrsdörfer, T. Giese, S. Endres, and G. Hartmann. 2002. 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.* 168:4531–4537.
- Lewis, S. R., S. P. Ellison, J. J. Dascanio, D. S. Lindsay, R. M. Gogal, Jr, S. R. Werre, N. Surendran, M. E. Breen, B. M. Heid, F. M. Andrews, et al. 2014. Effects of experimental sarcocystis neurona-induced infection on immunity in an equine model. *J. Vet. Med.* 2014:239495. doi:10.1155/2014/239495
- Lu, M., and R. Munford. 2016. LPS stimulates IgM production in vivo without help from non-B cells. *Innate Immun.* 22:307–315. doi:10.1177/1753425916644675
- Mayall, S., E. Siedek, and A. S. Hamblin. 2001. The anti-human CD21 antibody, BU33, identifies equine B cells. *J. Comp. Pathol.* 124:83–87. doi:10.1053/jcpa.2000.0425
- Moore, J. N., N. A. White, J. N. Berg, C. M. Trim, and H. E. Garner. 1981. Endotoxemia following experimental intestinal strangulation obstruction in ponies. *Can. J. Comp. Med.* 45:330–332. PMID: 7200383.
- Moore, J. N., and D. D. Morris. 1992. Endotoxemia and septicemia in horses: experimental and clinical correlates. *J. Am. Vet. Med. Assoc.* 200:1903–1914. doi:10.1182/blood.V99.5.169
- Moore, J. N., and M. L. Vandenplas. 2014. Is it the systemic inflammatory response syndrome or endotoxemia in horses with colic? *Vet. Clin. North Am. Equine Pract.* 30:337–51, vii. doi:10.1016/j.cveq.2014.04.003
- Nagai, Y., R. Shimazu, H. Ogata, S. Akashi, K. Sudo, H. Yamasaki, S. Hayashi, Y. Iwakura, M. Kimoto, and K. Miyake. 2002. Requirement for MD-1 in cell surface expression of RP105/CD180 and B-cell responsiveness to lipopolysaccharide. *Blood* 99:1699–1705. doi:10.1182/blood.V99.5.1699
- Spier, S. J., J. P. Lavoie, J. S. Cullor, B. P. Smith, J. R. Snyder, and W. M. Sischo. 1989. Protection against clinical endotoxemia in horses by using plasma containing antibody to an Rc Mutant E. coli (J5). *Circ. Shock.* 28:235–248.
- Stavnezer, J., and C. E. Schrader. 2014. IgH chain class switch recombination: mechanism and regulation. *J. Immunol.* 193:5370–5378.
- Tinker, M. K., N. A. White, P. Lessard, C. D. Thatcher, K. D. Pelzer, B. Davis, and D. K. Carmel. 1997. Prospective study of equine colic incidence and mortality. *Equine Vet. J.* 29:448–453. doi:10.4049/jimmunol.172.2.954
- Wagner, M., H. Poeck, B. Jahrsdoerfer, S. Rothenfusser, D. Prell, B. Bohle, E. Tuma, T. Giese, J. W. Ellwart, S. Endres, et al. 2004. IL-12p70-dependent th1 induction by human B cells requires combined activation with CD40 ligand and CpG DNA. *J. Immunol.* 172:954–963.
- Xu, H., L. N. Liew, I. C. Kuo, C. H. Huang, D. L. M. Goh, and K. Y. Chua. 2008. The modulatory effects of lipopolysaccharide-stimulated B cells on differential T-cell polarization. *Immunology.* 125:218–228.