RESEARCH ARTICLE

Expression level of human TLR4 rather than sequence is the key determinant of LPS responsiveness

Adeline M. Hajjar†*, Robert K. Ernst‡, Jaehun Yi§, Cathy S. Yam‖, Samuel I. Miller¶

1 Department of Comparative Medicine, University of Washington, Seattle, Washington, United States of America, 2 Department of Microbial Pathogenesis, University of Maryland, Baltimore, Maryland, United States of America, 3 Departments of Medicine, Microbiology and Genome Sciences, University of Washington, Seattle, Washington, United States of America

† hajjar@uw.edu

Abstract

To address the role of Toll-like receptor 4 (TLR4) single nucleotide polymorphisms (SNP) in lipopolysaccharide (LPS) recognition, we generated mice that differed only in the sequence of TLR4. We used a bacterial artificial chromosome (BAC) transgenic approach and TLR4/MD-2 knockout mice to specifically examine the role of human TLR4 variants in recognition of LPS. Using in vitro and in vivo assays we found that the expression level rather than the sequence of TLR4 played a larger role in recognition of LPS, especially hypoacylated LPS.

Introduction

Two coding human Toll-like receptor 4 (TLR4) single nucleotide polymorphisms (SNP; rs4986790 and rs4986791) resulting in Asp→Gly or Thr→Ile substitutions at amino acids 299 and 399, respectively, have been described [1]. In Europeans, the allele frequency of the double TLR4$^{D299G+T399I}$ allele is ~7% [2]. The earliest study on these coding TLR4 SNPs suggested that they resulted in decreased responsiveness to inhaled lipopolysaccharide (LPS) [1]. Another report demonstrated prevalence of the single TLR4$^{D299G}$ polymorphism in Africa (5–9% allele frequency), whereas the TLR4$^{D299G+T399I}$ allele was found to be prevalent in Europe (3–9% allele frequency) [3]. The authors proposed that the TLR4$^{D299G}$ variant initially arose to protect against cerebral malaria in Africa and that this allele was hyperresponsive to LPS, in contrast to the previous study. They proposed that the compensatory T399I mutation that reduced LPS responsiveness arose in Europe to protect from sepsis. That study also showed that the single TLR4$^{T399I}$ variant is very rare. Other in vitro studies also demonstrated reduced responsiveness of TLR4$^{D299G+T399I}$ to hexa- or penta-acylated LPS [4, 5].

Multiple association studies have linked these SNPs to a wide variety of diseases (early studies reviewed in [6]). Published reports describe association with sepsis, meningococcal disease, cerebral malaria, Crohn’s disease, ulcerative colitis, respiratory syncytial virus, atherosclerosis, rheumatoid arthritis, malignant melanoma, and metabolic syndrome, although others show no association for the same diseases suggesting complex genetic and epigenetic interactions [1, 4, 7–17]. Two additional reports suggested that the TLR4$^{D299G}$ variant is protective against
increased systolic blood pressure with increased obesity as well as against periodontitis, though both failed to examine the co-segregating rs4986791 SNP and thus actual genotype cannot be determined [18, 19]. Since the data in the literature is association data and even this data in some instances is incomplete and conflicting, we sought to develop a model system that would more clearly isolate the role of the coding TLR4 SNPs in response to LPS and disease states.

Thanks to the success in generating mice that display human-like responses to LPS through the expression of human TLR4 and MD-2 from human genomic BACs [20], we generated transgenic mice expressing either the TLR4D299G or the TLR4D299G+T399I human allele and compared them to mice expressing human TLR4WT. Although our comparisons were limited by the copy number of lines randomly generated, we found that the expression level of human TLR4 was the largest determinant of LPS response and that the actual sequence of human TLR4 did not reveal obvious differences in LPS recognition.

Materials and methods

Reagents

Ultrapure O111:B4 EC LPS was purchased from InvivoGen (San Diego, CA), and ODN1826 from Coley Pharmaceuticals (prior to acquisition by Pfizer, Düsseldorf, Germany). Monophosphorylated lipid A (MPL) was purchased from Avanti Polar Lipids Inc (Alabaster, AL). Antibodies for flow cytometry were purchased from Becton Dickinson (Franklin Lakes, NJ), eBioscience (San Diego, CA) or BioLegend (San Diego, CA). Anti-huTLR4 clones HTA125 and TF901 as well as anti-muTLR4 clone MTS510 were used for surface expression analysis.

PA and YP LPS preparation

**Bacterial growth conditions and strains.** The strains used in this study are *P. aeruginosa* (PAO-1) and *Y. pestis* (KIM6-). *P. aeruginosa* was grown in Lysogeny broth (LB) supplemented with 1mM MgCl2 at 37˚C with shaking and *Y. pestis* was grown in brain heart infusion (BHI) supplemented with 1mM MgCl2 at 37˚C with shaking.

**LPS and lipid A isolation.** LPS was extracted by the hot phenol/water method [21]. Freeze-dried bacterial pellets were resuspended in endotoxin-free water at a concentration of 10 mg/ml. A volume of 12.5 ml of 90% phenol (Fisher Scientific, Pittsburgh, PA) was added and the resultant mixture was vortexed and incubated for 60 minutes in a hybridization oven at 65˚C. The mixture was cooled on ice and centrifuged at 12,096 x g at room temperature for 30 minutes. The aqueous phase was collected and an equal volume of endotoxin-free water was added to the organic phase. The extraction was repeated and aqueous phases were combined and dialyzed against Milli-Q purified water to remove residual phenol and then freeze-dried. The resultant pellet was resuspended at a concentration of 10 mg/ml in endotoxin-free water and treated with DNase (Qiagen, Venlo, Limburg) at 100 µg/ml and RNase A (Qiagen, Venlo, Limburg) at 25 µg/ml and incubated at 37˚C for 1 hour in a water bath. Protease K (Qiagen, Venlo, Limburg) was added to a final concentration of 100 µg/ml and incubated for 1 hour in a 37˚C water bath [22]. The solution was then extracted with an equal volume of water-saturated phenol. The aqueous phase was collected and dialyzed against Milli-Q purified water and freeze-dried as above. The LPS was further purified by the addition of chloroform/methanol 2:1 [vol:vol] to remove membrane phospholipids [23] and further purified by an additional water-saturated phenol extraction and 75% ethanol precipitation to remove contaminating lipoproteins [24]. For Mass Spectrometry (MS) structural analysis 1 mg of purified LPS was converted to lipid A by mild-acid hydrolysis with 1% sodium dodecyl sulfate (SDS) (Sigma, St Louis, MO) at pH 4.5 as described previously [25].
MALDI-TOF mass spectrometry. Lipid A isolated by small-scale lipid A isolation procedures was analyzed on a AutoFlex Speed MALDI TOF mass spectrometer (Bruker Daltonics, Billerica, MA). Data was acquired in reflection negative and positive modes with a Smartbeam laser with 1 kHz repetition rate and up to 500 shots were accumulated for each spectrum. Instrument calibration and all other tuning parameters were optimized using Agilent Tuning mix (Agilent Technologies, Foster City, CA). Data was acquired and processed using flexControl and flexAnalysis version 3.3 (Bruker Daltonics, Billerica, MA).

Mice

Ethics statement. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All protocols were approved by the Institutional Animal Care and Use Committee of the University of Washington.

Husbandry. All mice were bred in-house at the University of Washington. C57BL6/J mice were originally obtained from the Jackson Laboratory (Sacramento, CA). Mice were maintained in a specific pathogen free (SPF) facility in standard ventilated cages on autoclaved corn cob bedding with an automatic watering system and fed irradiated standard rodent chow with a 12 hr on/12 hr off light cycle. Several methods of euthanasia were used, depending on experimental manipulation. Mice were euthanized by CO₂ narcosis, followed by a secondary method, when only bones were collected. However, for experiments using splenocytes (including those where bones were also collected), mice were euthanized by sharp cervical dislocation. Finally, for experiments where mice had received intraperitoneal LPS prior to euthanasia, mice were injected IP with an overdose of Beuthanasia-D (Schering-Plough Animal Health Corp., Union, NJ).

Generation. Human genomic BAC RP11-150L1 was modified by introducing point mutations using lambda Red mediated recombination and galK positive/negative selection to generate the D299G and T399I variants as described [26]. Primers used for recombineering are shown in Table 1. The entire TLR4 coding region was sequenced in the modified BACs prior to injection into fertilized B6C3 x B6 oocytes by the Transgenic Core at the University of Washington. Founders were backcrossed to B6 mice for 5 generations followed by backcrossing to TLR4/MD-2 DKO mice on a C57BL/6J background for at least another 5 generations. Throughout the figures and text, KO or TLR4 KO refers to a functional TLR4 KO, i.e. it is a TLR4/MD-2 DKO mouse that also expresses human MD-2, but not human TLR4. Only males are used in these studies because the human MD-2 BAC is integrated on the Y-chromosome limiting our ability to study females [20].

BAC copy number. Copy number of the TLR4 BAC was determined using real-time PCR as previously described [20]. Primers used for amplification of TLR4 amplified both mouse and human TLR4 with equivalent efficiency allowing us to use a WT B6 mouse as a calibrator (copy number = 2) in the Pfaffl method to calculate the relative copy number of the transgene [27].

Flow cytometry

Bone-marrow-derived macrophages (BMDM) were generated and stained as described for surface TLR4 expression using anti-human TLR4 clones HTA125 and TF901 and anti-mouse TLR4 clone MTS510 and appropriate isotype controls (mouse IgG2a and IgG1, and rat IgG2a respectively) [20]. Whole splenocyte stimulation and intracellular cytokine analysis was performed as described [20] with macrophage/monocyte population identified as CD11b + CD11c low/negative (neg) CD3 neg CD19 neg cells.
Table 1. Primer sequences.

| Oligo   | Sequence 5'–3'                                                                 | Info                          |
|---------|--------------------------------------------------------------------------------|-------------------------------|
| 307     | TTTGACCA TTTGAA GAA TCCCGA TTAGCAT ACT TAGAC TACT ACCTCGATG–galK (CCTGGAGAA TAAATCATCGGA) | 5' oligo D299G                |
| 308     | CCAGGGAA ATGA AGA AACAT TTGACA AAA ATAC TAA AAGTCAAAT AATA–galK (TCAGCA CTGTCCTG TCCTT) | 3' oligo D299G                |
| 309     | TTTGACCA TTTGAA GAA TCCCGA TTAGCAT ACT TAGAC TACT ACCTCGATG–galK (CCTGGAGAA TAAATCATCGGA) | D299G sense                   |
| 310     | CAGGGAA ATGA AGA AACAT TTGACA AAA ATAC TAA AAGTCAAAT AATA–galK (TCAGCA CTGTCCTG TCCTT) | D299G antisense               |
| 311     | AAATGCT TGAGTT TCA AAGG TTGCTCTTC AAGT GTTT TGGGACCA–galK (CCTGGAGAA TAAATCATCGGA)   | 5' oligo T399I                |
| 312     | AAATGCT TGAGTT TCA AAGG TTGCTCTTC AAGT GTTT TGGGACCA–galK (CCTGGAGAA TAAATCATCGGA)   | 3' oligo T399I                |
| 313     | ACTACATG TGGAAAT TGA ACCAT TGAAGCTCA AGA TAAATCTT TAGGCTG–galK (TCAGCA CTGTCCTG TCCTT) | T399I sense                   |
| 314     | ACTACATG TGGAAAT TGA ACCAT TGAAGCTCA AGA TAAATCTT TAGGCTG–galK (TCAGCA CTGTCCTG TCCTT) | T399I antisense               |
RT-PCR

Relative TLR4 expression levels were determined by real-time PCR as described [20] using an ABI 7300 Real Time PCR System and the Brilliant II SYBR Green QPCR reagents (Agilent Technologies Inc., Santa Clara, CA). Total RNA was isolated from cells or tissues using Trizol, DNase treated, and reverse transcribed using oligo dT priming and Superscript II (Invitrogen, Carlsbad, CA). A no-RT control confirmed RNA amplification. β-actin was used to normalize the data between samples.

Luminex

Serum samples were filtered in a 96-well 1.2 µm membrane HTS plate (Millipore, Billerica, MA) and diluted 1:4 or 1:12 in serum diluent. Cytokines (IL-1β, IL-6, IL-10, IL-12p40, IL-12p70, TNFα) were quantified using a Bio-Plex 200 system with Bio-Plex Pro Assay Kits (Bio-Rad, Hercules, CA) following the manufacturer’s instructions as described [20].

Statistics

Prism software (GraphPad, La Jolla, CA) was used for all statistical analyses. Multiple comparisons were performed using 1-way ANOVA followed by Bonferroni’s Multiple Comparison test.

Results

Generation of human TLR4 SNP mice

Initially, we modified the human TLR4 BAC to introduce the D299G mutation using BAC recombineering methods [26]. We then further modified this BAC to introduce the T399I mutation and generate the double D299G/T399I allele (often labeled as 399 in the figures). The BACs were then injected into fertilized oocytes at the University of Washington Transgenic Core (http://depts.washington.edu/compmed/transgenic/). Chimeric pups were identified by coat color and backcrossed to B6 mice and subsequently to TLR4/MD-2 double-KO mice, such that mice differed only in the sequence of human TLR4. The mice were also bred to humanized MD-2 mice [20]. Thus, in addition to human TLR4, these mice also express human MD-2 and lack mouse TLR4 and mouse MD-2. We generated and further characterized two lines for each TLR4 allele.

Expression of human TLR4 variants

We first examined copy number using methods as described in [20] and found that our new lines consisted of 4 and 7 copies of huTLR4D299G and 1 and 8 copies of huTLR4D299G+T399I, as compared to our previously generated 2- and 4-copy lines of huTLR4WT. While increasing BAC copy number from 1 to >48 correlates with increased expression (we did not have lines with identical copy numbers for comparison), when independent lines of same copy number were examined differences in staining intensity could be seen [28]. Therefore, we next examined expression of TLR4 in bone-marrow derived macrophages (BMDM) by staining for surface expression (Fig 1A–1C) and by RNA expression (Fig 1D). Increased copy number clearly resulted in increased RNA (Fig 1D) although protein expression appears to plateau by 7 copies, with no increase in the 8-copy line (Fig 1A and 1C). For this analysis, we also stained cells with a second clone (TF901; S1 Fig) that confirmed our results with clone HTA125, due to potential differences between antibodies directed at human TLR4 [29].
To determine whether we had restored *E. coli* (EC) LPS responsiveness in the splenic macrophage/monocyte population expressing the variant huTLR4 alleles, we used primary splenocyte intracellular cytokine staining (Fig 2) as described [20, 30]. An example of the gating strategy and TNF production in the macrophage/monocyte population is shown in S2 and S3 Figs. Compared to the percentage of cells producing TNF in TLR4 KO splenocytes, all humanized TLR4 lines showed an increase in TNF+ cells following EC LPS stimulation (Fig 2A). The

---

**Fig 2. All TLR4 SNP primary splenocyte macrophage/monocyte populations respond to EC LPS as measured by intracellular cytokine staining.** (A) Percent of mac/mono cells that are TNF+ in response to 1000 ng/ml EC LPS or (B) 10 μg/ml CpG. (C) Percent of mac/mono TNF+ in response to EC LPS from (A) divided by the percent TNF+ in response to CpG from (B). † above genotype show results compared to KO. †† P<0.01, ††† P<0.001, †††† P<0.0001, ns = not significant. Brackets show significant pairwise comparisons. Combined data from 7 experiments with each symbol representing a separate experiment for each genotype. Note that not each genotype was tested in each experiment. huWT = huTLR4<sup>WT</sup>, D299G = huTLR4<sup>D299G</sup>, T399I = huTLR4<sup>D299G+T399I</sup>, number after dash shows copy number of huTLR4 transgene.

---

https://doi.org/10.1371/journal.pone.0186308.g002
absolute percent positive varied from day to day, as did the response to the positive control CpG (Fig 2B; each symbol is from a different mouse spleen on a different day). Therefore, we normalized the results by dividing the percentage of TNF+ cells in response to LPS by the percent TNF+ in response to CpG (Fig 2C). This correction helped reveal decreased TNF production by the 1-copy compared to 8-copy TLR4<sup>D299G+T399I</sup> line.

We also examined the primary splenic responses to hypoacylated LPS or monophosphorylated lipid A (MPL; Fig 3, We used a penta-acylated preparation of <i>Pseudomonas aeruginosa</i> (PA) LPS [31], as well as tetra-acylated <i>Yersinia pestis</i> (YP) LPS [20]. Unlike the response to EC LPS, all humanized TLR4 mice, including huTLR4<sup>WT</sup>, showed reduced activity in response to hypoacylated LPS, as compared to WT B6 mice. In addition, following correction by the response to CpG, all lines except the 1-copy TLR4<sup>D299G+T399I</sup> line showed significantly increased response to PA LPS or MPL compared to the KO responses. The 1-copy TLR4<sup>D299G+T399I</sup> line did not respond to either PA or YP LPS even though we observed a response to EC LPS (Fig 2).

**In vitro cytokine responses**

We next generated bone-marrow-derived macrophages (BMDM), and stimulated cells with either PA, YP LPS, or EC LPS, and measured IL-6, CCL5, and IL-10 cytokines in the supernatants 24 hr later by Luminex (Fig 4). All humanized BMDM secreted very low levels of cytokines in response to PA or YP LPS, in contrast to WT B6 cells. In fact, the responses were not significantly different from TLR4 KO responses, although there was a trend towards higher values in the 7-copy TLR4<sup>D299G</sup> and 8-copy TLR4<sup>D299G+T399I</sup> lines compared to lower copy numbers. All humanized lines and the KO line were significantly reduced for IL-6 production, as compared to WT B6 cells, when stimulated with EC LPS. However, all humanized lines with the exception of the 1-copy TLR4<sup>D299G+T399I</sup> line had increased IL-6 production compared to the KO. In contrast to the previous assays, the 1-copy TLR4<sup>D299G+T399I</sup> line did not respond to EC LPS.
**TLR4 SNP and LPS response**

**In vivo cytokine responses**

To determine whether differential recognition of hypoacylated LPS could be measured *in vivo*, we extended our studies into mice, and injected mice intraperitoneally (IP) with either EC or PA LPS (Fig 5 and Fig 6). In response to EC LPS, IL-6 and TNF were increased at 1 hr and decreased by 6 hr, although B6 maintained high IL-6 levels at 6 hr (Fig 5). IL-10 was also increased at 1 hr in all genotypes compared to KO though absolute levels were very low. At 6 hr, IL-12p40, CCL5 and CXCL9 were all increased. However, in all cases no significant differences were observed between the humanized lines indicating that the sequence of TLR4 did not alter responses to EC LPS.

Mice were also injected with hypoacylated PA LPS (Fig 6). When comparing the values of IL-6 at 1 hr to the EC response, it is obvious that PA LPS induced less IL-6 at 1 hr, although a small response was also observed in KO mice, potentially indicating that non-TLR4 contaminants were present in the preparation despite repurification [32]. By 6 hr, the levels of IL-6 were comparable between PA and EC LPS in B6 mice; however, the humanized TLR4 lines all had reduced levels that were not significantly different from KO mice. There is a trend, however, towards higher levels in the TLR4 SNP mice compared to human TLR4 WT for both IL-6 and IL-12p40 at 6 hr, though the results are not significant.

**Discussion**

There is a large body of literature that continues to expand examining the association of TLR4 SNPs with disease. However, no study has clearly, reproducibly, and mechanistically linked the common coding TLR4 SNPs (rs4986790 and rs4986791) with disease. TLR4 and MD-2 are coreceptors that bind directly to the lipid A component of LPS [33, 34]. The structure of lipid
A varies between bacterial species and is also regulated in response to culture/environmental conditions or infection of a host [35, 36]. Highly endotoxic lipid A found in *E. coli* and *Salmonella* is predominantly hexa-acylated and bisphosphorylated. This lipid A can be detoxified by the removal of one phosphate resulting in mono-phosphoryl lipid A (MPLA), a known vaccine.

**Fig 5. Serum cytokine responses to 100 μg EC LPS 1 + 6 hr post IP injection.** Mice of the indicated genotypes were injected IP with EC LPS and then bled from the retro-orbital sinus 1 hr after injection. At 6 hr, the mice were euthanized for a terminal sample collection. Shown are combined data from 3 experiments, with 1 experiment containing 1 and 6 hr time points whereas the other 2 had either the 1 or 6 hr time point. All genotypes were included in all experiments. Data are plotted on a log scale for easier visualization due to large variation. Line indicates mean for genotype. * above symbols shows results compared to B6 whereas † is compared to KO. [https://doi.org/10.1371/journal.pone.0186308.g005](https://doi.org/10.1371/journal.pone.0186308.g005)

**Fig 6. Serum cytokine responses to 50 μg PA LPS 1 + 6 hr post IP injection.** Mice of the indicated genotypes were injected IP with PA LPS and then bled from the retro-orbital sinus 1 hr after injection. At 6 hr, the mice were euthanized for a terminal sample collection. Shown are combined data from 2 experiments. Data are plotted on a log scale for easier visualization due to large variation. * above symbols shows results for that genotype compared to B6 whereas † is compared to KO. [https://doi.org/10.1371/journal.pone.0186308.g006](https://doi.org/10.1371/journal.pone.0186308.g006)
An adjuvant that results in a selective decrease in MyD88-dependent responses while TRIF-dependent responses are maintained [37, 38]. These results suggest that the structure of the lipid A molecule alters signaling. *Pseudomonas aeruginosa* contains lipid A that is penta-acylated although cystic fibrosis isolates produce hexa-acylated molecules [39]. Similarly, *Yersinia pestis* produces hexa-acylated lipid A at room-temp but a tetra-acylated molecule at 37°C [40, 41]. These hypoacylated molecules induce reduced inflammatory responses selectively through human TLR4/MD-2 compared to mouse TLR4/MD-2 again demonstrating that the structure of lipid A alters signaling through TLR4/MD-2 [20, 31]. Similarly, TLR4 and MD-2 modification (by targeted mutagenesis) alters recognition of lipid A [42].

The crystal structure of TLR4<sup>D299G+T399I</sup> in complex with *E. coli* LPS demonstrated conformational changes at the site of the D299G polymorphism though not at the site of LPS binding [43]. One *in vitro* study that showed equivalent LPS binding between the D299G variant and WT TLR4 suggested that decreased inflammatory cytokine production mediated by huTLR4<sup>D299G</sup> is due to decreased recruitment of MyD88 and TRIF [44]. Our studies here do not reveal any obvious mechanistic differences in natural LPS recognition, including hypoacylated LPS, by the TLR4 variants, although one study suggests that the TLR4<sup>D299G+T399I</sup> variant is impaired in dimerization in response to MPLA [29]. In fact, our results confirm what others have clearly demonstrated *in vitro* and in mouse studies [45–48], that increased expression of TLR4 results in increased responsiveness to LPS. Our results also suggest that non-coding TLR4 SNPs (e.g. rs2770150, rs7873784, rs10759932) that could result in altered expression levels of TLR4 are likely more important in disease association than the coding SNPs.

Supporting information

**S1 Fig. TLR4 expression using TF901 clone.** (A) BMDM from each of the genotypes indicated above plots were stained with anti-huTLR4 clone TF901 or isotype control. Boxed number in each plot shows ΔMFI of TLR4 (red histogram for huTLR4, blue histogram in B for muTLR4) vs. isotype control (filled grey histogram). Histograms from 2 separate experiments are shown. (B) B6 mouse BMDM stained with anti-muTLR4. (C) Each symbol represents a separate BMDM preparation. All cells express huMD-2; red squares depict huTLR4WT, light blue triangles huTLR4D299G (299), and dark blue inverted triangles huTLR4D299G+T399I (399). X (KO) does not express TLR4. Open symbols have lower copy numbers than closed symbols. Brackets show significant pair-wise comparisons using 1-way ANOVA followed by Bonferroni’s Multiple Comparison test. *P*<0.05.

(TIF)

**S2 Fig. Gating strategy to examine primary splenocyte responses.** Data from a single experiment shown in Fig 2 are presented to demonstrate the gating strategy used to identify the macrophage/monocyte population as previously published (Ref 20).

(TIF)

**S3 Fig. TNF production in the macrophage/monocyte population from S2 Fig.** TNF histograms of the macrophage/monocyte population stimulated with indicated ligands. The % of macrophages/monocytes producing TNF is shown in each histogram.

(TIF)

**Acknowledgments**

We thank Michael Weaver and Christopher B Wilson for helpful suggestions at the start of this project. We also thank Alicia Brasfield, Lisa Nguyen, Miaolu Tang, and Kevin Mears for technical assistance, as well as Stacie Chan for preparation of solutions.
Author Contributions
Conceptualization: AMH RKE SIM.
Data curation: AMH RKE.
Formal analysis: AMH RKE.
Funding acquisition: AMH SIM.
Investigation: AMH RKE JY CSY.
Methodology: AMH RKE.
Project administration: AMH RKE.
Resources: AMH RKE.
Supervision: AMH RKE.
Validation: AMH RKE.
Visualization: AMH RKE.
Writing – original draft: AMH RKE.
Writing – review & editing: AMH RKE SIM.

References
1. Arbour NC, Lorenz E, Schutte BC, Zabner J, Kline JN, Jones M, et al. TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. *Nat Genet*. 2000; 25(2):187–91. https://doi.org/10.1038/76048 PMID: 10835634
2. Smirnova I, Hamblyn MT, McBride C, Beutler B, Di Rienzo A. Excess of rare amino acid polymorphisms in the Toll-like receptor 4 in humans. *Genetics*. 2001; 158(4):1657–64. PMID: 11514453
3. Ferwerda B, McCall MB, Alonso S, Giamarellos-Bourboulis EJ, Mouktaroudi M, Izagirre N, et al. TLR4 polymorphisms, infectious diseases, and evolutionary pressure during migration of modern humans. *Proc Natl Acad Sci U S A*. 2007; 104(42):16645–50. https://doi.org/10.1073/pnas.0704828104 PMID: 17925445
4. Rallabandi P, Awomoyi A, Thomas KE, Phalipon A, Fujimoto Y, Fukase K, et al. Differential activation of human TLR4 by *Escherichia coli* and *Shigella flexneri* 2a lipopolysaccharide: combined effects of lipid A acylation state and TLR4 polymorphisms on signaling. *J Immunol*. 2008; 180(2):1139–47. PMID: 18178854
5. Rallabandi P, Bell J, Boukhvalova MS, Medvedev A, Lorenz E, Arditi M, et al. Analysis of TLR4 polymorphic variants: new insights into TLR4/MD-2/CD14 stoichiometry, structure, and signaling. *J Immunol*. 2006; 177(1):322–32. PMID: 16785528
6. Schroder NW, Schumann RR. Single nucleotide polymorphisms of Toll-like receptors and susceptibility to infectious disease. *Lancet Infect Dis*. 2005; 5(3):156–64. https://doi.org/10.1016/S1473-3099(05)01308-3 PMID: 15766650
7. Ferwerda B, McCall MB, Verheijen K, Kullberg BJ, van der Ven AJ, Van der Meer JW, et al. Functional consequences of Toll-like receptor 4 polymorphisms. *Mol Med*. 2008; 14(5–6):346–52. https://doi.org/10.2119/2007-00135.Ferwerda PMID: 18231573
8. Douville RN, Lisitsyn Y, Hirschfeld AF, Becker AB, Kozyrskyj AL, Liem J, et al. TLR4 Asp299Gly and Thr399Ile polymorphisms: no impact on human immune responsiveness to LPS or respiratory syncytial virus. *PLoS One*. 2010; 5(8):e12087. https://doi.org/10.1371/journal.pone.0012087 PMID: 20711470
9. Fageras Bottcher M, Hmani-Aifa M, Lindstrom A, Jenmalm MC, Mai XM, Nilsson L, et al. A TLR4 polymorphism is associated with asthma and reduced lipopolysaccharide-induced interleukin-12(p70) responses in Swedish children. *J Allergy Clin Immunol*. 2004; 114(3):561–7. https://doi.org/10.1016/j.jaci.2004.04.050 PMID: 15366557
10. von Aulock S, Schroder NW, Guenzius K, Traub S, Hoffmann S, Graf K, et al. Heterozygous toll-like receptor 4 polymorphism does not influence lipopolysaccharide-induced cytokine release in human whole blood. *J Infect Dis*. 2003; 188(6):938–43. https://doi.org/10.1086/378095 PMID: 12964127
11. Tulic MK, Hurrelbrink RJ, Prele CM, Laing IA, Upham JW, Le Souef P, et al. TLR4 polymorphisms mediate impaired responses to respiratory syncytial virus and lipopolysaccharide. J Immunol. 2007; 179(1):132–40. PMID: 17579031

12. Awomoyi AA, Rallabandi P, Pollin TI, Lorenz E, Sztein MB, Boukhvalov MS, et al. Association of TLR4 polymorphisms with symptomatic respiratory syncytial virus infection in high-risk infants and young children. J Immunol. 2007; 179(5):3171–7. PMID: 17709532

13. Schwartz DA, Cook DN. Polymorphisms of the Toll-like receptors and human disease. Clin Infect Dis. 2005; 41 Suppl 7:S403 –7.

14. Weyrich P, Staiger H, Stancakova A, Machicao F, Machann J, Schick F, et al. The D299G/T399I Toll-like receptor 4 variant associates with body and liver fat: results from the TULIP and METSIM Studies. PLoS One. 2010; 5(11):e13980. https://doi.org/10.1371/journal.pone.0013980 PMID: 21125016

15. Schneider S, Hoppmann P, Koch W, Kemmerer S, Schmaderer C, Renders L, et al. Obesity-associated hypertension is ameliorated in patients with TLR4 single nucleotide polymorphism (SNP) rs4986790. J Inflamm (Lond). 2015; 12:57.

16. Schneider S, Hoppmann P, Koch W, Kemmerer S, Schmaderer C, Renders L, et al. Obesity-associated hypertension is ameliorated in patients with TLR4 single nucleotide polymorphism (SNP) rs4986790. J Inflamm (Lond). 2015; 12:57.

17. Zhou J, Zhang X, Liu S, Wang Z, Chen Q, Wu Y, et al. Genetic association of TLR4 Asp299Gly, TLR4 Thr399Ile, and CD14 C-159T polymorphisms with the risk of severe RSV infection: a meta-analysis. Influenza Other Respir Viruses. 2016; 10(3):224–33. https://doi.org/10.1111/irv.12378 PMID: 26901241

18. Westphal O JK. Bacterial lipopolysaccharides: extraction with phenol–water and further applications of the procedure. Methods in Carbohydrate Chemistry. 1965;(5):83–91.

19. Fischer W, Koch HU, Haas R. Improved preparation of lipoteichoic acids. Eur J Biochem. 1983; 133(3):523–30. PMID: 6190649

20. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem. 1957; 226(1):497–509. PMID: 13428781

21. Caroff M, Tacken A, Szabo L. Detergent-accelerated hydrolysis of bacterial endotoxins and determination of the anomeric configuration of the glycosyl phosphate present in the isolated lipid A fragment of the Bordetella pertussis endotoxin. Carbohydr Res. 1988; 175(2):273–82. PMID: 2900666

22. Warming S, Costantino N, Court DL, Jenkins NA, Copeland NG. Simple and highly efficient BAC recombinase using galK selection. Nucleic Acids Res. 2005; 33(4):e36. https://doi.org/10.1093/nar/gni035 PMID: 15731329

23. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 2001; 29(9):e45. PMID: 11328886

24. Chandler KJ, Chandler RL, Broeckelmann EM, Hou Y, Southard-Smith EM, Mortlock DP. Relevance of BAC transgene copy number in mice: transgene copy number across multiple transgenic lines and correlations with transgene integrity and expression. Mammm Genome. 2007; 18(10):693–708. https://doi.org/10.1007/s00335-007-9056-y PMID: 17882484

25. Yamakawa N, Ohto U, Akashi-Takamura S, Takahashi K, Saitoh S, Tanimura N, et al. Human TLR4 polymorphism D299G/T399I alters TLR4/MD-2 conformation and response to a weak ligand monophosphoryl lipid A. Int Immunol. 2013; 25(1):45–52. https://doi.org/10.1093/intimm/dxs084 PMID: 22962435

26. Yam CS, Hajjar AM. Whole spleen flow cytometry assay. Bio-protocol. 2013; 3(15):e834.
31. Hajjar AM, Ernst RK, Tsai JH, Wilson CB, Miller SI. Human Toll-like receptor 4 recognizes host-specific LPS modifications. *Nat Immunol*. 2002; 3(4):354–9. https://doi.org/10.1038/ni777 PMID: 11912497

32. Hirschfeld M, Ma Y, Weis JH, Vogel SN, Weis JJ. Cutting Edge: Repurification of lipopolysaccharide eliminates signaling through both human and murine Toll-like receptor 2. *J Immunol*. 2000; 165(2):618–22. PMID: 10878331

33. Beutler B, Rietschel ET. Innate immune sensing and its roots: the story of endotoxin. *Nat Rev Immunol*. 2003; 3(2):169–76. https://doi.org/10.1038/nri1004 PMID: 12563300

34. Park BS, Lee JO. Recognition of lipopolysaccharide pattern by TLR4 complexes. *Exp Mol Med*. 2013; 45:e66. https://doi.org/10.1038/emm.2013.97 PMID: 24310172

35. Miller SI, Ernst RK, Bader MW. LPS, TLR4 and infectious disease diversity. *Nat Rev Microbiol*. 2005; 3(1):36–46. https://doi.org/10.1038/nrmicro1068 PMID: 15608698

36. Trent MS, Stead CM, Tran AX, Hankins JV. Diversity of endotoxin and its impact on pathogenesis. *J Endotoxin Res*. 2006; 12(4):205–23. https://doi.org/10.1179/096805106X118825 PMID: 16953973

37. Mata-Haro V, Cekic C, Martin M, Chilton PM, Casella CR, Mitchell TC. The vaccine adjuvant monophosphoryl lipid A as a TRIF-biased agonist of TLR4. *Science*. 2007; 316(5831):1628–32. https://doi.org/10.1126/science.1138963 PMID: 17569868

38. Persing DH, Coler RN, Lacy MJ, Johnson DA, Baldridge JR, Hershberg RM, et al. Taking toll: lipid A mimetics as adjuvants and immunomodulators. *Trends Microbiol*. 2002; 10(10 Suppl):S32–7. PMID: 12377566

39. Ernst RK, Moskowitz SM, Emerson JC, Kraig GM, Adams KN, Harvey MD, et al. Unique lipid A modifications in *Pseudomonas aeruginosa* isolated from the airways of patients with cystic fibrosis. *J Infect Dis*. 2007; 196(7):1088–92. https://doi.org/10.1086/521367 PMID: 17763333

40. Kawahara K, Tsukano H, Watanabe H, Lindner B, Matsuura M. Modification of the structure and activity of lipid A in *Yersinia pestis* lipopolysaccharide by growth temperature. *Infect Immun*. 2002; 70(8):4092–8. https://doi.org/10.1128/IAI.70.8.4092-4098.2002 PMID: 12117916

41. Rebil R, Ernst RK, Gowen BB, Miller SI, Hinnebusch BJ. Variation in lipid A structure in the pathogenic *yersiniae*. *Mol Microbiol*. 2004; 52(5):1363–73. https://doi.org/10.1111/j.1365-2958.2004.04059.x PMID: 15165239

42. Maeshima N, Evans-Atkinson T, Hajjar AM, Fernandez RC. *Bordetella pertussis* Lipid A recognition by Toll-like receptor 4 and MD-2 is dependent on distinct charged and uncharged interfaces. *J Biol Chem*. 2015; 290(21):13440–53. https://doi.org/10.1074/jbc.M115.653881 PMID: 25837248

43. Ohto U, Yamakawa N, Akashi-Takamura S, Miyake K, Shimizu T. Structural analyses of human Toll-like receptor 4 polymorphisms D299G and T399I. *J Biol Chem*. 2012; 287(48):40611–7. https://doi.org/10.1074/jbc.M112.404608 PMID: 23055527

44. Figueiroa L, Xiong Y, Song C, Piao W, Vogel SN, Medvedev AE. The Asp299Gly polymorphism alters TLR4 signaling by interfering with recruitment of MyD88 and TRIF. *J Immunol*. 2012; 188(9):4506–15. https://doi.org/10.4049/jimmunol.1200202 PMID: 22474023

45. Kalis C, Kanzler B, Lemoa A, Poltorak A, Galanos C, Freudenberg MA. Toll-like receptor 4 expression levels determine the degree of LPS-susceptibility in mice. *Eur J Immunol*. 2003; 33(3):798–805. https://doi.org/10.1002/eji.200332431 PMID: 12616500

46. Bihl F, Salez L, Beaubier M, Torres D, Lariviere L, Laroche L, et al. Overexpression of Toll-like receptor 4 amplifies the host response to lipopolysaccharide and provides a survival advantage in transgenic mice. *J Immunol*. 2003; 170(12):6141–50. PMID: 12794144

47. Roy MF, Lariviere L, Wilkinson R, Tam M, Stevenson MM, Malo D. Incremental expression of Tlr4 correlates with mouse resistance to *Salmonella* infection and fine regulation of relevant immune genes. *Genes Immun*. 2006; 7(5):372–83. https://doi.org/10.1038/sj.gene.6364309 PMID: 16738669

48. Du X, Poltorak A, Silva M, Beutler B. Analysis of Tlr4-mediated LPS signal transduction in macrophages by mutational modification of the receptor. *Blood Cells Mol Dis*. 1999; 25(5–6):328–38. https://doi.org/10.1006/bcmd.1999.0262 PMID: 10660480