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EDUCATION

Oklahoma State University, Center for Health Sciences
Department of Pharmacology & Physiology
PhD Biomedical Sciences
Expected Graduation, May 2014

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M.S. Biomedical Research
Expected Graduation, December 2011
Thesis: "The Impact of the Asp299Gly and Thr299Ile Polymorphisms on Toll-like Receptor 4 Signal Transduction"

University of the Virgin Islands, St. Thomas, USVI
B.S. Biology (Cum Laude)
May 2008

RESEARCH EXPERIENCE

University of Maryland, School of Medicine
Department of Microbiology & Immunology
Research Graduate Assistant (September 2009-July 1st, 2011)
Overall project consisted of looking at molecular mechanisms of TLR4 variants and their effects on LPS binding to the TLR4/CD14/MD2 complex, TLR4 interactions with MD2, as well as LPS-elicited activation of MyD88 and TRIF dependent signaling pathways. To complete this project, both cellular and molecular techniques were used, which include cell culture (HEK293T/293 and bone marrow derived macrophages (BMDM)), transient/stable transfection, plasmid purification, western blotting, immuno-precipitation, Real-time PCR, confocal microscopy, and FACS analysis, while also following good laboratory practices.

Morehouse College & the Centers for Disease Control and Prevention
Project: IMHOTEP

Summer Intern (2008)
In 2008, I completed the Project: IMHOTEP public health internship, a competitive internship sponsored by Morehouse College in conjunction with the Centers for Disease Control and Prevention. During the first two weeks, an advanced training session in public health introductory courses was completed, which included Epidemiology, Biostatistics, and International Health. My placement in the Division of HIV/AIDS Prevention in the Program Evaluation Branch allowed me to work alongside many public health professionals whose guidance allowed me to complete a literature review entitled " An Assessment of Evaluation Capacity for Community-Based Organizations: A Literature Review."

TEACHING EXPERIENCE

University of Maryland, School of Medicine

- Guest Lecturer for undergraduate senior Applied Cellular and Molecular Biology (MEDT 454) on "Two-Component Regulatory Systems"
  Fall 2009 & Fall 2010

- Teaching Assistant for undergraduate junior Cellular and Molecular Biology (MEDT 451)
  August 2009-December 2009

PRESENTATIONS

American Association of Immunologists
98th Annual Meeting, San Francisco, CA
May 2011
Oral/Poster Presentations: "Asp299Gly polymorphism decreases the TLR4-elicited activation of MyD88- and TRIF-dependent signaling pathways." Leandra K. Figueroa and Andrei E. Medvedev. University of Maryland, School of Medicine

University of Maryland, Graduate Student Association
33rd Annual Graduate Research Conference, Baltimore, MD
April 2011
Oral Presentation: "The D299G Polymorphism inhibits Signal Transduction by Interfering with the MyD88- and TRIF-dependent Signaling Pathways"

American Association of Immunologists
97th Annual Meeting, Baltimore, MD
May 2010
Poster Presentation: "Asp299Gly TLR4 polymorphism impairs sensing of *Escherichia coli, Rickettsia akari*, and LPS-mediated activation of MyD88- and TRIF-dependent signaling pathways." A. Medvedev, C. Song, M. Quevedo, F. Qiu, and L. Figueroa. University of Maryland, School of Medicine

HONORS & AWARDS

- American Association of Immunologists
  AAI Trainee Award
  May 2011

- Federation of American Societies for Experimental Biology (FASEB)
  Student Minority Travel Award
  May 2011

- Best student for Project: IMHOTEP
  Summer 2008

- One of two nominees from the University of the Virgin Islands for the Jack Kent Cooke Scholarship Foundation
  Undergraduate Nominee, Spring 2008

- National Dean's List, 2006-2007; University of the Virgin Islands

- Induction into the International Golden Key Honor Society, University of the Virgin Islands, St. Croix Chapter, 1996

PUBLICATIONS

ABSTRACTS

"Asp299Gly polymorphism decreases the TLR4-elicited activation of MyD88- and TRIF-dependent signaling pathways." Leandra K. Figueroa and Andrei E. Medvedev. University of Maryland, School of Medicine; J. Immunol 2011, 186:116.17.

"Asp299Gly TLR4 polymorphism impairs sensing of *Escherichia coli, Rickettsia akari*, and LPS-mediated activation of MyD88- and TRIF-dependent signaling pathways." A. Medvedev, C. Song, M. Quevedo, F. Qiu, and L. Figueroa. University of Maryland, School of Medicine; J. Immunol 2010, 184:37.17.

"The D299G Polymorphism inhibits Signal Transduction by Interfering with the MyD88- and TRIF-dependent Signaling Pathways." Leandra K. Figueroa and Andrei E. Medvedev. University of Maryland, School of Medicine; 33rd Annual UMB Graduate Research Conference Abstract Book; pg. 9.

ABSTRACT

Toll-like receptors (TLRs) sense microbial pathogens to activate innate immune cells and prime adaptive immunity. Asp299Gly (D299G) and Thr399Ile (T399I) TLR4 polymorphisms underlie an increased risk of certain inflammatory and infectious diseases, but the mechanisms by which they affect TLR4 signaling are poorly understood. In this study, I determined the impact of the D299G and T399I polymorphisms on TLR4 expression and interactions with myeloid differentiation factor 2 (MD2), LPS binding and activation of the myeloid differentiation primary response gene (MyD) 88- and TIR domain-containing adapter inducing IFN-β (TRIF)-dependent pathways. Complementation of human embryonic kidney (HEK) 293 cells with transfected wild-type (WT) or mutant TLR4 variants expressing yellow fluorescent protein (YFP) revealed comparable TLR4 expression, TLR4-MD2 interactions, and LPS binding. 293/CD14/MD2 cells expressing D299G or T399I YFP-TLR4s had deficient LPS-induced MyD88 recruitment to D299G YFP-TLR4, phosphorylation of TBK1, IRF3 and p38, activation of NF-κB- and IRF3-driven reporters, and induction of IL-8 and IFN-β mRNA. In contrast to WT YFP-TLR4, expression of the D299G YFP-TLR4 in immortalized $TLR4^{-/-}$ mouse macrophages failed to elicit LPS-mediated induction of TNF-α and IFN-β genes. Thus, the D299G and T399I polymorphisms do not affect TLR4 expression, TLR4-MD2 interaction and LPS binding, but impair LPS-induced activation of the MyD88- and TRIF-dependent pathways, suggesting their interference with TLR4 dimerization and assembly of docking platforms within the TIR domains.

The Impact of the Asp299Gly and Thr399Ile Polymorphisms on Toll-like Receptor 4 Signal Transduction

By

Leandra K. Figueroa

Thesis submitted to the faculty of the Graduate School of the University of Baltimore in partial fulfillment of the requirements for the degree of Master of Science 2011

DEDICATION

I would like to dedicate my thesis to my parents, Juan and Catherine Figueroa, who have supported me throughout the years. I love you.

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Confocal studies: Drs. Adam Puche (Department of Anatomy and Neurobiology (University of Maryland), Gregory Melikian and Yuri Kim (*Now in the Department of Pediatrics, Emory University, Atlanta, GA)

Committee Memebers: Drs. Ivana Vucenik (Department of Medical and Research Technology), Dhan Kalvakolanu, Bret Hassel, Andrei E. Medvedev (Department of

Microbiology and Immunolgy) and Alan Cross (Department of Medicine) (University of Maryland, School of Medicine, Baltimore, MD)

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LIST OF ABBREVIATIONS

PAMPs	pathogen-associated molecular patterns
PRRs	pattern recognition receptors
TLRs	Toll-like receptors
NOD	nucleotide-binding oligomerization domain
NLRs	Nod-like receptors
RIG-I	retinoic acid-inducible gene-I
IFN	interferon
IFI16	interferon-inducible protein 16
AIM-2	Absent in Melanoma
ALRs	AIM2-like receptors
LRR	leucine-rich repeat
LRRFIP	LRR flightless-interacting protein
DAI	DNA-dependent activator of IFN regulatory factors
IL	interleukin
R	receptor
TIR	Toll-IL-1R resistance
EST	expressed-sequence tag
hToll	human toll
dToll	*Drosophila* toll
CD	cluster of differentiation
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
Th	T helper
LPS	lipopolysaccharide
DCs	dendritic cells
NK	natural killer
DAMPs	danger-associated molecular patterns
(F)	fusion
RSV	respiratory syncytial virus
HSP	heat shock protein

MyD88	myeloid differentiation primary response gene (88)
HMGB-1	high mobility group box-1
MD2	myeloid differentiation factor 2
MHC	major histocompatibility complex
TNF-α	tumor necrosis factor-alpha
CTL	cytotoxic T cells
LBP	LPS-binding protein
sCD14	soluble CD14
mCD14	membrane CD14
LTA	lipotechoic acid
MALP-2	Mycoplasma macrophage-activating lipopeptide-2
LDL	low-density lipoprotein
CR3	complement receptor 3
CHO	Chinese hamster ovary
COX-2	cyclooxygenase-2
TRIF	TIR-domain-containing adapter-inducing IFN-β
Ub	ubiquitin
F	phenylalanine
L	leucine
V	valine
M	methionine
I	isoleucine
S/Ser	serine
N/Asn	asparagine
E	glutamic acid
G	glycine
K	lysine
R	arginine
Q	glutamine
AP-1	activator protein 1

IRF	interferon regulatory factor
ER	endoplasmic reticulum
Y	tyrosine
MAL	MyD88 adapter-like
IRAK	IL-1R-associated kinase
DD	death domain
Myddosome	MyD88, IRAK4 and IRAK2 death domain complex
A	alanine
H	histidine
D	Aspartic acid
T/Thr	threonine
W	tryptophan
P	proline
TRAF	TNFR-associated factor
TAK	TGF-β-activated kinase
MAPKs	mitogen-activated protein kinases
ERK	extracellular signal-regulated kinase
JNK	c-Jun N-terminal kinase
TRAM	TRIF-related adapter molecule
RIP	receptor interacting protein
TANK	TRAF family member-associated NF-kappa-B activator
TBK	TANK-binding kinase
ERC	endocytic recycling compartments
PRAT4A	protein associated with TLR4
SNPs	single nucleotide polymorphisms
UTI	urinary tract infection
D299G	Asp299Gly
T399I	Thr399Ile
HEK	human embryonic kidney
WT	wild-type

YFP	yellow fluorescent protein
FACS	fluorescent activated cell sorting
SA-APC	streptavidin-allophycocyanin
GFP	green fluorescent protein
HA	hemagglutinin
iBMDMs	immortalized bone marrow-derived macrophages
DMSO	dimethyl sulphoxide
TBS	tris-buffered saline
Abs	antibodies
HRP	horseradish-peroxidase
DMEM	Dulecco's modification of Eagle's medium
Min	minute
Secs	seconds
LB	Luria-Bertani
h	hour
AMP	ampicillin
RT	room temperature
BSA	bovine serum albumin
cDMEM	complete DMEM
PMSF	phenylmethylsufonylfluoride
DTT	dithiothreitol
NAF	sodium fluoride
TBST	TBS with 10% Tween

INTRODUCTION

I. Toll-like receptors as principal membrane-associated sensors of pathogen-associated molecular patterns (PAMPs) and endogenous "danger" molecules

A. *Drosophila* Toll and identification of Toll-like Receptors

The immune system, which consists of the innate and adaptive branches, functions to detect and eliminate microbial pathogens and endogenous "danger" molecules to maintain host homeostasis (1). Whereas in adaptive immune cells, T cell antigen and B cell antigen receptors are formed by somatic gene re-arrangements that create a wide range of receptor specificities (2,3), innate immunity was long considered to be "non-specific." In his ground-breaking paper, Charles Janeway proposed that innate immune recognition is mediated by a set of germ-line encoded pattern recognition receptors (PRRs) detecting evolutionary conserved pathogen-associated molecular patterns (PAMPs) that cannot be easily changed because of their significance for microbial survival and infectivity (1). He also proposed that PRR recognition by innate immune cells provides signals (secretion of cytokines and up-regulation of major histocompatibility complex (MHC) and co-stimulatory molecules) that are necessary for initiation and orchestration of adaptive immune responses (1). Subsequent studies proved principal postulates of this theory and identified several classes of PRRs, including membrane-associated Toll-like receptors (TLRs) and C type lectins (4-7), and cytosolic PRRs, nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (8-11), and retinoic acid-inducible gene-I (RIG-I)-like helicases (12). In addition, sensors of cytoplasmic microbial DNA have been discovered, including interferon (IFN)-inducible protein 16

(IFI16), a family of Absent in Melanoma-2 (AIM-2)-like receptors (ALRs) (13,14), leucine-rich repeat (LRR) flightless-interacting protein (LRRFIP) (15), and DNA-dependent activator of interferon (IFN) regulatory factors (DAI) (16).

The advent of TLR biology in the 1990's was preceded by the discovery of the Toll gene from *Drosophila melangonaster* (15) that was found to be critical for the dorsal-ventral patterning in *Drosophila* embryos and antifungal host defense in adult flies (**Figure 1**)[1]. It was found that in response to fungal infection, endogenous cytokine pro-spatzle undergoes proteolytic cleavage by serine endoproteases Easter (17) and Spatzle-processing enzyme (18), yielding mature spatzle that binds to Toll and activates the transcription factor Dorsal (19) to induce expression of antimicrobial peptides (20) cecropins (21), diptericin (22), drosocin, attacin (23) and drosomycin (19). The cytoplasmic domain of *Drosophila* Toll is evolutionary conserved and homologous to interleukin (IL)-1 receptor (R) cytoplasmic domain and regions in mammalian TLRs and adapter proteins, and was named Toll-IL-1 receptor (TIR) domain that is important for signaling and host defense (24).

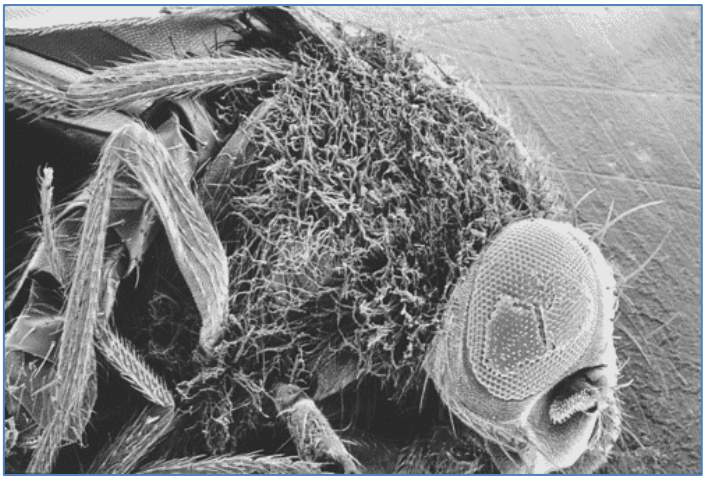

Figure 1 Toll-deficient *Drosophila melangonaster* (fruit fly) succumbs to *Aspergillus fumigatus* infection. The Toll gene present in *Drosohpila melangonaster* was found to be critical for antifungal host defense in adult flies, and Toll-deficiency, as shown, leads to uncontrolled fungal growth and death. Lemaitre B, Nicolas E, Michaut L, Reichhart JM, and Hoffmann JA. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell*. 1996; 86: 973-83.

[1] Permission was obtained from JA Hoffman and Elsevier Publications for use of this figure.

Subsequent studies by Janeway and Medzhitov resulted in the identification of the human ortholog, human toll (hToll), which was highly homologous to *Drosophila* toll (dToll) (25). Transfection-based expression of constitutively-active TLR4 (due to replacement of LRR domains with cluster of differentiation (CD)-4 dimerization interface that enables signaling) in THP1 cells resulted in typical innate-like responses, *e.g.*, activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), expression of pro-inflammatory cytokines IL-1, IL-6, and IL-8, and up-regulation of co-stimulatory molecules, *e.g*. CD80 (25). RNA expression profiling revealed the presence of hToll (TLR4) mRNA in monocytes, macrophages, dendritic cells, γ/δ T cells, T helper (Th)1 and Th2 α/β T cells, a small intestinal epithelial line and a B-cell line (25).

Notably, concurrent positional cloning studies by Bruce Beutler and colleagues identified the Pro712His mutation in *tlr4* responsible for LPS hypo-responsiveness of C3H/HeJ mice and showed the deletion of the entire *Tlr4* locus in another LPS hypo-responsive mouse strain, C57BL/10ScCR (26). Consequently, transfection-based complementation of TLR4-negative cell lines and macrophages with TLR4 was found to impart LPS sensitivity (27), while $TLR4^{-/-}$ mice failed to respond to LPS, succumbing to Gram-negative bacterial infections (28,29). These results unambiguously identified TLR4 as the principal membrane-associated receptor for Gram-negative lipopolysaccharide (LPS). Subsequent cloning of mammalian TLRs discovered a family of ten receptors that recognize distinct PAMPs and endogenous "danger" molecules.

B. Structural Organization and Pattern Recognition of TLRs

TLRs are membrane-associated sensors of microbes and endogenous "danger" molecules that mediate a first line of host immune defense by stimulating production of antimicrobial peptides, inflammatory cytokines, chemokines and IFNs, activating expression of MHC, adhesion and co-stimulatory molecules and regulating phagocytosis (30-36). TLRs are type I transmembrane glycoproteins expressed by a variety of cell types, including macrophages, neutrophils, dendritic cells (DCs), basophils, natural killer (NK) cells (37) and non-immune cells, *e.g*. keratinocytes, epithelial and endothelial cells (38,39). In addition, T- and B-lymphocytes have also been reported to express TLRs (40). All TLRs express an N-terminal ectodomain containing multiple LRRs involved in ligand recognition and co-receptor interactions, a single transmembrane helix and a C-terminal cytoplasmic tail with the TIR signaling domain (41,42) (**Figure 2**).

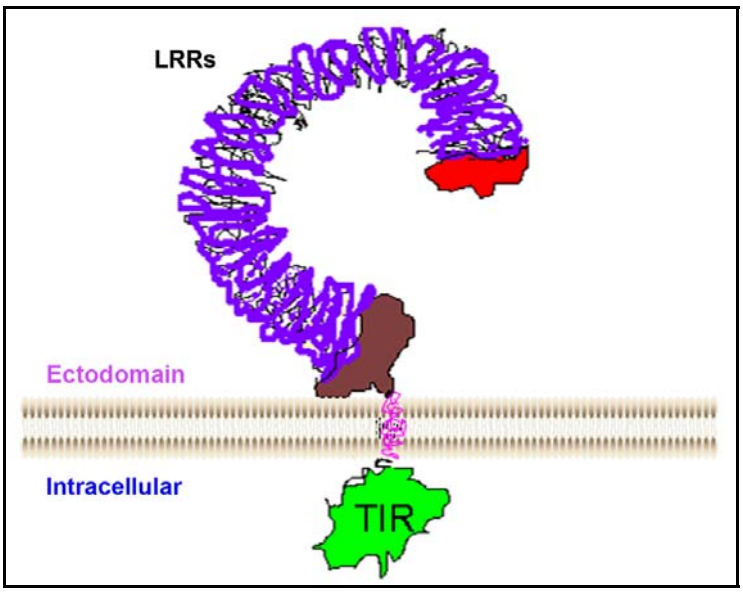

Figure 2 TLR Structural Organization. TLRs consist of an N-terminal ectodomain expressing LRRs, involved in ligand recognition and co-receptor interactions, a hydrophobic transmembrane domain comprised of an alpha-helix of ~ 20 amino acid residues, and an intracellular TIR domain necessary for signal transduction.

TLRs recognize a diverse array of microbial components, including nucleic acids, proteins, lipids, and polysaccharides (43), as well as endogenous danger-associated molecular patterns (DAMPs), which are normally sequestered in TLR-inaccessible cellular compartments but become exposed and accessible to TLRs during cell stress, inflammation or infection (44) (**Figure 3**). TLR2 recognizes tri- or di-acylated lipoproteins[2] from Gram-positive bacteria in association with TLR1 or TLR6, respectively (45,46). It also senses mycobacterial lipoarabinomannan (41), zymosan, a component of yeast cell walls (46) and envelope proteins of measles virus (46). TLR4 is the main receptor for LPS produced by Gram-negative bacteria, but also detects other, structurally unrelated PAMPs such as mannan, a plant polysaccharide (46), the fusion (F) protein of respiratory syncytial virus (RSV) (41,46) and *Chlamydial* heat shock protein (HSP) 60 (47). TLR5 senses extracellular bacterial flagellin, a structural protein that forms a major portion of flagella, which contributes to virulence by enabling bacterial motility during host invasion (46). Human TLR10 is highly expressed in the B cell lineage, suggesting a role for it in B cell function (48), while mouse TLR10 is a pseudogene, due to the presence of gaps and retroviral insertions (48). Although the ligands for TLR10 remain largely unknown, a recent study demonstrated that TLR10 cooperates with TLR2 to sense triacylated lipopeptides, as well as other bacterial and fungal agonists shared by TLR1, but not TLR6 (49). Computational modeling and mutational analysis of human TLR10 revealed the presence of the TLR2 dimerization interface and lipopeptide-binding channel similar to that found in TLR1. Similar to TLR2/1,

[2] Lipoproteins are an assembly of lipids and proteins

TLR2/10 complexes were found to recruit the proximal adapter myeloid differentiation primary response gene 88 (MyD88) to the activated receptor complex. However, TLR10, alone or in cooperation with TLR2, fails to activate NF-κB–, IL-8–, or interferon (IFN)-β–driven reporters. These data indicate that human TLR10 cooperates with TLR2 in sensing microbes and fungi but possesses a function distinct from that of other TLR2 subfamily members (49). TLR11, expressed only in mice, is important for protection against uropathogenic *E. coli* (50) and recognizes a profilin-like molecule from *Toxoplasma* (51).

While cell surface-expressed TLRs recognize lipids, proteins, and polysaccharides, endosomal TLRs sense bacterial, fungal or viral nucleic acids, *e.g.* TLR9 detects hypomethylated CpG motifs present in bacterial, viral and fungal DNA (46), while TLR3, TLR7 and TLR8 recognize viral double stranded (ds) RNA (TLR3) and single stranded (ss) RNA (TLR7 or TLR8), respectively (46) (**Figure 3**).

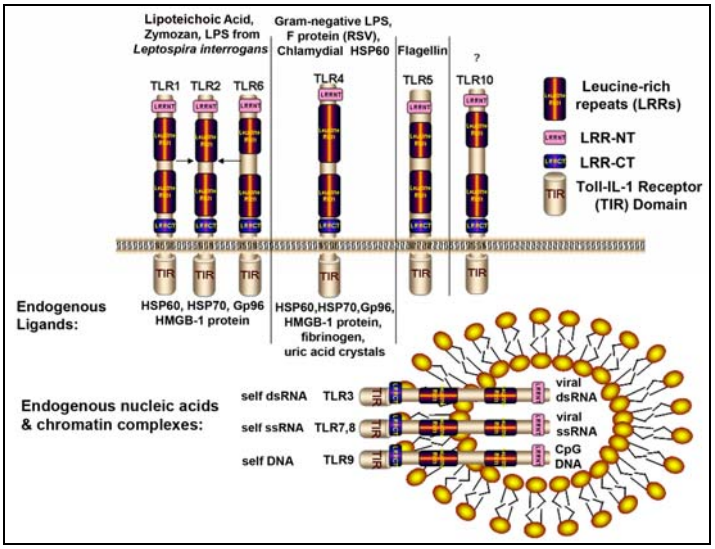

Figure 3 TLRs recognize exogenous and endogenous ligands. TLRs recognize a variety of PAMPs during the innate immune response. TLRs detecting bacterial and fungal lipids and peptides are localized on the cell surface, while those recognizing bacterial, viral and fungal nucleic acids are located intracellulary, in endosomes. TLRs also sense host-derived DAMPs, such as HSP60, HSP70, fibrinogen and self dsRNA, ssRNA and DNA.

Besides the recognition of PAMPs, TLRs also sense an array of endogenous DAMPs (52). The release of DAMPs, including heat shock proteins (HSPs), high mobility group box 1 (HMGB-1), host nucleic acids and chromatin, after necrosis or late apoptosis followed by necrosis, signals cell damage to the innate immune system (52), resulting in TLR sensing of DAMPs and initiation of inflammatory responses (52). TLR2 has been reported to recognize several endogenous ligands including HSP60, HSP70, HMGB-1, and fragments of hyaluronic acid (37,52). TLR4 recognizes similar DAMPs including HSP22, HSP60, HSP70, fibrinogen, and HMGB-1 (37,52). Endosomal TLRs also recognize endogenous ligands such as self dsRNA (TLR3), ssRNA (TLR7, 8) and DNA (TLR 9) (37), nucleosomes, and chromatin (52). TLR recognition of this enormous selection of PAMPs and DAMPs often occurs in cooperation with co-receptors, such as CD14 (TLR2, TLR3, TLR4) and myeloid differentiation factor 2 (MD2) (TLR4) (53-57).

Due to their expression in antigen-presenting cells, TLRs up-regulate expression of MHC, co-stimulatory and accessory molecules and induce secretion of cytokines, enhancing the 1st signal (antigen presentation by MHC molecules) and providing the 2nd signal (cytokines and co-stimulatory molecules) to initiate adaptive immune responses (42,58,59). Non-immune cells, including epithelial and endothelial cells, also express TLRs (38,39) and can secrete antimicrobial peptides (60,61) and up-regulate expression of adhesion molecules in response to most TLR ligands (38,42). TLRs can induce adhesion of leukocytes by up-regulating expression of selectins on the endothelium, and by increasing selectin-integrin interactions via the production of cytokines and chemokines (*e.g.* tumor necrosis factor-α (TNF-α),

IL-6 and IL-8) (42). TLR activation also plays an important role in dendritic cell migration through TLR-mediated downregulation of inflammatory chemokine receptors and upregulation of lymphoid chemokine receptors (42). Stimulation of immature DCs with TLR ligands leads to downregulation of CCR6 and upregulation of CCR7, (62,63), increasing DC migration into draining lymph nodes for antigen presentation (64), which leads to T-lymphocyte activation and differentiation into Th1 (58), Th2 (65) and cytotoxic T cells (CTL) (66) in response to antigen (42). Apart from cytokine and chemokine production, TLRs also initiate adaptive immune responses through production of type I IFN-β and type I IFN-inducible genes, which promote proliferation of memory T cells, prevent T cell apoptosis (67,68) and allow B-cells to undergo isotype switching and differentiation into plasma cells (69).

II. Sensing of Gram negative Bacterial LPS by TLR4

A. Gram-negative Bacterial LPS, a TLR4 Ligand

One of the ligands for TLR4 is LPS, the major structural component of all Gram-negative bacteria and a potent activator of the immune system (70). Exposure to minute amounts of LPS alerts the host innate immune system to microbial infection and initiates powerful host immune responses that, if unchecked and dysregulated, can lead to septic shock and multi-organ failure (70,71). LPS consists of three regions, a repeating O-polysaccharide side chain responsible for serologic specificity, a more conserved "core" KDO (3-deoxy-D-manno-octulosonic acid) oligosaccharide that is covalently linked to a hydrophobic glycolipid portion, lipid A (71). Lipid A is considered the major mediator of biological effects of LPS (46) and its acylation patterns determine LPS bioactivity. Bioactive LPS contains six fatty acids composed of 12 or 14 carbons, known as hexa-acylated lipid A (72), while a variation in the number of acyl chains decreases its endotoxic potential (hepta-acylated LPS) or confers TLR4 antagonistic activity (tetra/penta-acylated LPS), such as the case for lipid IVa or eritoran (70).

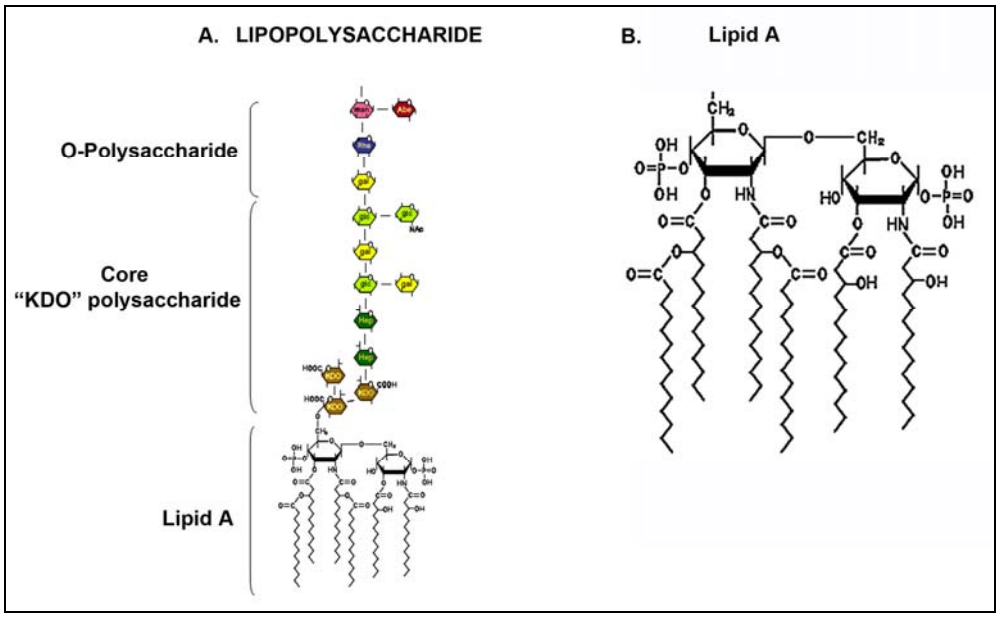

Figure 4 Structure of *Escherichia coli* LPS. **A.** LPS, the major structural component of Gram-negative bacteria, consists of an O-polysaccharide side chain with variable O-antigen, a core oligosaccharide KDO and a lipid A portion consisting of two phosphorylated glucosamines and six fatty acid chains (hexa-acylated LPS). **B.** Hexa-acylated LPS is a strong TLR4 agonist, while variations in the number of acyl chains decrease the LPS bioactivity or render it into a TLR4 antagonist.

Endotoxin-rich bacterial membranes are readily accessible to a serum lipid-transfer protein[3], LPS-binding protein (LBP), a 60 kDa glycoprotein that contains a lipid A binding site, allowing for high affinity interactions (Kd = 1 nM) with lipid A moieties in LPS micelles (73,74), catalyzing its extraction and disaggregation into monomeric LPS (75,76). LBP then transfers monomeric LPS to soluble CD14 (sCD14) or membrane CD14 (mCD14), in which CD14 then transfers LPS to the TLR4:MD2 complex (75-77). In CD14-negative cells, such as endothelial and epithelial cells, sCD14 interacts with LBP:LPS complexes and transfers LPS to the TLR4/MD2 complex (78,79). The utilization of LBP by other TLRs is less clear and

[3] LBP present in human serum at concentrations ranging from 5-10 μg/ml (81)

appears to be ligand-specific. For instance, Finberg et al. reported that zymosan-dependent activation of TLR2 in human cell lines requires CD14, but not LBP (80), while Schroder et al showed that both LBP and CD14 play an important role in TLR2 recognition of Gram-positive lipoteichoic acid (LTA), triacylated and diacylated lipoproteins and TLR2-driven immune responses (81,82).

B. Accessory molecules used for TLR ligand recognition

Accessory molecules, or co-receptors, are cell surface or soluble proteins that act by promoting interaction of PAMPs with true signal-transducing receptors, such as TLRs (54). MD2, CD14, CD36 and CD11b/CD18 are four such molecules used by TLRs for the recognition of PAMPs and DAMPs. MD2, a soluble protein, confers LPS responsiveness to TLR4. It constitutively interacts with the ectodomain of TLR4, and presents LPS to TLR4 upon cell stimulation, promoting dimerization of TLR4-MD2 receptor complexes (see below) and signaling (54-56).

Another accessory molecule, CD14, is expressed on the surface of myelomonocytes as a glycosylphosphatidylinositol-anchored glycoprotein or secreted as a soluble form (sCD14). CD14 has no signaling domain (53) and is mainly implied in the transfer of monomeric LPS to TLR4:MD2 (53). CD14 is also implicated in the recognition of TLR2/TLR1 agonists, glycolipids and triacylated lipoproteins (83) and TLR2/TLR6 agonists, LTA, zymosan and diacylated lipoproteins (83).

CD36, a double-spanning transmembrane molecule, is a class B scavenger receptor that binds polyanionic ligands of both pathogen and self-origin and mediates

phagocytosis and endocytosis (84). Through the endocytic uptake of altered self-components, including oxidized phospholipids, apoptotic cells and amyloid proteins, CD36 is important for the clearance of these molecules (84-87). CD36 was demonstrated to function as a TLR2-TLR6 co-receptor for *Staphylococcus aureus*-derived LTA, mycoplasma macrophage-activating lipopeptide-2 (MALP-2) (54,88) and diacylated lipopeptides (54,88). In addition, a recent study by Stewart et al. indicates that CD36 associates with TLR4 and TLR6 to recognize oxidized low-density lipoprotein (LDL) and amyloid-β peptide (84).

CD11b/CD18, also known as complement receptor 3 (CR3), has been reported to facilitate microbial recognition by TLRs. CR3, a member of the β2 integrin family, is a heterodimeric transmembrane glycoprotein that consists of CD11b (α-chain) non-covalently associated with CD18 (β-chain) (89). CR3, expressed on the plasma membrane of mononuclear phagocytes, NK cells (90) and minor subsets of $CD5^+$ B cells and $CD8^+$ T cells (91), regulates macrophage activation following LPS binding (92,93) and associates with CD14 (94). Transfection of Chinese hamster ovary (CHO) cells with CR3 imparted LPS responses (92,95), and a study in CD11b/CD18-deficient mice suggested that CD11b/CD18, along with CD14, facilitate signaling of certain TLR4-dependent genes in response to LPS, including IL-12 p40, cyclooxygenase-2 (COX-2) and IL-12 p35 (93). While CD11b/CD18 acts in concert with CD14 and TLR4 for LPS-inducible gene expression, recent studies have implicated that CD11b also has the ability to negatively regulate TLR signaling by facilitating degradation of signaling adapters, MyD88 and TIR domain-containing adapter-inducing IFN-β (TRIF) (96). CR3 deficiency was shown to decrease TLR-

triggered activation of tyrosine kinases Syk and Src, which phosphorylate MyD88 and TRIF, thereby forming docking sites for Cbl-l, an E3 ubiquitin (Ub) ligase responsible for degradation of these adapters (96). Thus, further studies will be required to clarify the regulatory role of CR3 in TLR signaling.

C. Binding of LPS to MD2-TLR4 receptor complex

LPS binding to TLR4 involves LBP, CD14, and MD2 (72). Before LPS binds to TLR4/CD14/MD2 complex, the primary interface between TLR4 and MD2 is formed at two chemical regions, the A and B patches[4], which are provided by the N-terminal and central LRR domains of TLR4, respectively (55). During bacterial infection, LBP extracts LPS from Gram-negative bacterial outer membranes or LPS micelles and catalyzes the transfer of monomeric LPS to sCD14 or mCD14, which in turn presents monomeric LPS to the TLR4:MD2 complex (75-77), leading to TLR4 homo-dimerization and activation (72). MD2 contains two, anti-parallel β-sheets, which form a large hydrophobic β-cup structure, where LPS binds (53). Of the six acyl chains[5] present in hexa-acylated LPS, R3', R3'', R2', R2'' and R3 are buried within the hydrophobic pocket of MD2 and the remaining R2 chain is partially exposed, allowing for hydrophobic interactions with TLR4 core residues, phenylalanine (F) 440 and 463, and leucine (L) 444, the residue located at the edge of the receptor (55). Once LPS is bound, the main dimerization interface of MD2, consisting of several

[4] A and B patches, provided by the N-terminal and central domains of TLR4 are located within LRRs 1-12.

[5] LPS consists of six acyl chains R3', R3'', R2', R2'', R3 and R2

residues (V82, M85, L87, I124 and F126)[6], forms hydrophobic interactions with the TLR4 residues F440, F463 and L444, ultimately stabilizing this complex (55). Along with hydrophobic interactions, TLR4 residues S416, N417, E439[7] form hydrogen bonds with MD2 residues G123, K125 and R90[8], while glutamine (Q) 436 from TLR4 forms a hydrogen bond with the 3-hydroxyl group attached to the R2 LPS chain (55). The two phosphate groups of lipid A interact with positively charged residues of TLR4 (K388, R264, K341, K362) and MD2 (K58, S118 and K122) (55), thereby contributing to TLR4 dimerization events (53,55).

The pattern of LPS acylation, *e.g*. tetra-, penta- or hepta-acylated LPS, is largely responsible for its ability to activate TLR4-mediated responses. For instance, penta-acylated LPS differs from hexa-acylated LPS in that it contains five fatty acyl chains, resulting in distinct interaction with MD2 and ~ 100 fold less endotoxic activity (55,97). Mutagenesis of F126 residue of MD2 prevented LPS-induced dimerization and signaling, indicating its critical importance (98). Binding of hexa-acylated LPS initiates structural changes in MD2 and MD2-assisted, F126-mediated presentation of LPS to the neighboring TLR4-MD2 complex, whereas binding of penta-acylated LPS exposes F126 to solvent area, preventing its interactions with ligand or other protein residues (55). These changes in the ability of MD2 to interact with penta-acylated LPS prevent a structural shift in the F126 loop, the movement of critical MD2

[6] Amino acid residues of MD2 involved in hydrophobic interaction with TLR4: Valine (V), Methionine (M), Leucine (L), Isoleucine (I) and Phenylalanine (F)

[7] Amino acid residues of TLR4 involved in hydrogen bonding with MD2: Serine (S), Asparagine (N) and Glutamic Acid (E),

[8] Amino acid residues of MD2 involved in hydrogen bonding with TLR4: Glycine (G), Lsyine (K) and Arginine (R).

residues (G123, I124, K125) to suitable positions, and the lack of proper LPS presentation to promote TLR4 dimerization events (55).

III. TLR Signal Transduction

A. TLR Signal Transduction Paradigm

TLRs recognize PAMPs and DAMPs via their LRR-containing ectodomains, often in cooperation with co-receptors, such as CD14, MD2 or CD36. TLR recognition of their respective ligands initiates homo-dimerization (*e.g.,* TLR4) or hetero-dimerization (*e.g.*, TLR1-TLR2 or TLR6-TLR2) that brings together intracellular TIR domains and induces conformational changes and post-translational modifications (97,99,100). These processes result in formation of docking interfaces to provide a scaffold for subsequent recruitment of adapter proteins and kinases, leading to activation of downstream pathways (101-103) that culminate in activation and nuclear translocation of transcriptional factors. Binding of transcription factors NF-κB, activator protein 1 (AP-1), IRF3 and IRF7 to the respective promoter elements within target genes turns on expression of inflammatory mediators, MHC, adhesion and co-stimulatory molecules, which initiate innate host defense and prime adaptive immune responses (104).

B. Post-translational modifications of TLRs

Important post-translational modifications of TLRs that are necessary for receptor activity include N-linked glycosylation, tyrosine phosphorylation, and ubiquitination. TLRs undergo N-linked glycosylation, which plays a critical role in protein folding, trafficking, LPS binding and receptor complex stabilization (105-107). N-linked glycosylation takes place in the endoplasmic reticulum (ER) where oligosaccharyl transferase catalyzes transfer of a $Glc_3Man_9GlcNAc_2$ oligosaccharyl

moiety from a dolichol-linked pyrophosphate donor to the Asn side chain within a consensus sequence (Asn-X-Thr/Ser) (106,107). This post-translational modification occurs mostly in regions of the ectodomain not involved in ligand binding (108), but also involves the ascending lateral surface where ligand interaction takes place (100). TLR1-TLR10 vary in the number of N-linked glycosylation sites, for instance, TLR2, TLR3 and TLR4 express four, fifteen and nine N-linked glycosylation sites, respectively (100). Site-directed mutagenesis of TLR2 N-linked glycosylation sites revealed the importance of this post-translational modification in biosynthesis and secretion of the TLR2 ectodomain (109) and showed significant differences in the location and degree of conservation between glycosylation sites across different TLRs (109). Inhibition of glycosylation with tunicamycin and mutations in two TLR3 N-linked glycosylation sites prevented TLR3-induced NF-κB activation, (110), without affecting protein expression, indicating the importance of this post translational modification for TLR3 signaling (110). N-linked glycosylation sites of TLR4 and MD2 are necessary for optimal LPS-mediated TLR4 signaling (105). TLR4 mutants carrying substitutions in Asn^{526} and Asn^{575} failed to be transported to the cell surface, and, subsequently, cells expressing these variants failed to bind LPS, whereas TLR4 glycosylation mutants expressing Asn^{35}, Asn^{497} and Asn^{624} showed no deficiencies in cell surface expression or LPS binding compared to wild-type TLR4-expressing transfectants (105). Despite retaining the ability to be expressed on the cell surface and bind to LPS, the triple mutant N35A/N173A/N205A TLR4 exhibited diminished capacities to mediate LPS-induced IL-8 promoter-driven luciferase reporters and IL-8

expression (105). These studies suggest that different N-linked glycosylation sites in the TLR ectodomains have non-redundant mechanisms of control of TLR signaling.

Another important post-translational modification is tyrosine phosphorylation of the TIR domain of TLR2, TLR3, TLR4 and TLR5, which is essential for initiation of downstream signaling (97,111-113). Mutations of tyrosine (Y) residues in the TIR domains were reported to abolish TLR2-dependent[9] transactivation of NF-κB and binding of phosphoinositide 3-kinase (PI3K) p85 subunit to TLR2 (111), TLR3-dependent[10] signaling in response to dsRNA (112), TLR4-inducible activation of p38, NF-κB and cytokine expression (114) and flagellin-induced TLR5-dependent[11] activation of p38, NF-κB signaling and IL-8 production (113).

TLRs also undergo ubiquitination, a covalent modification involved in the regulation of protein-protein interactions and function (K63-linked ubiquitination), and proteasomal degradation (K48-linked ubiquitination) (97). TLR3, 4, 5 and 9, but not TLR2, were shown to interact with two RING fingers and double-RING-finger-linked 3A (Triad3A), an E3 ubiquitin ligase that attaches K48-linked poly-unbiquitin (Ub) moieties to these TLRs, targeting them for proteasomal degradation and leading to down-regulating of TLR-elicited responses (115). Ubiquitination of TLRs is thought to act as a regulatory mechanism to prevent excessive signaling that can be detrimental and lead to diseases such as sepsis and multi-organ failure (116,117).

[9] Mutation as double mutant of TLR2 tyrosine residues Y616 and Y761

[10] TLR3 mutations in Y759 and Y858

[11] TLR5 mutation in Y798

Park et al. showed that signal-transducing capabilities of TLR9, but not TLR7, are regulated by cathepsin-mediated proteolytic cleavage in endosomal compartments, which is a prerequisite for TLR9 signaling (118). In contrast, in a recent study, Ewald et al. showed that TLR7, as well as TLR3 are processed in a manner analogous to TLR9, thereby suggesting proteolytic cleavage of endosomal TLRs as a potential regulatory mechanism for recognition of nucleic acids and signaling (119). Collectively, these results strongly suggest that post-translational modifications of TLR2, TLR3, TLR4, TLR5, TLR7 and TLR9 are crucial for receptor expression, localization and function, parameters that ensure fine-tuned and proper innate immune response.

C. TLR signaling pathways

TLRs signal via two major pathways: MyD88-dependent pathway, used by all TLRs except TLR3, and TRIF-dependent pathway, used only by TLR3 and TLR4 (120). Upon recognition of their respective ligands, TLRs dimerize, bringing TIR domains into close proximity and forming docking platforms to enable recruitment of TIR domain-containing adapter proteins. TLR4 first signals via the MyD88 pathway from the cell surface (120). TLR4-associated adapter, MyD88 adapter-like (Mal), functions as a bridging adapter to mediate MyD88 recruitment and interactions with the TLR4 receptor signalosome via TIR-TIR domain interactions (121-123). This forms a scaffold interface and enables recruitment of IL-1R-associated kinase (IRAK) 4, 1 and 2, which associate with MyD88 intermediate and death domains, respectively (43). Recently, the crystal structure of MyD88, IRAK4 and IRAK2 death domain (DD) complex (Myddosome) was obtained (124), revealing a hierarchical assembly,

in which MyD88 recruits IRAK4 and the MyD88-IRAK4 complex recruits IRAK2 or IRAK1, which is thought to bring the IRAK kinase domains into close proximity for phosphorylation and activation. Myddosome represents a single-stranded, left-handed helix of DDs comprised of six MyD88 molecules at the two bottom layers, four IRAK4 proteins in the middle layer and four IRAK2 in the top layer (124). Once MyD88 is recruited to the activated receptor complex, it oligomerizes and recruits IRAK4 to form the MyD88-IRAK4 complex involving several critical residues. Specifically, V43 and alanine (A) 44 residues of MyD88 interact with R47, Y48, histidine (H) 52, aspartic acid (D) 73 and threonine (T) 76 residues of IRAK4 to form the MyD88-IRAK4 complex. The MyD88-IRAK4 complex then recruits IRAK2 in which Y6, tryptophan (W) 62, and M66 residues of IRAK2 insert into the IRAK4 region composed of F25, proline (P28), Q29, Q59, R54, E92, F94, A95 and P96 residues (124). Clustering of IRAK4 results in its auto-phosphorylation and activation of kinase activity, leading to IRAK4→IRAK1/2 phosphorylation and activation of IRAK1/2 (46,125,126). Phosphorylated IRAK1 engages a downstream adapter protein, TNFR-associated factor (TRAF)-6, which is an E3 Ub ligase that undergoes K63-linked poly-ubiquitination, promoting direct recruitment of TGF-β-activated kinase (TAK)-1 (99). IRAK1 is also subject to K63-linked poly-ubiquitination by Pellinos that promotes recruitment of IκB kinase (IKK)-γ, via recognition of K63-linked Ub moieties on IRAK1 by the recognition motif within IKK-γ (127). TAK1 activates mitogen-activated protein kinases (MAPKs) and the IKK complex, and recruitment of IKK kinases to IRAK1 situates TAK1 and IKK kinases into close proximity, facilitating TAK1-mediated IKK activation (99). IKK-β

phosphorylates IκB proteins, targeting them for K48-linked ubiquitination and proteosomal degradation, relieving NF-κB dimers to translocate to the nucleus (43,97). Extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 MAP kinases activate the transcription factors E twenty-six like (Elk), AP-1 and Activating transcription factor 2 (ATF2), respectively, leading to their nuclear translocation (99), binding to promoter regions of target genes and transcription of pro-inflammatory cytokines (*e.g.* IL-6 and TNF-α) and chemokines (*e.g.* IL-8) (97).

While MyD88-dependent signaling occurs via recruitment of MyD88 to plasma membrane-associated Mal-TLR4 for production of pro-inflammatory cytokines, the TRIF-dependent pathway is induced from endosomes after endosomal translocation of TLR4 and a sorting adapter, TRIF-related adapter molecule (TRAM) (120). After TLR4 and TRAM are localized to the endosomal compartment, the TRIF pathway is initiated by recruitment of TRIF to TRAM-TLR4 (43), enabling recruitment of a downstream adapter TRAF3 (120,128-130). TRIF signals activation of kinases IKK-ε and TRAF family member-associated NF-kappa-B activator (TANK)-binding kinase (TBK)-1 that phosphorylate and activate the transcription factor IRF3 (43,121,131). IRF3 translocates to the nucleus and, together with NF-κB, activates IFN-β (132). Apart from induction of a type I IFN, the TRIF-dependent pathway mediates late activation of MAP kinases and NF-κB, and up-regulation of co-stimulatory molecules (132-135). Besides TLR4, TLR3 is the only other TLR that signals through the TRIF-dependent pathway after bacterial dsRNA activation (120,136-140). TLR7, TLR8 and TLR9 activate type I IFNs via recruitment of the MyD88- IRAK4-IRAK1 signaling module, which then interacts with TRAF6, leading

to activation of NF-κB and production of pro-inflammatory cytokines, or activates TRAF3 and IKK-α, leading to induction of IRF3 and IRF7, respectively (140,141).

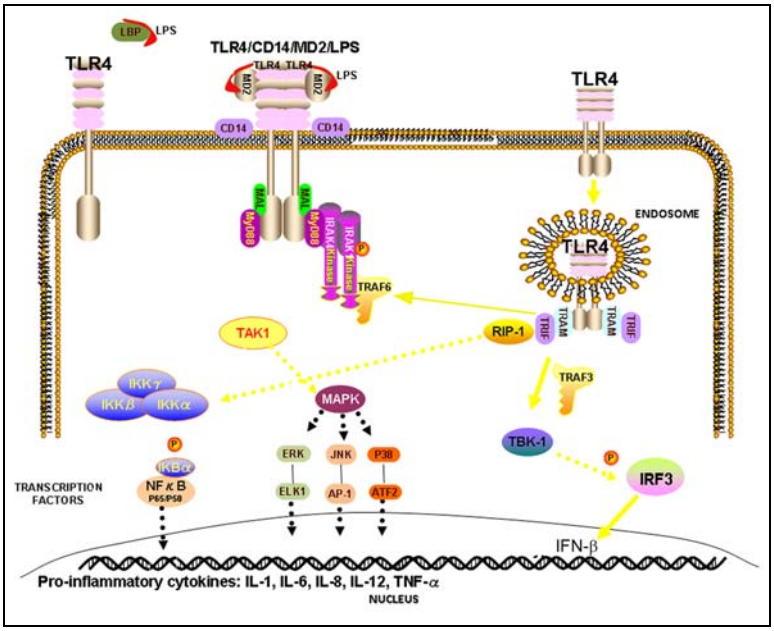

Figure 5 MyD88- and TRIF-dependent signaling pathways in Toll-like receptor signal transduction. Upon signaling activation, the intracellular adapters MyD88 and TRIF are recruited, which in turn, activate the downstream kinases IRAK4, IRAK1 (via MyD88) and TBK1 (via TRIF). This leads to transcription of genes that encode for inflammatory cytokines, chemokines, type I IFNs, MHC and co-stimulatory molecules.

D. TLR trafficking

TLR trafficking represents an important mechanism responsible for proper receptor localization to corresponding sites within the cell, which regulates the ability of TLRs to recognize their specific ligands (97,142). TLR1, TLR2, TLR4-6 are localized to the plasma membrane, while TLR3, TLR7, TLR8 and TLR9 recognize their ligands within late endosomes or lysosomes (97). TLR4/CD14/MD2 complex rapidly cycles from the Golgi to the plasma membrane until the complex is involved in LPS recognition at the cell surface (143). After dissociation of TLR4 from the MyD88 signalosome, the receptor trafficks to early/sorting endosomes (144) through

dynamin and clathrin-dependent pathways, which allows for TRIF-dependent signaling, crosstalk with antigen presentation machinery (144) and TLR4 degradation for down-regulation of NF-κB signaling (115). A GTPase, Rab11a, was found to co-localize with TLR4 in endocytic recycling compartments (ERC), as well as recruiting TLR4 to phagosomes for TRIF-dependent signaling (144). TLR trafficking is also carried out by chaperone proteins gp96, protein associated with TLR4 (PRAT4) A and Unc-93 homolog B (UNC93B). Gp96 is an ER paralog of the HSP90 that chaperones several cell surface and intracellular TLRs, as well as other molecules, such as integrins (145). Remarkably, gp96 deficiency leads to the loss of expression of TLR1-TLR2, TLR3, TLR4, TLR5 and TLR7, or improper protein folding (TLR9) (145,146), providing multiple TLR knockout phenotypes (145,147). PRAT4A is an ER protein that is involved in the trafficking of TLR1, TLR2 and TLR4 to the plasma membrane and mediates trafficking of TLR9 to the endosome/lysosome (148). A multispan transmembrane protein, UNC93B, interacts with transmembrane domains of TLR3, TLR7 and TLR9, and enables trafficking of these intracellular proteins to endosomes for signaling (149). Thus, multiple regulations of TLR trafficking serve as a complex fine-tuning mechanism that controls proper TLR localization, expression and signaling.

III. Polymorphisms within TLR4

A. TLR4 polymorphisms are linked to infectious and inflammatory diseases

Accumulating evidence has supported the role of dysregulated TLR signaling in the pathogenesis of infectious and inflammatory diseases, including sepsis, rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis and allergy (150). Several single nucleotide polymorphisms (SNPs) in the coding and promoter regions have been identified in human *TLR4*. TLR4 SNPs have been linked to increased incidence of certain infectious and inflammatory diseases, such as Gram-negative infections (151) and sepsis (116), RSV bronchiolitis (152), disseminated candidiasis, and inflammatory bowel disease (153), but at the same time, their carriage lowers a risk of atherosclerosis (154) and rheumatoid arthritis (155).

Several TLR4 polymorphisms have been identified in the 5'-promoter region of *TLR4*. Sequencing of the *TLR4* promoter in Swedish blood donors with mild or severe urinary tract infection (UTI), identified eight TLR4 promoter sequence variants: G4038A, G3612A, G3002A, G2604A, A2570G, G2081A, A2026G and T1607C (156), three of which were also identified in TLR4 promoter polymorphic association studies with myocardial infarction ((157) and systemic responsiveness (158). Ragnarsdottir et al reported that single or multiple TLR4 promoter polymorphisms influenced expression dynamics *in vitro* and innate immune responses in patients with UTIs, especially in response to *E. coli* infection (156). In contrast, the presence of the A2570G, T2431C, A2026G and T1607C TLR4 polymorphisms showed no association with myocardial infarction (157) or LPS responsiveness (A2026 and T1607C) (158).

Two SNPs have been identified in the coding region of *TLR4*, which are responsible for single amino acid substitutions in the ectodomain, from aspartic acid to glycine at position 299, Asp299Gly (D299G); and from threonine to isoleucine at position 399, Thr399Ile (T399I) (117,159). TLR4 polymorphisms are rare in Asian populations, such as Chinese and Japanese, while American and Canadian Caucasians represent frequency alleles of D299G ranging from 6-10% and T399I from 5-10% (160). The presence of D299G has been associated with blunted responsiveness to inhaled LPS and decreased LPS-mediated production of cytokines by airway epithelial cells and alveolar macrophages (117), Gram-negative bacteremia (151) and sepsis (116,117). The D299G TLR4 polymorphism was also linked to Mediterranean spotted fever caused by *Rickettsiae conorii* (161), and blunted responses to *Rickettsiae akari* were seen in human embryonic kidney (HEK) 293 cells expressing the D299G TLR4 variant (162). The two TLR4 SNPs also exist in a co-segregated form, D299G/T399I, which confers the most severe LPS hyporesponsiveness compared to hypomorphic responses elicited by the single alleles (163) and is associated with an increased risk of sepsis (150), severe RSV infection (152) and myocardial infarction (164). Genome-wide association studies on the role of TLR4 SNPs in predisposition to infectious and inflammatory diseases have provided conflicting results, mainly due to diverse populations and small sample numbers used for each study. For example, conflicting data have been published regarding association of the D299G polymorphism with tuberculosis. While TLR4 was reported to recognize HSP65 from *M. tuberculosis* to elicit immune responses (165), other groups were unable to reproduce these results and the extent to which the

D299G mutation affects sensing of *M. tuberculosis* and susceptibility to and severity of tuberculosis is unclear. Newport et al. (166) found no association between the presence of the D299G and incidence of tuberculosis in a Gambian population. However, Ferwerda et al. (167) reported that HIV patients expressing this SNP from Tanzania had an increased risk of developing active tuberculosis, which suggest that association between D299G and tuberculosis may be dependent on prior immune deficiencies and ethnic backgrounds (166-168). Human subjects carrying the T399I polymorphism either exhibit a milder LPS-hyporesponsive phenotype or do not manifest it at all (117). The presence of the T399I and D299G TLR4 polymorphisms was also linked to premature birth rates in a Finnish population (169) and an increased risk of RSV infections in full-term infants (170). Most recently, the carriage of the D299G TLR4 has been linked to an increased risk of endometriosis (171), recurrent tonsillitis and/or tonsillar hypertrophy (172), increased prevalence of infections in cirrhotic patients (173), and susceptibility to age-related macular degeneration (174). More correlations between the presence of the D299G polymorphism and increased susceptibility to several diseases have been found, such as infections with RSV in a cohort of Israeli children (152), Crohn's disease (153), ulcerative colitis (153), myocardial infarction (164) and increased total body fat, visceral fat, liver fat and decreased insulin sensitivity in non-diabetic Caucasians (175), suggesting that D299G may contribute to diabetes risk. While these TLR4 SNPs have been associated with infectious diseases, they have been reported to confer beneficial effects such as protection against atherogenesis (154) and rheumatoid arthritis (155).

B. Molecular Basis for Altered Signaling of TLR4 Polymorphic Variants

The molecular mechanisms by which the TLR4 polymorphisms affect receptor functions are largely unknown. Arbour et al. originally reported that compromised LPS-mediated responses elicited by the D299G TLR4 variant is associated with decreased expression levels of the mutant receptor compared to expression of wild-type (WT) TLR4 (117). Likewise, Prohinar et al. reported 2-fold reduced expression of D299G/T399I TLR4 *vs*. WT TLR4 upon co-expression with MD2, and greater than10-fold reduction in D299G/T399I TLR4 expression levels when it was expressed without MD2, paralleling differences in cellular LPS responsiveness (176). A study of RSV-induced responses revealed that D299G and T399I TLR4 polymorphic variants fail to efficiently translocate to the cell surface, leading to a decrease in NF-κB signaling and production of IFNs, IL-8, and IL-10 (177). In contrast, Rallabhandi et al. reported that despite equal total and cell sutface expression of WT, D299G or T399I TLR4s in HEK293T/CD14/MD2 transfectants, the polymorphic TLR4 species were compromised in their ability to elicit NF-κB activation and cytokine gene expression in response to LPS, F protein from RSV or *Chlamydia* HSP60 (163).

There are several possible mechanisms by which polymorphisms could affect TLR4 responsiveness under conditions of equal receptor expression, including ligand binding, TLR4 dimerization or co-receptor interactions, leading to altered activation of downstream signaling. In this study, I sought to determine the impact of the D299G and T399I polymorphisms on TLR4 expression, interactions with MD2, LPS binding to the TLR4/CD14/MD2 complex and recruitment and activation of signaling

adapters and kinases. An improved understanding of the molecular mechanisms by which these mutations alter TLR4 signaling is expected to facilitate the development of new therapeutic strategies for treatment of diseases such as systemic inflammatory response syndrome (178), acute respiratory distress syndrome, and airway diseases (116,150,179).

HYPOTHESIS

Due to the location of the D299G and T399I polymorphisms in the TLR4 ectodomain involved in ligand recognition and co-receptor interactions, these polymorphisms could impact LPS binding to the TLR4/MD2/CD14 complex, TLR4-MD2 interactions and/or TLR4 homo-dimerization, thereby altering docking TIR domain platform assembly and suppressing recruitment and activation of key adapters and kinases of the MyD88- and TRIF-dependent pathways.

SPECIFIC AIMS

Molecular mechanisms by which the D299G and T399I polymorphisms alter TLR4 signal transduction are poorly understood. The magnitude of TLR4 signaling depends on receptor expression levels (180,181), and TLR4 expression and trafficking is regulated by chaperons Gp96 and canopy 3 homolog (CNPY3) that interact with the ectodomain of TLR4 (142,145,182) where the polymorphisms are located (30,117,159). Thus, it is possible that the D299G and T399I polymorphisms could interfere with TLR4-chaperon interactions, affecting TLR4 trafficking and expression. Since the D299G and T399I polymorphisms are present in the TLR4 ectodomain involved in ligand recognition and co-receptor interactions (55,98), these mutations could affect LPS binding to the TLR4/CD14/MD2 complex and/or impact TLR4 interactions with MD2. LPS recognition by TLR4/MD2 induces dimerization events that bring the intracellular TIR domains into close proximity, creating docking platforms to enable recruitment of TIR domain-containing adapters MyD88 and TRIF. Thus, the presence of the D299G or T399I polymorphisms in the ectodomain could alter dimerization of TLR4 proteins, compromising recruitment of adapters/kinases and activation of the MyD88 and/or TRIF signaling pathways.

Therefore, I **hypothesized** that the D299G and T399I polymorphisms could impact LPS recognition, TLR4-MD2 interactions or TLR4 homo-dimerization, thereby altering assembly of the docking TIR domain platforms and suppressing recruitment and activation of key adapters and kinases of the MyD88- and TRIF-dependent signaling pathways. The following **Specific Aims** have been designed to test my hypothesis (**Figure 7**):

1. Elucidate the effect of D299G and T399I polymorphisms on TLR4 expression.

I used transient and stable transfection of HEK293 cells with expression plasmids encoding CD14/MD2 co-receptors and WT or mutant YFP-TLR4 variants to examine total TLR4 expression, using fluorescent and confocal microscopy, immunoprecipitation and immunoblotting.

2. Define whether the D299G and T399I polymorphisms affect LPS binding to the TLR4/CD14/MD2 receptor complex and TLR4-MD2 interactions.

Biotinylated LPS was added to 293/CD14/MD2 tranfectants expressing WT or mutant YFP-TLR4 variants, and LPS binding was revealed using streptavidin-allophycocyanin (SA-APC) by fluorescence-activated cell sorting (FACS). To study interactions of YFP-TLR4s with Flag-tagged MD2 proteins, I employed co-immunoprecipitation and immunoblot analyses.

3. Determine the impact of the D299G and T399I polymorphisms on LPS-induced, TLR4-elicited activation of MyD88- and TRIF-dependent signaling pathways.

I studied how polymorphisms affect LPS-mediated activation of MyD88-dependent (recruitment of MyD88 to TLR4, expression of IL-8 and TNF-α genes) and TRIF-dependent (phosphorylation of TBK1 and IRF3, induction of IFN-β promoter-driven luciferase reporter and expression of IFN-β mRNA) signaling, as well as phosphorylation of p38 MAP kinase and activation of NF-κB, which are dependent on both pathways.

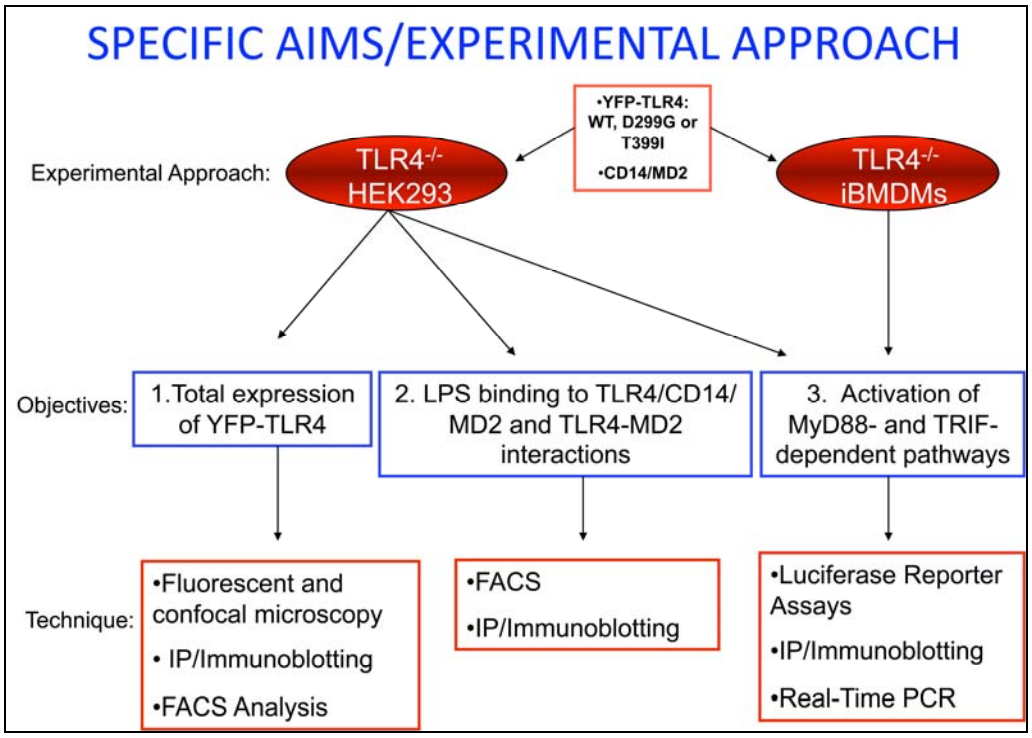

Figure 6 Schematic of specific aims and experimental approaches.

RESEARCH PLAN

OVERALL STRATEGY

My main strategy involved transfection-based complementation of TLR4-deficient cells with expression plasmids previously designed in Dr. Medvedev's laboratory that encode WT or polymorphic YFP-TLR4 variants fused at the C-terminus to yellow fluorescent protein (YFP) (162). Fusion of YFP to C-terminus of TLR4 does not alter receptor cellular localization and expression, and preserves TLR4 signaling functions (143,183). Two model systems were used to test the hypothesis by performing transfection-based complementation with WT or mutant TLR4 species: (i) TLR4-negative HEK293 cells (27,143), to achieve high transfection efficiencies and to facilitate protein-protein interactions studies, and (ii) WT or $TLR4^{-/-}$ immortalized bone marrow-derived macrophage cell lines, to verify findings in cells with the macrophage phenotype.

First, expression vectors encoding WT or polymorphic (D299G and T399I) YFP-TLR4 variants were introduced into $TLR4^{-/-}$ HEK293T (transient transfection) or HEK293 (stable transfection) cells, along with co-transfection of Flag- or hemagglutinin (HA)-tagged MD2- and CD14-expressing plasmids, to impart LPS sensitivity (27). HEK293 cells have high transfection efficiencies (27,184), facilitating studies to examine the impact of the polymorphisms on total protein expression of WT vs. D299G and T399I YFP-TLR4 variants and their interactions with MD2. I used fluorescent and confocal microscopy to examine YFP-TLR4 expression based on YFP fluorescence. These results were confirmed by immunoprecipitation and immunoblot analyses of levels of transfected YFP-TLR4s

using antibody against green fluorescent protein (GFP) that cross-reacts with YFP (143,183,185).

To study LPS binding to the TLR4/CD14/MD2 complex, 293/CD14/MD2 transfectants expressing WT or polymorphic TLR4s or pcDNA3-transfected HEK293 (negative control) cells were treated with biotinylated LPS, and LPS bound to TLR4/CD14/MD2 complex was detected by streptavidin-APC (SA-APC) reagent using FACS, similar to published reports (143,186). To study interactions of YFP-TLR4s with Flag-tagged or HA-tagged MD2, I utilized co-immunoprecipitation and immunoblotting, whereby YFP-TLR4 proteins were immunoprecipitated with anti-GFP antibody, and subjected to immunoblot analyses with anti-Flag or anti-HA antibodies to detect TLR4-associated MD2 proteins.

MyD88 recruitment was analyzed by co-immunoprecipitation based on LPS-inducible association of transfected AU1-MyD88 to WT or D299G YFP-TLR4 variants in 293/YFP-TLR4 transfectants expressing CD14 and HA-MD2. We also used HEK293 cells transfected with plasmids encoding CD14, MD2 and WT or mutant YFP-TLR4 to analyze LPS-inducible activation of kinases and transcription factors involved in MyD88- and TRIF-dependent signaling pathways, and expression of MyD88- and TRIF-dependent cytokines. Activation of TBK-1 and MAP kinases was analyzed by their phosphorylation, which strongly correlates with induction of kinase activity (187-189), based on immunoblot detection of phosphorylated kinase species with the corresponding phosphospecific antibodies. Activation of NF-κB was assessed based on LPS-inducible activation of NF-κB-dependent luciferase reporter (pGL2-endothelial cell-leukocyte adhesion molecule (ELAM), as measured by, NF-

κB-driven induction of luciferase activity. I also used reporter assays to study the effect on D299G and T399I polymorphisms on LPS-induced, TLR4-driven activation of IFN-β promoter-dependent p125 luciferase reporter (121). Stimulation of MyD88-dependent (IL-8, TNF-α) or TRIF-dependent (IFN-β) cytokine genes was examined by real-time quantitative (q) PCR.

Secondly, I studied the impact of the D299G and T399I polymorphisms on TLR4 signaling in cells with the macrophage phenotype, using $TLR4^{+/+}$ and $TLR4^{-/-}$ immortalized mouse bone marrow-derived macrophage (iBMDMs) cell lines (kindly provided by Drs. Douglas Golenbock and Katherine Fitzgerald, UMass Medical School, Worcester, MA) to carry out transfection-based complementation with WT or mutant YFP-TLR4 variants and study LPS-mediated expression of cytokine genes. iBMDMs were originally obtained by oncogene transformation of the respective primary BMDMs (190-193) and show TLR4-inducible responses similar to those observed in primary BMDMs (190-193). As a control for TLR specificity of the observed effects of the D299G and T399I polymorphisms, I used cell stimulation with TLR-independent stimuli, such as TNF-α.

MATERIALS AND METHODS

Reagents

G418 disulfate salt was purchased from Sigma (St. Louis, MO) for selection of stable transfectants, trypsin-EDTA was from Invitrogen (Carlsbad, CA) and used for splitting cells, trypan blue (Mediatech, Inc., Manassas, VA) was used to determine cell viability, and dimethyl sulphoxide (DMSO) (Sigma) was used for making freezing medium to preserve cell lines. Biotinylated LPS from Invivogen (San Diego, CA), and SA-APC from (Biolegend, San Diego, CA) were used for LPS binding experiments; human tumor necrosis factor (TNF)-α (Cell Signaling Technologies, Danvers, MA) was used as a TLR4-independent control. Highly purified, lipoprotein-free *Escherichia coli* K235 lipopolysaccharide (LPS) was kindly provided by Dr. Stephanie Vogel (University of Maryland School of Medicine) for cell stimulation.

Western blot reagents included Laemmli's sample buffer, 2-mercaptoethanol, protein assay reagents A, B and S (all from Bio-Rad, Hercules, CA), 4-20% Tris-Glycine gels (Invitrogen), Immobilon-P transfer membrane-polyvinylidene fluoride (PVDF) (Millipore, Billerica, MA), precision plus protein standards dual color, 10% Tris/Glycine/SDS buffer as a running buffer, 10% Tris/Glycine buffer (all from Bio-Rad) and 20% methanol (Sigma-Aldrich, St. Louis, MO) for making a transfer buffer, 5% blotting grade blocker non-fat dry milk (Bio-Rad) for blocking, Tris-buffered saline (TBS, Mediatech, Inc., Manassas, VA) and 10% Tween 20 (TBST) (Bio-Rad) for washing, ECL Plus Western blot detection system (Amersham, Pittsburg, PA), and stripping buffer (sterile water, 2-Mercapthanol, 10% SDS (Sigma), 0.5 M Tris-

HCl (Quality Biological, Inc., Gaithersburg, MD). The following antibodies (Abs) were used: anti-green fluorescent protein (GFP) Ab (Invitrogen), monoclonal anti-Flag (M2) and M2-horseradish-peroxidase (HRP) (Sigma), anti-active (phosphorylated) and anti-total p38 (Promega, Madison, WI), anti-phospho-IRF3 (Ser 396), anti-total TBK-1 and anti-phospho-TBK1 (Cell Signaling), anti-mouse IgG-HRP, anti-rabbit IgG-HRP Abs (Cell Signaling), anti-TLR4 (H80), anti-β-tubulin (H-235), anti-phospho, anti-total IRF3 (FL-425) and anti-HA (F7) HRP (all from Santa Cruz Biotechnology, Santa Cruz, CA) and AU1 mouse antibody (Covance, Princeton, NJ).

Luciferase assay reagents included passive lysis buffer, luciferase assay substrate/buffer and stop & glo substrate/buffer (dual luciferase reporter assay system, Promega). FACS reagents included non-enzymatic cell dissociation buffer (Mediatech, Inc.), streptavidin- allophycocyanin (SA-APC) (Biolegend), CD56-APC (BD Biosciences, San Diego, CA), and 2% paraformaldehyde (USB, Cleveland, OH). Trizol (Invitrogen), choloroform (J.T. Baker, Phillipsburg, NJ), molecular grade 2-propanol and 75% ethanol (Sigma-Aldrich) were used for RNA extraction. Nuclease-free water (Promega), DNase and 10X DNAse buffer (Promega) were utilized for DNase treatment, reverse transcription system (25 mM $MgCl_2$, 10X RT buffer, 10 mM dNTPs, random primers, RNasin inhibitor, AMV reverse transcriptase) (Promega) were used for cDNA synthesis, and real-time PCR reagents included iQ SYBR green supermix (Bio-Rad). Reagents for confocal microscopy included poly-L-lysine hydrobromide (Sigma) for slide coating, phenol-free Dulbecco's Modification of Eagle's Medium (DMEM) High Glucose 1X (Invitrogen), 2% paraformalydehyde

(Sigma-Aldrich), 0.1 M PBS, 10 mM NaOH and HCl (all from Sigma) for fixing and fluorescent mounting media (made with DABCO anti-fade mounting media, Sigma).

Transformation and isolation of plasmid DNA

The corresponding samples of plasmid DNA (10 ng) were added to 50 µl of DH5α chemically competent cells (Invitrogen) and incubated on ice for 30 minutes (min). The mixture was heat-shocked at 42^{o} C for 30 sec, placed on ice for 2 min and then 950 µl of Luria-Bertani (LB) Broth (Quality Biological, Inc.) was added to transformation reactions. After incubation for 1 hour (h) at 37^{o} C on a shaker (~300 rpm), 30 µl and 270 µl of bacterial suspensions were plated onto LB-ampicillin (AMP, Research Products International, Mt. Prospect, IL) agar plates (LB Agar-USB and Amp-50 µg/ml) and grown overnight at 37^{o} C. Individual colonies were picked and placed into a tube with 3 ml of LB broth containing 50 µg/ml AMP (Research Products International), mixture was incubated for 4 h at 37^{o} C on a shaker (~300 rpm), followed by inoculation into a sterile flask containing 500 ml of LB broth with AMP (50 µg/ml) and incubation for 20 h at 37^{o} C on a shaker (~300 rpm). Bacterial cells were harvested by centrifugation at 6000 x g for 15 min and plasmid DNA was isolated using the Endo-free plasmid maxi-prep kits (Qiagen). Briefly, bacterial pellets were suspended in 10 ml of buffer P1 (suspension buffer with RNAse A) and then 10 ml of buffer P2 (lysis buffer) was added, mixed thoroughly and incubated at room temperature (RT) for 5 min. After incubation, 10 ml of buffer P3 (neutralization buffer) was added to lysates, mixed and poured into QIAfilter Cartridges and incubated at RT for 10 min. Then 2.5 ml of buffer ER (endotoxin removal buffer) was added to filtered cell lysates and incubated on ice for 30 min,

after which lysates were added to a QIAGEN-tip 500 equilibrated with buffer QBT, and allowed to pass by gravity flow. The QIAGEN-tip was washed twice with 30 ml of Buffer QC (wash buffer) and then DNA eluted with 15ml of buffer QN (elution buffer). DNA was precipitated with 10.5 ml 2-isopropanol (Sigma) and centrifuged at 15,000 x g for 30 min at 4^{o} C. Pellets were washed twice with 70% ethanol, centrifuged at 15,000 x g for 10min, air dried and suspended in Tris-EDTA buffer (Qiagen). DNA concentrations were determined using the BioRad SmartSpec Plus spectrophotometer at wavelengths of 260 nm and 280 nm for DNA and protein, respectively.

Restriction Analysis

DNA samples (1 μg) were incubated for 2 h at 37^{o}C with restriction enzyme 1 (20 units), restriction enzyme 2 (20 units), corresponding buffer for double digest (2 μl), bovine serum albumin (BSA) (2 μl), and nuclease-free water (up to 20 μl). Products were then analyzed on a 1% agarose gel (1X TAE-Quality Biological, Inc., ultrapure Agarose-Invitrogen) to confirm the correct insert size. The following restriction enzymes for each plasmid and size of products were used: a) pcDNA3-CD14: Hind III/Xbal I, insert size of 1.1 kb, b) pcDNA3-YFP-TLR4 encoding WT, D299G, T399I, D299G/T399I, and P714H variants: BamH I/Xho I, insert size of 2.5 kb, c) pEFBOS-HA-MD2: Xho I/BamH I, insert size of 535 bp, e) pEFBOS-Flag-MD2: Not I/Xho I, insert size of 500 bp f) pcDNA3-AU1-MyD88: BamHI/ EcoRI, insert size of 920 bp, g) pGL2-ELAM-Luciferase: HindIII/ KpnI, 6.2kb, h) p125-Luciferase: NotI/ HindIII, 560bp.

Cell Culture

Human embryonic kidney (HEK) 293 and HEK293T cells generated by transformation of HEK cells with sheared adenovirus 5 DNA (194) were obtained from ATCC (Manassas, VA). $TLR4^{+/+}$ and $TLR4^{-/-}$ iBMDMs were described previously (190-193) and kindly provided by Drs. Douglas Golenbock and Katherine Fitzgerald (UMass Medical School, Worcester, MA). HEK293 and HEK293T cells were cultured in DMEM (Mediatech) with 4.5g/L glucose containing 1% L-glutamine (Sigma), 10% heat-inactivated fetal bovine serum (Sigma), 1% penicillin/streptomycin and 0.1% Hepes (Mediatech, Inc.) (complete DMEM, cDMEM) at 37° C in a 5% CO_2 incubator. Stably transfected HEK293 cell lines were cultured in cDMEM containing 0.5 mg/ml G418 (Sigma).

Transient and stable transfections

Transient transfections of HEK293T cells were carried out using superfect transfection reagent (QIAGEN, Valencia, CA) according to the manufacturer's protocol and as reported (195). For reporter assays, HEK293 cells were plated in 24-well plates ($2x10^5$ cells/well) in 2 ml of cDMEM and incubated for 20 h at 37^o C in a 5% CO_2 atmosphere. Thereafter, cells were transfected with a mixture of superfect (5 μl/well) and plasmids pcDNA3-CD14 (50 ng), pEFBOS-Flag-MD2 (50 ng), pcDNA3-YFP-TLR4 encoding WT or mutant TLR4 variants (150 ng), pTK-Renilla luciferase (10 ng), pGL2-ELAM-luciferase (300 ng, NF-κB reporter assays) or p125-Luc (300 ng, IFN β promoter reporter assays) and adjusted with pcDNA3 (empty vector) to total amount of 1 μg (all amounts are given per well). For immunoprecipitation and Western blot analyses, HEK293T cells were plated in 100

mm dishes ($5x10^6$ cells) in 5 ml of cDMEM and incubated for 20 h at 37^o C in a 5% CO_2 incubator. Cells were then transfected with a mixture of superfect (60 µl/dish) and plasmids pcDNA3-CD14 (2.0 µg), pEFBOS-MD2 (2.0 µg), pcDNA3-YFP-TLR4 encoding WT or mutant TLR4 variants (6.0 µg) (all plasmid amounts are given as µg/dish). For negative controls, cells were transfected with pcDNA3-CD14 (2.0 µg), pEFBOS-MD2 (2.0 µg) and pCDNA3 (6.0 µg) (control 1) or pcDNA3 (10.0 µg) (control 2). For transfection in 6-well plates, HEK293T cells were plated ($1x10^6$ cells/well) in 2 ml of cDMEM. Cells were transfected with a mixture of superfect (10 µl/well) and plasmids pcDNA3-CD14 (0.5 µg), pEFBOS-MD2 (0.5 µg), pcDNA3-YFP-TLR4 encoding WT or mutant TLR4 variants (1.0 µg) or pcDNA3 (2.0 µg, a negative control). Transfection mixtures, consisting of superfect reagent and corresponding plasmids, were vortexed for 20 s and incubated at RT for 30 min. Fresh cDMEM was added to mixtures and 600 µl/well distributed to cells. Cells were incubated at RT for 3 h in a 5% CO_2 incubator, washed twice with 1X PBS, fresh cDMEM was added (2 ml per well) and cells were recovered for 20 h. After recovery, cells were lysed and extracts used for western blot and immunoprecipitation.

For stable transfection, HEK293 cells were transfected using superfect transfection reagent as specified above. Cells were plated into 75 cm^2 T-flasks and cultured for 48 h in cDMEM containing 1 mg/ml G418, and were subsequently trypsinized and transferred into new flasks. Cells surviving G418 selection were analyzed by fluorescent and confocal microscopy, FACS and immunoblotting to confirm YFP-TLR4 expression. HEK293 stable cell lines expressing WT, D299G or

T399I YFP-TLR4 species were plated in 100 mm TC dishes ($5x10^6$ cells/dish) in cDMEM and incubated for 20 h at 37° C in a 5% CO_2 incubator. Cells were then transfected with a mixture of superfect (60 µl/dish) along with plasmids encoding pcDNA3-AU1-MyD88 (6.0 µg/dish), pcDNA3-CD14 (2.0 µg/dish) and pEFBOS-HA-MD2 (2.0 µg/dish).

iBMDMs were transfected with expression plasmids encoding human WT or polymorphic YFP-TLR4 species, using lipofectamine 2000 (Invitrogen). $TLR4^{-/-}$ or $TLR4^{+/+}$ iBMDMs were plated in 6-well plates ($2x10^6$ cells/well) in 2 ml of cDMEM. For transfection of $TLR4^{-/-}$ iBMDMs, plasmid DNA (4.0 µg/well) was diluted in 50 µl serum-free DMEM and mixed gently. Lipofectamine 2000 (10 µl/well) was diluted in 50 µl of cDMEM without antibiotics and incubated for 5 min at room temperature. DNA was combined with diluted lipofectamine 2000, mixed and incubated for 10 min at room temperature. Mixture was distributed to wells, incubated for 3 h at 37° C in a 5% CO_2 incubator and media was changed after 3-4 h.

Reporter Assays

HEK 293 cells (2×10^5 /well) were plated in 24-well plates and incubated overnight at 37° C in a 5% CO_2 incubator. Stably or transiently transfected HEK293 cells expressing YPF-TLR4 WT, D299G or T399I, along with CD14 and Flag-MD2 were treated for 5 h at 37° C with medium, LPS or TNF-α (100 ng/ml each) in 5% CO_2 atmosphere. After treatment, cells were washed, lysed with passive lysis buffer (Promega), and lysates (5 µl) were collected to measure firefly and Renilla luciferase activities, using the dual luciferase reporter assay system (Promega) on the Lumat LB 9507 luminometer (Berthold Technologies, Bad Wild, Germany). Firefly luciferase

activity was normalized to Renilla luciferase activity, and values in agonist-treated cells were normalized to those detected in medium-treated cells, and expressed as folds induction.

RNA Isolation

After treatment for 3 h with medium, LPS or TNF-α (100 ng/ml each), cells were washed with PBS and incubated with 1 ml Trizol reagent for 5 min at room temperature. Cell lysates were pipetted three times, transferred to Eppendorf tubes, and chloroform (200 µl/sample) was added. Samples were incubated for 3 min at RT and centrifuged for 15 min at 12,000 x g, 4^{o} C. Aqueous supernatant was transferred to fresh Eppendorf tubes and RNA precipitated with 500 µl of 2-isopropanol, incubated for 10 min at RT, and centrifuged (12,000 x g, 4^{o}C, 10 min). The pellet was then washed with 1 ml of 75% ethanol by centrifugation (7,500 x g, 4^{o} C, 10 min), ethanol was carefully removed, the pellet was air-dried for 10-15 min, and suspended in 30-50 µl of nuclease-free water. Samples were incubated for 1 h at 37^{o} C with RNase-free DNase (2 units, Promega), re-purified as specified above, and quantified spectrophotometrically. cDNA was prepared from 1 µg RNA using reverse transcription system (Promega) as follows: samples were incubated at 70^{o} C for 10 min to denature, spun down and 10 µl reverse transcriptase master mix (25 mM $MgCl_2$, 10X RT buffer, 10 mM dNTP mixture, random primers (0.5 µg), RNasin (0.5 µl), AMV RT (15 units) and nuclease-free water up to 20 µl) was added. Samples were treated for 1 h at 42^{o} C, 5min at 95^{o} C and cooled down to 4^{o} C, and adjusted to a volume of 200 µl with nuclease-free water. cDNA (5 µl) was then subjected to real-time qPCR with the corresponding gene-specific primers on a MyIQ Single Color

Real-Time PCR machine (Bio-Rad). PCR amplification was performed using 5 μl of cDNA, 0.3 μM forward and reverse primers, and SYBR green Supermix (Bio-Rad). The following primer pairs were used: human HPRT, forward, 5'-GCTGACCTGCTGGATTACATT-3'; reverse, 5'-GTTGAGAGATCATCTCCACCA; human IL-8, forward, 5'-CACCGGAAGGAACCATCTCACT-3'; reverse, 5'-TGCACCTTCACACAGAGCTGC-3'; human INF-β , forward, 5'-ACTGCCTCAAGGACAGGATG-3, reverse, 5'-AGCCAGGAGGTTCTCAACAA-3'; mouse TNF-α, forward, 5'-CCCAGGCAGTCAGATCATCTTC-3', reverse, 5'-GCTTGAGGGTTTGCTACAACA TG-3'; mouse HPRT, forward, 5'-GCTGACCTGCTGGATTACATT-3', reverse, 5'-GTTGAGAGATCATCTCCACCA-3'. For human IL-8 and HPRT amplification, PCR was carried out for 40 cycles each consisting of the following steps: denaturing for 10 sec at 95° C, annealing for 1 min at 55° C, extension for 30 sec at 72° C. For human IFN-β amplification, PCR was carried out for 40 cycles each consisting of the following steps: denaturing for 10 sec at 95° C, annealing for 1 min at 56° C, extension for 30 sec at 72° C. For mouse TNF-α, IFN-β and HPRT amplification, PCR was carried out for 40 cycles each consisting of the following steps: denaturing for 10 sec at 95° C, annealing for 1 min at 55° C, extension for 30 sec at 72° C. Melting curve analysis was carried out to ensure specific amplification of the desired mRNA, as recommended by the manufacturer. Real-time PCR data were processed using $2^{-\Delta\Delta CT}$ method to calculate relative changes in gene expression (196,197), using the following equation: [delta][delta]C_t = [delta]$C_{t,sample}$ - [delta]$C_{t,reference}$. (196,198).

[delta]$C_{t,sample}$ is the value for any sample normalized to the endogenous gene, while [delta]$C_{t,reference}$ is the value of the control values also normalized by the endogenous genes (198). This method involves comparing the targets *vs* control values, in which both are normalized to a suitable housekeeping gene. Amplification efficiencies of normalized targets and controls must be equal to use this method (198).

LPS Binding

HEK293T cells were detached using non-enzymatic cell dissociation buffer (0.5 ml/well of 6-well plates) and 1 x10^6 cells were treated for 30 min with medium or 1 μg/ml of biotin-LPS at 37^o C in 5% CO_2 atmosphere, with agitation. Cells were washed with PBS and incubated with or without 1 μg/ml SA-APC on ice for 30 min, with agitation every 5 min. After incubation, cells were re-washed 3 times with ice-cold PBS, and fixed for 15 min in 2% paraformaldehyde. HEK293T gated cells assessed for YFP (excitation and emission wavelengths: 495 nm and 515 nm) and APC (650 nm and 660 nm) were acquired using a BD LSR II Flow Cytometer (BD Biosciences). All samples were analyzed using Flow Jo Flow Cytometry Analysis Software (Tree Star, Inc., Ashland, OR).

Preparation of cell extracts and Western blot analyses

After treatment, cells were washed twice with ice-cold PBS, gently scraped, centrifuged (1400 x g, 10 min, 4^o C), and suspended with ice-cold lysis buffer containing 50 mM Tris-HCl (Quality Biological, Inc.), 1 mM phenylmethylsufonyl fluoride (PMSF, Sigma), 1 mM dithiothreitol (DTT), 1 mM sodium orthovanadate (Sigma), 50 mM sodium fluoride (NaF, Sigma), 2 mM EDTA (USB), 150 mM sodium chloride (NaCl, Promega), 1% Triton X-100 (Sigma), 20 mM β-glycerol

phosphate (Sigma) and protease inhibitor cocktail (Roche). Samples were rotated for 30 min at 4° C, centrifuged 14,000 x g, 4° C, 20 min) and protein concentration determined using Coomassie protein assay reagents (Bio-Rad). Twenty micrograms of samples were re-suspended in Laemmli's sample buffer (2X) containing 2-mercaptoethanol (100mM), loaded onto 4-20% polyacrylamide gels (Invitrogen) and separated for 1.5-2.0 h at 150 volts. Proteins were electro-transferred onto Immunobilon-P PDVF membranes (Millipore), incubated with TBST- 5% non-fat milk (Bio-Rad) to block non-specific binding and washed three times. Membranes were incubated overnight at 4° C with the respective primary antibodies diluted in TBST-5% non-fat milk, washed three times and incubated for 1 h at room temperature with secondary HRP- conjugated anti-mouse or anti-rabbit antibodies. Protein bands were visualized after incubation with the ECL plus Western blot detection reagents (Amersham) for chemiluminescent detection.

Immunoprecipitation

Cell lysates (0.5-1 mg total protein) were incubated for 2 h at 4° C with 20 µl of 50% slurry of protein G-agarose beads (Roche Diagnostics) to reduce co-immunoprecipitation of non-specific proteins. Pre-cleared cell lysates were incubated for 20 h at 4° C with α-GFP Ab (YFP-TLR4 interactions with MD2, MyD88) or α-AU1 Ab (MyD88 interactions with YFP-TLR4) (2 µg per sample each). Protein G-agarose beads were added (45 µl of 50% slurry /sample) and incubated for 4-6 h at 4° C, followed by washing 5 times with ice-cold lysis buffer containing 0.5% Triton X-100 by centrifugation (8000 x g, 4° C, 0.5 sec). Beads were suspended in 25 µl of

Laemmli sample buffer (Bio-Rad), boiled for 10 min, cooled down for 2 min, centrifuged and supernatants examined by immunoblotting.

Confocal Microscopy

Coverslips (22 x 22 mm, Fischer Scientific, Suwanee, GA) were coated with poly-L-lysine (0.1 mg/ml) for 30 min at RT, washed with PBS and sterilized for 30 min under UV light. Coverslips were placed in 6-well plates, cells plated (4×10^5 cells/well) on top of coverslips in 2 ml phenol-free cDMEM and cultured for 20 h at 37^o C in 5% CO_2 incubator. Transient or stable transfection of cells was performed using superfect transfection reagent, followed by gentle washing and then recovery for 48 h. Cells were fixed with 2% paraformaldehyde (pH 7.4), and mounted to glass slides using a DABCO-based anti-fade fluorescent mounting media. Images were acquired using an Olympus Fluoview 500 laser scanning confocal microscope (Olympus, Center Valley, PA) at excitation wavelength 515 nm and standard emission filters for YFP detection (YFP-TLR4).

RESULTS

Specific Aim 1: Elucidate the effect of the D299G and T399I polymorphisms on TLR4 expression

The working hypothesis for this Aim was that since the magnitude of LPS responses depends on TLR4 expression (180,181), the D299G and T399I polymorphisms could reduce TLR4 protein levels by affecting protein conformation and/or receptor trafficking, resulting in inhibited LPS signaling. The approach to test this hypothesis included transfection-based complementation of TLR4-deficient HEK293 cells (114,143,144,199) with WT or mutant YFP-TLR4 species, along with CD14 and Flag- MD2, and subsequent analyses of total TLR4 expression using immunofluorescence, confocal imaging, and FACS analyses. Expression levels of YFP-TLR4 proteins were determined based on fluorescence intensities of the C-terminal YFP fusion tag (exicitation and emission wavelengths of 495nm and 515 nm) and its immunoreactivity with anti-GFP antibody (cross-reacting with YFP, (143,183,185). C-terminally fused YFP does not affect TLR4 localization, trafficking and functional capabilities (143,183) and allows reliable detection of transfected proteins (143,183). **Figure 7 A** demonstrates comparable fluorescence intensities of WT, D299G and T399I YFP-TLR4 species in 293/CD14/MD2/TLR4 stable transfectants, whereas cells transfected with pcDNA3 empty vector did not produce fluorescent signals. FACS analysis showed 67.5, 61.4 and 67.7 median fluorescence intensity (MFI) values for 293T/CD14/MD2 cells transfected with WT, D299G and T399I YFP-TLR4s, respectively (**Figure 7B**), compared to 52.9 MFI observed in 293T cells transfected with pCDNA3. Similarly, 293/CD14/MD2 cells stably

expressing WT, D299G and T399I YFP-TLR4 species produced 87.4, 95.0 and 76.7 median fluorescence intensity (MFI) values, respectively, (**Figure 7C**) compared to 33.8 MFI observed for pCDNA3-transfected cells. Confocal imaging also showed comparable YFP fluorescence intensities of HEK293/YFP-TLR4 stable cell lines expressing WT, D299G or T399I YFP-TLR4s and co-transfected with vectors encoding CD14 and MD2 , whereas no YFP fluorescence was seen in cells transfected with pcDNA3 (**Figure 7D**). I observed similar fluorescence intensities of WT, D299G and T399I YFP-TLR4 localized in the plasma membrane and the intracellular membrane compartment (**Figure 7D**), likely to represent the Golgi and endoplasmic reticulum, as reported (142,143), indicating the the polymorphisms do not affect receptor cellular distribution.

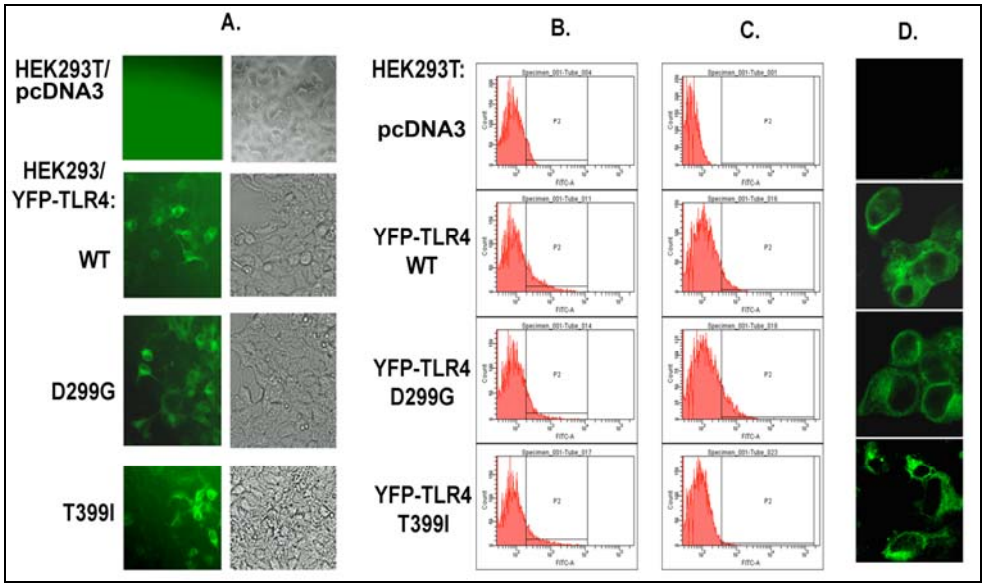

Figure 7 TLR4 D299G and T399I polymorphisms do not affect TLR4 total expression levels. HEK293 cells transiently (B) or stably (A, C or D) transfected with pcDNA3-YFP-TLR4 expression vectors encoding WT, D299G or T399I YFP-TLR4 and co-transfected with pcDNA3-CD14 and pEFBOS-Flag-MD2. HEK293T cells transiently transfected with pcDNA3 (empty vector) were used as control cells. After recovery, TLR4 expression was measured by A) fluorescent microscopy, B) FACS analysis and C) confocal microscopy. The results of a representative experiment are depicted: A) n=5, B) n=5 and C) n= 4.

Secondly, I employed immunoblotting to determine expression of YFP-TLR4 species and Flag-MD2 proteins in cell lysates from HEK293T cells transiently transfected with pcDNA3-CD14, pEFBOS-Flag-MD2 and pcDNA3-YFP-TLR4 vectors encoding WT vs. D299G species, using anti-GFP and anti-Flag antibodies. **Figure 8A** demonstrates comparable intensity of WT and D299G YFP-TLR4 bands immunoreactive with anti-GFP Ab (3rd and 4th bands from the left, top panel), as evidenced by densitometric quantification of these bands (0.74 and 0.78 arbitrary protein expression values, **Figure 8B**).

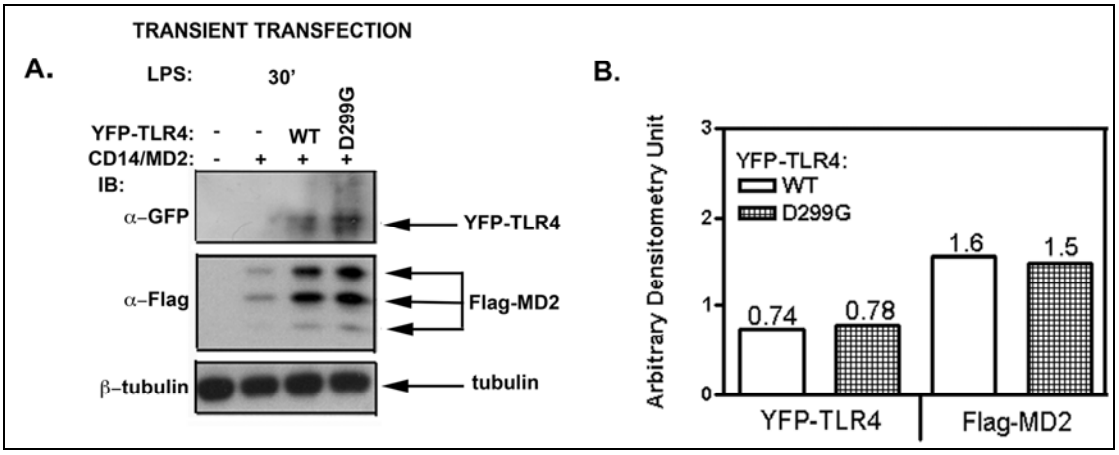

Figure 8 Immunoblot analysis of YFP-TLR4 and MD2 expression in 293T/CD14/MD2 cells overexpressing WT or D299G YFP-TLR4s. A**)** HEK293T cells were transiently co-transfected with pEFBOS-Flag-MD2, pcDNA3-CD14, along with pcDNA3-YFP-TLR4 vectors encoding WT or D299G species. Control cell cultures were transfected with pcDNA3 only or with pcDNA3-CD14 only. Whole cell lysates were prepared and analyzed by immunoblotting with anti-GFP (YFP-TLR4), anti-Flag (Flag-MD2) and anti-β-tubulin (protein loading) antibodies. B) Quantification of the results shown in (A). Shown are data of a representative (n=4) experiment.

Comparable intensities of Flag-MD2 protein bands immunoreactive with anti-Flag Ab were observed in cells expressing WT or D299G YFP-TLR4 variant, in which their densitometric quantification yeilded 1.6 vs. 1.5 arbitrary protein expression values (**Figure 8B**). No bands immunoreactive with either anti-GFP antibody were

seen in cell lysates prepared from control, pcDNA3- or pcDNA3-CD14/pEFBOS-Flag-MD2- transfected cells (**Figure 8A**, 1st and 2nd bands from the left, top panels), and cells transfected with pcDNA3 exhibited no anti-Flag-immunoreactive bands, indicating specific detection of TLR4 and MD2 proteins. Comparable levels of tubulin were observed in all samples, indicating equal protein loading (**Figure 8A**, bottom panel). Taken collectively, these results indicate that the D299G and T399I polymorphisms do not affect total TLR4 expression or receptor localization.

Specific Aim 2: Define whether the D299G and T399I polymorphisms affect LPS binding to the TLR4/CD14/MD2 receptor complex and TLR4-MD2 interactions

The working hypothesis for this Aim was that since the D299G and T399I polymorphisms are located in the TLR4 ectodomain (117) involved in ligand sensing and co-receptor interactions (53,69,72,97), these mutations could affect LPS binding to the TLR4/CD14/MD2 complex or TLR4-MD2 assembly. To test if the polymorphisms affect LPS binding, 293/CD14/MD2 transfectants expressing WT or mutant YFP-TLR4s were incubated with medium or biotinylated LPS, washed, and bound LPS was quantified by staining with SA-APC followed by FACS analyses. FACS was performed, using filter settings for YFP, to determine TLR4 expression levels, and for APC, to measure LPS binding[12]. As shown in **Figure 9 A and B**, analyses of APC fluorecence in 293T/CD14/MD2 transient transfectants complemented with WT, D299G or T399I YFP-TLR4 yielded 303, 239 and 203 MFI values, vs. 163 MFI seen in HEK293T cells transfected with pcDNA3.

[12] Excitation/emission wavelengths: YFP-495/515 nm and APC:650/660nm. (BD Biosciences)

Quantification of YFP-TLR4 expression revealed 72.2, 63.7 and 70.2 MFI values in 293/CD14/MD2 cells expressing WT, D299G and T399I species, respectively, whereas pcDNA3-transfected cells exhibited 52.9 MFI value.

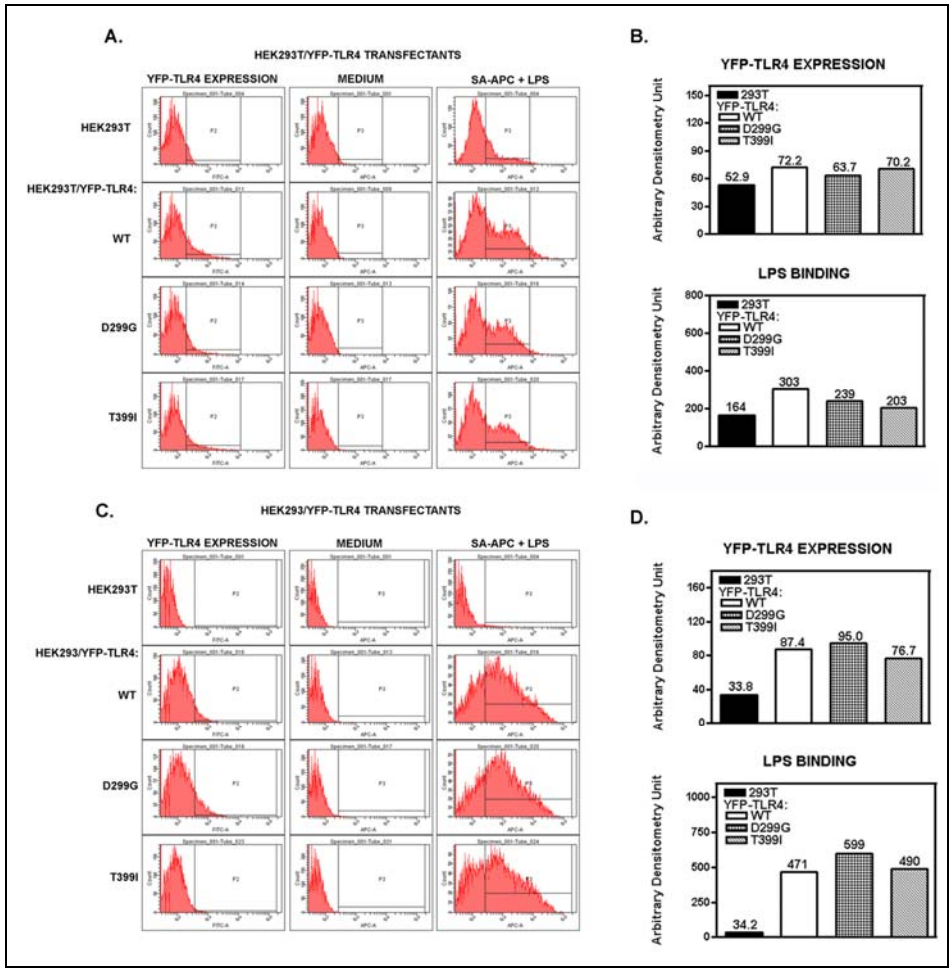

Figure 9 TLR4 polymorphisms do not affect LPS binding to TLR4/CD14/MD2. A) HEK293T cells transiently transfected with the respective plasmids encoding WT, D299G or T399I YFP-TLR4s, along with vectors encoding CD14/Flag-MD2 were incubated for 30 min at 37^{o} C with medium or biotinylated LPS (1µg/ml). After washing, cells were stained with SA-APC (1µg/ml), and analzyed by FACS, to detect LPS binding, based on APC fluorescence, and TLR4 expression, based on YFP fluorescence (APC: 650/660 nm and FITC-495/515 nm). Shown are the results of a representative (n=3) experiment. B) Quantification of results shown in (A). C) HEK293 cells stably expressing WT, D299G or T399I were transiently transfected with CD14 and Flag-MD2 and incubated for 30 min at 37^{o} C with medium or biotinylated LPS (1µg/ml). After washing, cells were stained with SA-APC (1µg/ml), and analyzed by FACS, to detect LPS binding (APC fluorescence) and TLR4 expression (YFP fluorescence). Shown are the results of a representative (n=2) experiment. D) Quantification of results shown in (C).

FACS analyses of APC fluorescence in the stable HEK293/YFP-TLR4 cell lines expressing WT or mutant TLR4s and transiently transfected with CD14/Flag-MD2 revealed 471, 599 and 490 MFI values for WT, D299G and T399I YFP-TLR4s, respectively, significantly exceeding 34.2 MFI observed in pCDNA3-transfected cells (**Figure 9C and D**). Quantification of YFP-TLR4 expression revealed 87.4, 95.0 and 76.7 MFI values in 293/CD14/MD2 cells expressing WT, D299G and T399I YFP-TLR4s, respectively, whereas control, pcDNA-transfected HEK293 cells showed 33.8 MFI (**Figure 9C and D**). Collectively, these results demonstrate comparable LPS binding to 293T/CD14/MD2 and 293/CD14/MD2 cells expressing similar amounts of WT, D299G or T399I YFP-TLR4 species, indicating that the D299G and T399I polymorphisms do not affect LPS binding to the TLR4/MD2/CD14 receptor complex.

My second approach involved studies to determine whether the D299G polymorphism affects TLR4-MD2 interactions. To this end, cell lysates prepared from 293T/CD14/MD2 transfectants complemented with WT or D299G YFP-TLR4s were immunoprecipitated with anti-GFP antibody to pull down YFP-TLR4s, and the amount of TLR4-interacting Flag-MD2 was determined by immunoblotting of GFP immune complexes with anti-Flag antibody. Immunoblot analyses of immunoprecipitated YFP-TLR4 proteins obtained from 293T/CD14/Flag-MD2 cells complemented with WT or D299G TLR4s revealed comparable intensities of protein bands immunoreactive with anti-GFP and anti-Flag antibodies (**Figure 10**, 3rd and 4th bands, two panels at the top). These data indicate similar amounts of Flag-MD2 interacting with WT or D299G YFP-TLR4 variants under conditions of similar TLR4 expression. No bands immunoreactive with anti-GFP antibody were seen in lysates

from cells not transfected with YFP-TLR4s, and no anti-Flag-immunoreactive bands were observed in cells lacking transfected Flag-MD2, indicating the specificity of immunoblot detection (**Figure 10,** 1st and 2nd bands, two panels at the top). Whole cell lysates obtained from 293T/CD14/Flag-MD2 cells complemented with WT or D299G YFP-TLR4 variant showed comparable intensities of bands immunoreactive with anti-Flag antibody, indicating equal total expression levels of Flag-MD2 proteins (**Figure 10**, 2nd panel from bottom). Similar levels of β-tubulin were detected in cell lysates prepared from 293/CD14/MD2 cells complemented with WT or mutant YFP-TLR4s, indicating equal protein loading (**Figure 10**, bottom panel). Thus, the D299G polymorphism does not significantly affect TLR4-MD2 interactions in unstimulated cells.

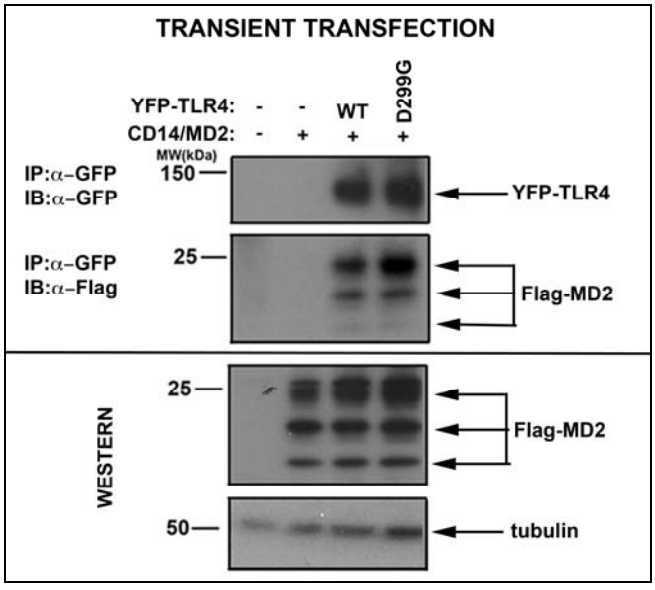

Figure 10 D299G polymorphism does not alter TLR4 interactions with MD2 under basal conditions. HEK293T cells were transiently transftected with vectors encoding WT or D299G YFP-TLR4 species, along with pcDNA3-CD14/pEFBOS-Flag-MD2. After recovery, cell lysates were prepared and immunoprecipitated with anti-GFP antibody (YFP-TLR4 pull-down), followed by immunoblot analyses with anti-Flag antibody (to determine Flag-MD2 interactions with YFP-TLR4s). Whole cell lysates were analyzed by immunoblotting, using antibodies against anti-Flag (Flag-MD2 detection) and β-tubulin (loading control). The data of a representative experiment (n=2) are shown.

Next, I sought to determine the impact of the D299G mutation on TLR4-MD2 interactions under conditions of LPS stimulation. Immunoblot analyses of immunoprecipitated YFP-TLR4 proteins obtained from 293T/CD14/Flag-MD2 cells overexpressing WT or D299G YFP-TLR4s revealed no decreases in the amount of Flag-MD2 proteins interacting with the mutant TLR4 variant (**Figure 11**, 3rd and 4th bands, top panel), under conditions of equal total Flag-MD2 expression (2nd panel from the top), even though in this particulate experiment, total D299G levels were somewhat lower compared to WT TLR4 variant. Immunoblot analyses of lysates from cells not transfected with YFP-TLR4s or Flag-MD2 showed no anti-GFP- or anti-Flag-reactive bands , indicating specific detection of the respective proteins (**Figure 11,** 1st and 2nd bands, two panels at the top).

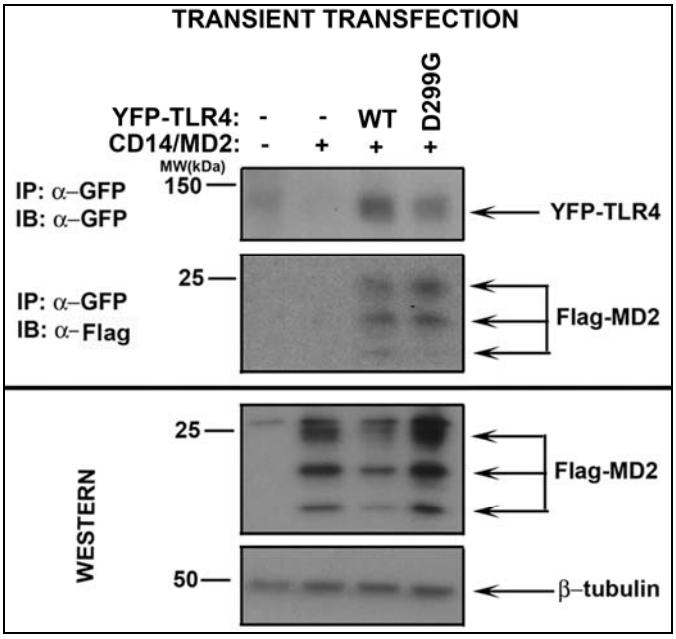

Figure 11 D299G polymorphism does not alter TLR4-MD2 interactions upon LPS treatment. HEK293T cells transiently transftected with vectors encoding WT or D299G YFP-TLR4 species, along with pcDNA3-CD14/pEFBOS-Flag-MD2, were treated with LPS (100 ng/ml) for 30 min. Cell lysates were immunoprecipitated with anti-GFP Ab, followed by immunoblot analyses with anti-Flag Ab. Whole cell lysates were analyzed by immunoblotting, using antibodies against Flag (Flag-MD2 detection) and β-tubulin (loading control). The data of a representative experiment (n=2) are shown.

Overall, our data indicate that the D299G and T399I polymorphisms do not affect LPS binding to the TLR4/CD14/MD2 receptor complex and that the D299G mutation does not alter TLR4 interactions with MD2 under basal conditions and upon LPS stimulation.

Specific Aim 3: Determine the impact of the D299G and T399I polymorphisms on LPS-induced, TLR4-elicited activation of the MyD88- and TRIF-dependent signaling pathways

The goal of this study was to determine how the D299G and T399I polymorphisms affect LPS-mediated, TLR4-elicited activation of the MyD88- and TRIF-dependent signaling pathways. The working hypothesis for Aim 3 was that these polymorphisms affect dimerization/assembly of the docking platforms within the TIR domain, resulting in impaired LPS-inducible recruitment of adapter proteins and kinases to the TLR4 signalosome and their deficient activation, leading to compromised activation of the MyD88- and TRIF-dependent signaling pathways. TLR4 uses both the MyD88- and TRIF-dependent signaling pathways (69,200,201), which control production of pro-inflammatory cytokines and type I IFNs (30,97), respectively. In order to examine LPS-inducible activation of MyD88- and TRIF-dependent pathways, several approches were used. To analyze MyD88-dependent signaling events, I studied LPS-mediated MyD88 recruitment to TLR4 and expression of MyD88-dependent IL-8 and TNF-α genes (1,97), whereas TRIF-dependent signaling outputs included phosphorylation of TBK1 and IRF3, activation of IFN-β promoter-driven luciferase reporter (p125-luciferase) (121), and expression of IFN-β mRNA (120). In addition, activation of signaling molecules as a result of

concomitant engagement of MyD88- and TRIF-dependent pathways was studied, including phosphorylation of p38 MAP kinase and induction of NF-κB-dependent pGL2-ELAM-luciferase reporter (27,202).

The following cell lines were used to carry out experiments: 1) HEK293T cells transiently transfected with pcDNA3-CD14/pEFBOS-Flag- or HA-MD2, along with expression vectors encoding WT or mutant YFP-TLR4 variants; 2) HEK293 cells stably expressing WT, D299G or T399I YFP-TLR4s and transiently transfected with vectors encoding CD14 and Flag- or HA-MD2; and 3) immortalized mouse iBMDMs obtained from WT mice ($TLR4^{+/+}$) or $TLR4^{-/-}$ mice to carry out transfection-based complementation with human WT or D299G YFP-TLR4 species.

3.1. Determine the impact of the D299G and T399I polymorphisms on LPS-inducible, TLR4-elicited expression of MyD88-dependent IL-8 and TNF-α genes

IL-8 and TNF-α gene expression is controlled via TLR4-mediated activation of the MyD88-dependent signaling pathway (97,99,203). To gain insights into how TLR4 SNPs affect LPS-mediated activation of the MyD88-dependent pathway, I first assessed LPS-mediated transcription of IL-8 gene in TLR4-deficient HEK293 cells complemented with CD14/MD2 co-receptors and WT or mutant YFP-TLR4s by transient or stable transfection. LPS stimulation for 3 h resulted in a 7.5-fold induction of IL-8 mRNA in 293T/CD14/MD2 cells stably transfected with WT YFP-TLR4, whereas overexpression of the D299G or T399I YFP-TLR4 species led to a significant downregulation in LPS-mediated IL-8 mRNA inducibility, accounting for 29% and 83% inhibition (**Figure 12A**). Likewise, 293T/CD14/MD2 cells transiently

transfected with D299G or T399I YFP-TLR4 showed a decrease in LPS-mediated induction of IL-8 gene expression compared to the robust response observed in WT YFP-TLR4-transfected cells , albeit the differences did not reach the level of statistical significance (data not shown).

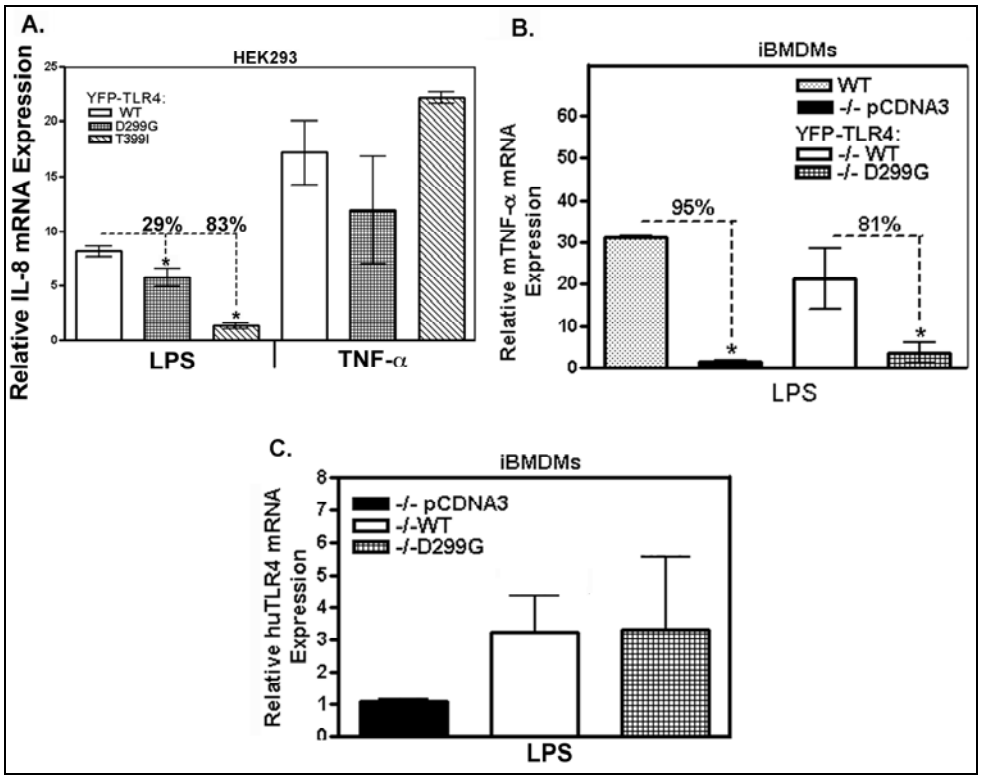

Figure 12 The impact of the D299G and T399I TLR4 polymorphisms on LPS-induced expression of MyD88-dependent genes. A) HEK293 cells stably expressing WT, D299G or T399I YFP-TLR4s were transeintly co-transfected with pcDNA3-CD14/pEFBOS-Flag-MD2. B) $TLR4^{+/+}$ iBMDMs were left untransfected ($TLR4^{+/+}$ cells) and $TLR4^{-/-}$ iBMDMs were transfected as shown. Cells were stimulated for 3 h with 100 ng/ml LPS, RNA was isolated, reverse-transcribed, and analyzed by real-time qPCR with primers specific for human HPRT and IL-8 (A) or mouse HPRT and TNF-α (B). C) Real-time qPCR quantification of TLR4 mRNA levels in iBMDMs complemented with WT or D299G YFP-TLR4s or transfected with pcDNA3 (a negative control). The results of a representative experiment (A; n=2, B; n=1 and C; n= 1 are depicted:; *$p<0.05$, ANOVA

To confirm the results in cells with the macrophage phenoptype, WT or TLR4$^{-/-}$ iBMDMs were employed. Consistent with the literature (204), LPS failed to up-regulate TNF-α mRNA in TLR4$^{-/-}$ iBMDMs, whereas WT iBMDMs expressing endogenous mouse TLR4 responded to LPS with a 30-fold increase in activation of TNF-α mRNA (**Figure 12B**). Transfection of TLR4$^{-/-}$ iBMDMs with expresison vector encoding human WT YFP-TLR4 resulted in ~20- fold induction of TNF-α mRNA, restoring the response to a 79% level compared to that seen in TLR4$^{+/+}$ iBMDMs. In contrast, overexpression of human D299G YFP-TLR4 in TLR4$^{-/-}$ iBMDMs led to only a 3.4-fold induction of TNF-α mRNA in response to LPS (**Figure 12B**). To determine transfection efficiencies, I used real-time qPCR quantification of human TLR4 mRNA in TLR4$^{-/-}$ iBMDMs complemented with human WT vs. D299G YFP-TLR4 variants. These results showed 3.2- and 3.3-fold increases in the expression levels of human TLR4 mRNA, respectively, as normalized to values obtained in iBMDMs complemented with pcDNA3 (**Figure 12C**), indicating similar expression levels of WT vs. mutant transfected TLR4 species. Taken collectively, these data demonstrate a significantly compromised capacity of the D299G TLR4 variant (81% inhibition compared to WT TLR4) to elicit LPS-mediated expression of TNF-α mRNA in iBMDMs.

3.2. Define if the D299G polymorphism affects recruitment of MyD88 to TLR4

TLR4-elicited activation of the MyD88-dependent pathway involves MyD88 adapter-like (MAL)-assisted recruitment of the signaling adapter protein MyD88 to the TLR4 TIR domains as one of the earliest events (97,123,205). To determine whether the D299G TLR4 polymorphisms affects MyD88 recruitment to TLR4, I

studied LPS-inducible association of transfected AU1-MyD88 with WT or D299G YFP-TLR4 variants in 293/YFP-TLR4 stable cell lines expressing the respective TLR4s and co-transfected with pcDNA3-CD14, pEFBOS-HA-MD2, and pcDNA3-AU1- MyD88, using co-immunoprecipitation. After treatment with medium or LPS, YFP-TLR4 proteins were immunoprecipitated from cell lysates with anti-GFP antibody, and the amount of recruited AU1-MyD88 was determined by Werstern blot analyses of TLR4 immune complexes with anti-AU1 antibody. **Figure 13A** demonstrates that LPS induced a robust recruitment of AU1-MyD88 to WT-YFP-TLR4 at 1 min post LPS stimulation (**Figure 13A**, 2nd from top panel, 5th lane from the left), whereas very little amounts of AU1-MyD88 was associated with the D299G YFP-TLR4 variant, as compared to control (**Figure 13B**). Immunoblot analyses of whole cell lysates with anti-HA and anti-AU1 antibodies revealed comparable expression levels of total HA-MD2 proteins (**Figure 13A**, 3rd panel from the top), while higher intensities of anti-AU1 immunoreactive bands were detected in samples obtained from D299G YFP-TLR4-expressing cells compared to those prepared from WT YFP-TLR4-expressing cells (**Figure 13**, 4th panel from the top, 4th and 6th lanes from the left). These data indicate that lower LPS-inducible AU1-MyD88 recruitment to D299G YFP-TLR4 cannot be accounted by lower total levels of interacting proteins. Overall, these results demonstrate that the presence of the D299G polymorphism impairs the ability of TLR4 to recruit MyD88 in response to LPS stimulation.

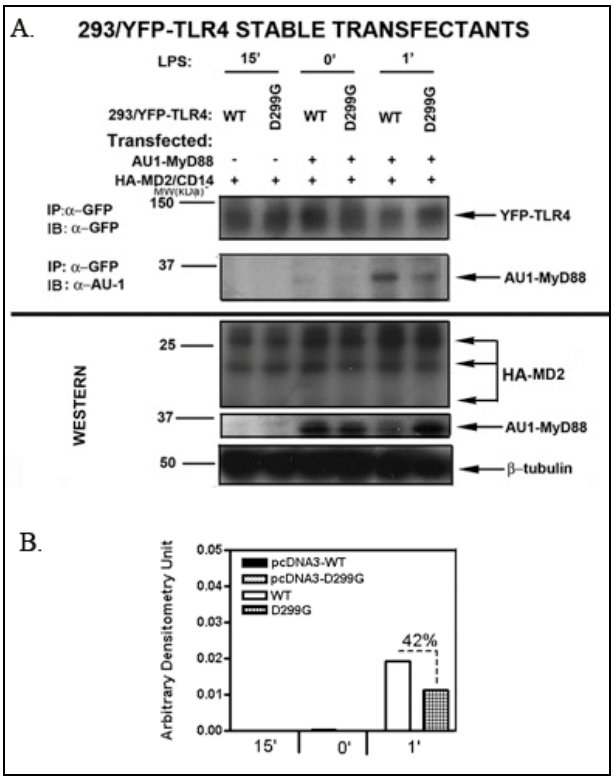

Figure 13 YFP-TLR4 D299G impaired recruitment of MyD88 to TLR4. A) HEK293 cells stably expressing YFP-TLR4 WT or D299G were transiently co-transfected with pcDNA3-CD14, pEFBOS-HA-MD2, and pcDNA3-AU1- MyD88. After recovery, cells were treated with medium or 100 ng/ml LPS for 0 and 1 min at 37^{o} C, and cell lystaes were prepared. YFP-TLR4 proteins were immunoprecipitated with anti-GFP Ab and analyzed by immunoblotting with anti-GFP anti-AU1 Ab. Whole cell lysates were examined by immunoblotting, using HA-HRP (HA-MD2), anti-AU1 (AU1-MyD88) and anti-β-tubulin antibodies. B) Quantification for results shown in (A). The results of a representative experiment (n= 2) are depicted.

3.3. Determine the impact of the D299G and T399I TLR4 polymorphisms on LPS-inducible activation of the TRIF-dependent signaling pathway

The TLR4-elicited, TRIF-dependent signaling pathway involves phosphorylation and activation of TBK1 that subsequently phosphorylates and activates IRF3, resulting in its nuclear translocation and activation of TRIF-dependent genes, such as IFN-β (97,99,132). To determine the impact of the D299G and T399I polymorphisms on TRIF-dependent signaling, I examined the following outputs: 1)

LPS-inducible phosphorylation of TBK1 and IRF3; 2) induction of IFN-β promoter-driven luciferase reporter, p125-luciferase, and 3) levels of IFN-β mRNA.

LPS-mediated, TLR4-driven phosphorylation of TBK1 and IRF3 was determined in 293T/CD14/MD2 cells transiently transfected with WT or D299G TLR4s, as well as in 293/YFP-TLR4 stable cell lines expressing WT or D299G YFP-TLR4s and co-transfected with vectors encoding CD14/Flag-MD2. Immunoblot analyses revealed that LPS stimulation causes the appearance of bands immunoreactive with anti-phospho-TBK1 and anti-phospho-IRF3 antibodies in cell extracts of 293T/CD14/MD2 cells transfected with WT YFP-TLR4, whereas significantly lower levels of these bands were seen in D299G YFP-TLR4-expressing cells after LPS stimulation (**Figure 14A**, 3rd panel from the top, and **Figure 14B**, 2nd panel from the top). Comparable expression levels of total TBK1 and IRF3 were seen in all samples, indicating that differences in phosphorylation statuses of these intermediates are not due to variations of their expression levels (**Figure 14A and B**, 4th and 3rd panels from the top, respectively). Total levels of WT and D299G YFP-TLR4 species and Flag-MD2 proteins were also comparable (**Figure 14A and B**, 1st and 2nd (A) panels from the top), indicating that defective phosphorylation of TBK1 and IRF3 mediated by D299G YPF-TLR4/MD2 complex cannot be attributed to variations in total protein levels of these receptor components.

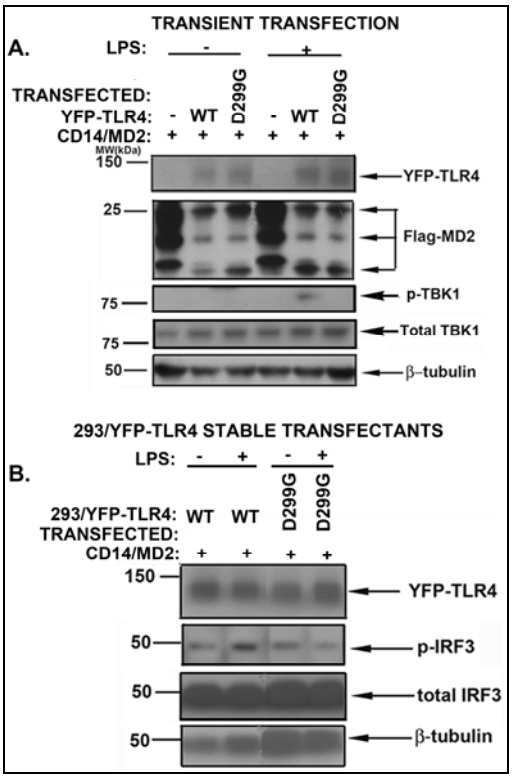

Figure 14 D299G YFP-TLR4 mediates deficient LPS-inducible phosphorylaiton of TBK1 and IRF3. **A**) HEK293T cells transiently transfected with plasmids encoding YFP-TLR4 WT or D299G, along with CD14 and Flag-MD2. After recovery, cells were treated with 1μg/ml LPS for 30 min and lysates were analyzed by immunoblotting with anti-GFP (YFP-TLR4), anti-Flag (Flag-MD2), anti-p-TBK1 and anti-TBK1 antibodies. **B**) HEK293 cells stably expressing YFP-TLR4 WT or D299G were transiently transfected with pcDNA3-CD14 and pEFBOS-HA-MD2. After recovery, cells were treated for with 1μg/ml LPS for 1 min and cell lysates were prepared and analyzed by immunoblotting with antibodies against GFP (YFP-TLR4), p-IRF3 and IRF3. Shown are the data of a representative experiment.

To determine the impact of TLR4 SNPs on IRF3 transactivation, I studied LPS-mediated activation of TRIF-controlled, IRF3-dependent IFN-beta promoter-driven luciferase reporter (p125-Luc) gene expression (203,206). LPS treatemnt of 293T/CD14/MD2 cells expressing WT YFP-TLR4 led to 4.1-fold induction of p125-Luc, whereas 1.4 and 1.1-folds activation were seen in cells expressing D299G or T399I YFP-TLR4 polymorphs, respectively (**Figure 14A**). Both D299G and T399I TLR4 polymorphs significantly lost their ability to activate IFN-β-promoter reporter,

accounting for 65% and 73% inhibition of this response compared to that observed in cells expressing WT YFP-TLR4 (**Figure 15A**). As a control, I used TLR-independent cell stimulation with TNF-α that resulted in 3-, 4-, and 3-fold induction of p125-Luc in 293T/CD14/MD2 cells expressing WT, D299G and T399I YFP-TLR4s, respectively (**Figure 15 A**). These results indicate that the D299G and T399I TLR4 polymorphs are incapacitated to elicit LPS-induced activation of IFN-β promoter-driven luciferase reporter, without affecting TLR-independent cell responses to TNF-α.

Along with transactivation studies, TLR4-inducible induction of IFN-β mRNA was studied in HEK293 cells stably expressing WT, D299G or T399I YFP-TLR4s. LPS stimulation of HEK293 cells led to a 2-fold induction in WT YFP-TLR4, while cells expressing D299G or T399I YFP-TLR4 led to a 1.2- and 1-fold induction, respectively (**Figure 15B**). Thus, the D299G and T399I TLR4 polymorphs are compromized in their abilities to mediate LPS-inducible IFN-β gene expression, accounting for 43% and 32% inhibition of this response compared to WT YFP-TLR4.

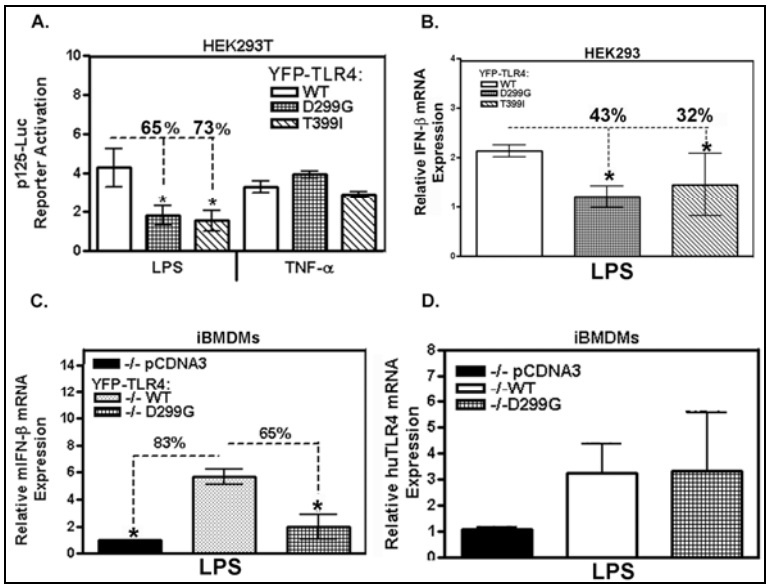

Figure 15 The effect of the D299G and T399I TLR4 polymorphisms on LPS-mediated activation of p125-Luc reporter (A) and induction of IFN-β mRNA (B and C). A) HEK293T cells were transiently transfected with WT, D299G or T399I YFP-TLR4 species, along with pcDNA3-CD14 and pEFBOS-MD2. In addition, cells were co-transfected with p125-Luc and pTK-RL vectors. After recovery, cells were stimulated for 5 h with LPS or TNF-α (TLR4-independent control) (100 ng/ml each). Cell lysates were analyzed for firefly and Renilla luciferase activities. B) HEK293 cells stably expressing WT, D299G or T399I YFP-TLR4 species were transiently transfected with pcDNA3-CD14 and pEFBOS-MD2. C) iBMDMs $TLR4^{+/+}$ cells were left untransfected and $TLR4^{-/-}$ iBMDMs were transfected as shown in the figure. After recovery, cells were stimulated for 3 h with 100 ng/ml LPS. RNA was isolated, reverse-transcribed and subjected to real-time qPCR analyses with primers specific for human HPRT and IFN-β (B) or mouse HPRT and IFN-β (C). D) Real-time qPCR quantification of TLR4 expression levels in iBMDMs complemented with WT or D299G YFP-TLR4 was analyzed using human TLR4-specific primers. iBMDMs complemented with pcDNA3 were used as a negative control for TLR4 expression. The results of a representative experiment are depicted; *$p<0.05$, ANOVA.

To determine the impact of the TLR4 SNPs on TRIF-mediated signaling in cells with the macrophage phenotype, TLR4-inducible induction of IFN-β mRNA was analyzed in $TLR4^{-/-}$ iBMDMs complemented with WT or D299G YFP-TLR4s introduced by transfection. LPS stimulation of iBMDMs complemented with WT vs. D299G YFP-TLR4s led to 6- and 2-folds induction of IFN-βmRNA, respectively, whereas pcDNA3-transfected cells did not show increases in IFN-β mRNA compared to the no treatment control, indicating their failure to respond to LPS (**Figure 15C**). Complementation of $TLR4^{-/-}$ iBMDMs with D299G YFP-TLR4s yielded 65%

inhibition of LPS-inducible IFN-β gene expression compared to the response seen in WT YFP-TLR4-transfected cells. Real-time qPCR quantification of human TLR4 transcript showed a 3.3- and 3.3-fold increases in TLR4 mRNA levels in iBMDMs complemented with YFP-TLR4 WT or D299G variant, as compared to iBMDMs complemented with pcDNA3 (**Figure 15D**). These data show deficiencies of the D299G and T399I TLR4 polymorphs in their ability to activate TRIF-dependent IFN-β gene expression, which were not due to variations in expression levels of transfected YFP-TLR4.

3.4. Assessment of the capacity of WT *vs.* muatant TLR4 variants to mediate activation of MyD88- and TRIF-dependent activation of p38 MAP kinase and NF-κB

Both the MyD88- and TRIF-dependent signaling pathways mediate TLR4-elicited activation of MAP kinases and NF-κB (203,206), albeit TRIF-mediated signaling results in delayed activation of these intermediates (76). To determine how TLR4 SNPs affect activation of NF-κB, LPS-mediated activation of a transfected NF-κB-dependent pELAM-luciferase reporter was examined in HEK293T cells complemented with CD14/MD2, along with WT, D299G or T399I YFP-TLR4 variants following transfection of the respective expression vectors. LPS challenge caused 14.8-, 5.8- and 6.4-fold increase in activation of pELAM-Luc reporter in HEK293T cells transiently transfected with WT, D299G or T399I YFP-TLR4, respectively (**Figure 16A**). These data show that compared to WT, D299G and T399I YFP-TLR4 variants exhibit 61% and 56% inhibition in its ability to activate the NF-κB reporter in response to LPS. As a control for TLR4-independent

responses, cell stimulation with TNF-α was used, resulting in 5.4-, 5.2- and 5.3-fold induction of pELAM-luciferase reporter, in presence of WT, D299G or T399I TLR4 polymorphs, respectively. LPS stimulation caused 6-, 4- and 2-fold increase in activation of pELAM-Luc reporter in HEK293/YFP-TLR4 stable cell lines expressing WT, D299G, or T399I YFP-TLR4 polymorphs and co-transfected with CD14/MD2, accounting for 29% and 61% inhibition (**Figure 16B**). As a control for TLR4-independent responses, cell stimulation with TNF-α was used, resulting in 11-, 10- and 15-fold induction of pELAM-luciferase reporter, indicating comparable responses in cells expressing WT or mutant TLR4 polymorphs. Taken collectively, these results indicate that the TLR4 polymorphic D299G and T399I variants are deficienct in their capacity to respond to LPS-inducible transactivation of NF-κB.

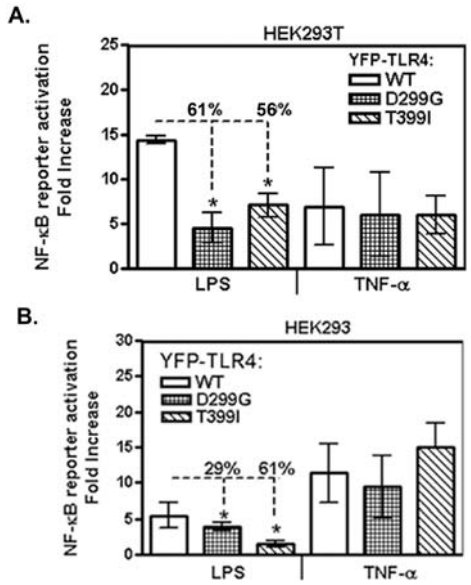

Figure 16 D299G and T399I TLR4 polymorphisms decrease LPS-inducible activation of NF-κB-dependent pELAM-luciferase reporter. A. HEK293T cells transiently transfected with WT, D299G or T399I YFP-TLR4 species, along with pcDNA3-CD14 and pEFBOS-Flag-MD2. B) HEK293 cells stably expressing WT, D299G or T399I YFP-TLR4 species, transiently transfected with pcDNA3-CD14 and pEFBOS-Flag-MD2. In addition, cells in A and B were co-transfected with pGL2-ELAM-Luc and pTK-RL. After recovery, cells were stimulated for 5 h with 100 ng/ml LPS or TNF-α (TLR4-independent control). Cell lysates were analyzed for firefly and Renilla luciferase activities. The results of a representative (n=3, A; n=2, B) experiment are depicted. *$p<0.05$, ANOVA.

In the next series of experiments, I determined whether the presence of the D299G TLR4 SNP impacts LPS-mediated activation of p38 MAP kinase. Since activation of p38 MAP kinase involves its phosphorylation (188,189), LPS-mediated p38 phosphorylation was examined phosphor-specific antibodies in immunoblot analyses of cell lysates obtained from 293 cells transfected with WT or D299G YFP-TLR4s, along with CD14 and Flag-MD2. LPS stimulation of 293T/CD14/MD2 transfectants expressing WT YFP-TLR4 for 30 min induced marked phosphorylation of p38, whereas the D299G YFP-TLR4 variant failed to mediate this response (**Figure 17A**, 3rd panel from the top). Immunoblot analyses revealed higher levels of D299G YFP-TLR4 compared with WT YFP-TLR4, and comparable levels of Flag-MD2, total p38 and β-tubulin (**Figure 17A**, 1st, 2nd, 4th and 5th panels, respectively). In subsequent experiments, I used 293/YFP-TLR4 stable cell lines expressing WT or D299G YFP-TLR4 that were transiently co-transfected with pcDNA3-CD14 and pEFBOS-Flag-MD2. Similar to the data obtained in transient transfectants, LPS stimulation for 30 min resulted in robust phosphorylation of p38 in 293/CD14/MD2 cells expressing WT YFP-TLR4, whereas D299G YFP-TLR4-expressing cells showed significantly decreased LPS-inducible response (**Figure 17B,** 3rd panel from the top**)**. Immunoblot analyses revealed higher levels of D299G YFP-TLR4 and Flag-MD2 in the corresponding transfectants compared to cells expressing WT YFP-TLR4 in combination with Flag-MD2 (**Figure 17B**, 1st and 2nd panels). Comparable expression levels were seen in total p38 and β-tubulin (**Figure 17B**, 4th and 5th panels), indicating that observed differences were not due to variations in total p38 levels or sample loading. Taken together, these data indicate that the D299G TLR4

variant is deficient in eliciting p38 phosphorylation, and that deficient p38 phosphorylation observed in LPS-stimulated 293/CD14/MD2/D299G YFP-TLR4 cells was not due to lower TLR4 levels, variations in total p38 expression proteins or differences in sample loading.

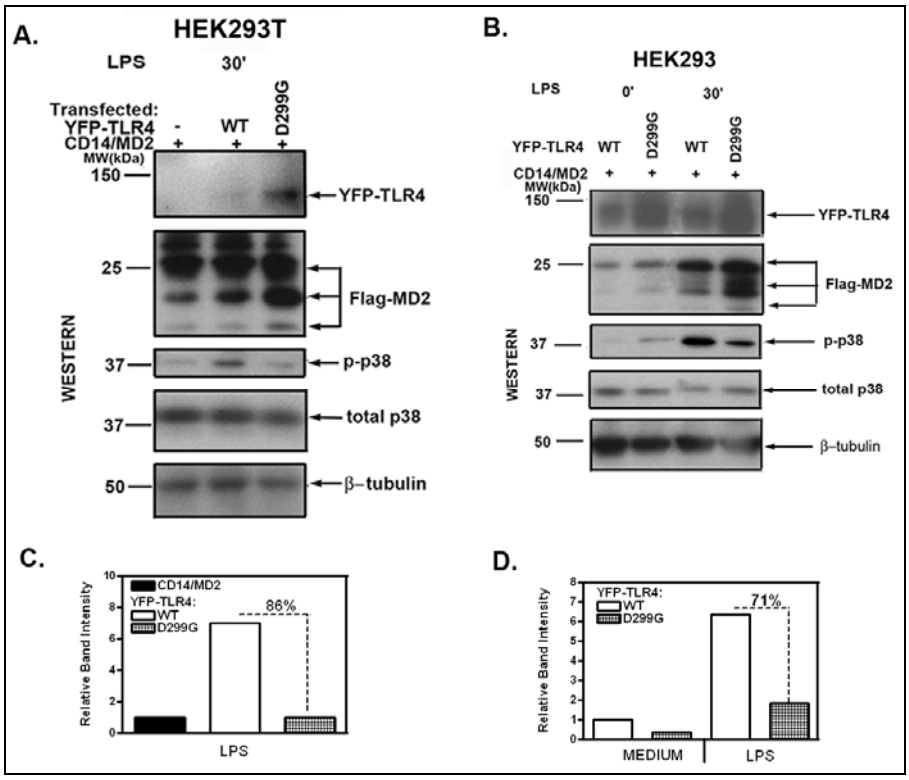

Figure 17 The presence of the D299G polymorphism impairs the ability of TLR4 to mediate LPS-induced p38 phosphorylation. **A**) HEK293T cells were transiently transfected with WT or D299G YFP-TLR4 species, along with pcDNA3-CD14 and pEFBOS-Flag-MD2. **B**) HEK293 cells stably expressing WT or D299G YFP-TLR4 were co-transfected with pcDNA3-CD14 and pEFBOS-Flag-MD2. Cells were stimulated for 30 min with LPS (1μg/ml) and cell lysates were analyzed by immunoblotting with antibodies against GFP (YFP-TLR4), Flag (Flag-MD2), p-p38 and total p38. **C**) and **D**): Quantification for results shown in (A) and (B). Shown are the data of a representative experiment: A) n= 2 and B) n=2.

DISCUSSION

TLRs play an important role in innate immune host defense via detection of microbial pathogens and host-derived "danger" signals (37,41,46,52). TLRs activate several important innate immune responses, including up-regulation of adhesion, MHC and co-stimulatory molecules, production of inflammatory cytokines, chemokines and IFNs, expression of antimicrobial peptides, and regulation of phagocytosis (38,39,42) (58-61). In addition to activating innate host defenses, TLRs initiate and orchestrate adaptive immune responses by controlling Th polarization, via influencing the pattern of cytokines produced by antigen-presenting cells, and by modulating T- and B-cell responses, due to TLR expression on these cell types (58,65-69). To restrain excessive TLR activation, TLR signaling is subject to multilayer regulation through various checkpoints, *e.g*. phosphorylation, glycosylation and ubiquitination, protein degradation, and regulation of signaling platform assembly by splice variants of adapter proteins and kinases (97,105,111,115,118,119,207). Furthermore, the presence of polymorphic mutations within TLRs significantly affects their ability to signal, as evidenced by altered capacities of polymorphic TLR2, TLR4, TLR5 and TLR9 variants to respond to agonist stimulation *in vitro* and control microbial infections *in vivo* (105,109,111,114,118,119).

Several SNPs in the promoter regions and coding sequence of human *TLR4* have been discovered and associated with an increased risk of certain inflammatory and infectious diseases (117,150,151,153,169). Two SNPs in the human *TLR4* coding sequence, A896G and C1196T, yield the D299G and T399I mutations in the

TLR4 ectodomain that have been linked to sepsis (169), Gram-negative infections (151), RSV bronchiolitis (152), Mediterranean spotted fever caused by *Rickettsiae conorii* (161), tuberculosis in HIV patients from Tanzania (167) and inflammatory bowel disease (153). The D299G and T399I TLR4 variants exhibit blunted receptor signaling in response to several agonists, including LPS, F protein from RSV, and chlamydial HSP60 (41,47,117,163), but the molecular mechanisms responsible for their signaling deficiencies remain poorly characterized. Contradictory data have been reported regarding the impact of these polymorphisms on TLR4 expression. While two groups reported decreased expression of the D299G TLR4 variant in primary airway epithelial cells (117) and in HEK293 cells (176), Rallabhandi et al. found that the D299G TLR4 variant elicits decreased cell activation in response to LPS, F protein from RSV and chlamydial HSP60 under conditions of comparable expression of WT and polymorphic TLR4 species in HEK293 cells (163).

To study the molecular mechanisms by which the D299G and T399I polymorphisms impact TLR4 signal transduction, I hypothesized that these ectodomain-localized polymorphisms could impact ligand recognition, interactions between TLR4 and MD2 and/or TLR4 homo-dimerization, thereby altering docking TIR domain platform assembly and suppressing recruitment and activation of key adapters and kinases of the MyD88- and TRIF-dependent pathways. To test this hypothesis, I sought to delineate their impact on total TLR4 expression (**Aim 1**), LPS binding to the TLR4/MD2/CD14 complex and TLR4-MD2 interactions (**Aim 2**), and recruitment and activation of key adapter protein, kinases, transcription factors and cytokines elicited via MyD88- and TRIF-dependent signaling pathways (**Aim 3**) . In

my studies, I used transfection-based complementation of TLR4-deficient HEK293 cells (163), and iBMDMs with YFP-TLR4, taking advantage of the fact that this C-terminally fused tag does not affect receptor localization and function (143,183) while facilitating studies on TLR4 expression and protein-protein interactions.

The magnitude of LPS responses correlates with total expression levels of TLR4 (180,181). TLR4 trafficking to the cell surface or endosomes depends on interactions of the TLR4 ectodomain with chaperones, including gp96 (145) and PRAT4A, also called canopy 3 homolog (CNPY3) (147,148,208). Indeed, gp96 deficiency was shown to preclude expression of multiple TLRs, including TLR4 (147). Since the D299G and T399I polymorphisms are located in the ectodomain of TLR4 (117,142,145,182), they may impact TLR4-gp96-CNPY3 complex assembly, thereby altering TLR4 trafficking and expression. In addition, although these polymorphisms are not located in the vicinity of the nine glycosylation sites within the TLR4 ectodomain (105), it is possible that they might impose conformational changes affecting the accessibility of these glycosylation sites to glycosyltransferases, affecting TLR4 glycosylation at the sites that have been reported to influence TLR4 expression and trafficking (209). In this study, comparable total expression of transfected WT *vs*. polymorphic D299G and T399I YFP-TLR4 variants was desmonstrated by three different approaches, fluorescent and confocal microscopy, FACS and immunoblot analyses, and using both transient and stable transfection. These results support similar data obtained with transfected untagged TLR4 (163) and indicate that the D229G and T399I polymorphisms do not alter total TLR4 protein levels. I cannot formally rule out a possibility that despite the lack of the impact of

SNPs on total TLR4 expression, the D299G and T399I plymorphisms may selectively decrease cell surface expression of TLR4, as my efforts to assess cell surface expression of YFP-TLR4 species using FACS analyses with antibody against the TLR4 ectodomain fail to produce reliable results due to technical reasons. However, confocal imaging of WT vs. mutant YFP-TLR4 species in transfected HEK293 cells demonstrated their comparable localization on the plasma membrane and in cellular compartments, reported to be the Golgi apparatus and endoplasmic reticulum (142,143). These data suggest that the D299G and T399I TLR4 undergo unaltered trafficking, implying that the interactions of TLR4 with chaperonic proteins do not seem to be affected.

MD2 is a principal co-receptor molecule conferring LPS responsiveness via binding LPS and presenting it to TLR4 (118,210). MD2 gene knockout appears to be a phenocopy of the TLR4-deficient mouse, in terms of its LPS unresponsiveness (210). TLR4 and MD2 are assembled in the Golgi apparatus when coexpressed (143), and co-expression of MD2 with TLR4 enhances expression of cell surface TLR4 (105,211,212). Studies with $MD2^{-/-}$ embryonic fibroblasts revealed the inability of TLR4 to traffick to the plasma membrane and its predominant localization in the Golgi apparatus (210), suggesting that MD-2 expression is required for cell surface distribution of TLR4. Furthermore, studies in the B cell line Ba/F3 showed that surface localization of functional mouse TLR4 depends on fully glycosylated MD2 (54,70). Studies in epithelial cells showed that incubation of sMD2 with HEK293 cells transfected with TLR4 increases the cell surface levels of TLR4, supporting a model whereby sMD2 binds to TLR4 and increases TLR4 cell surface

levels by preventing TLR4 turnover through the endocytic pathway (213). These results suggest that MD2 could have a specific chaperone function with a potential to regulate TLR4 trafficking and localization. On the other hand, reports from other groups failed to confirm the requirements for MD2 for TLR4 trafficking and cell surface expression (214). Thus, controversy exists in the literature regarding the ability of MD2 to regulate TLR4 cell surface expression and trafficking. Because of the proposed importance of MD2 in regulating TLR4 expression and trafficking, and since the TLR4 ectodomain interacts with MD2, it is plausible that the D299G and T399I polymorphisms, albeit located outside of the TLR4-MD2 interactions interface (reported at the F440, F463 and L444 residues of TLR4 (55), could impose conformational changes, affecting TLR4 residues involved in MD2 interactions. Assuming altered TLR4-MD2 interactions as a consequence of the presence of SNPs in TLR4, it is also possible that TLR4 trafficking/cellular distribution could be affected. To address these potential outcomes, I studied whether the D299G polymorphism associated with the strongest inhibition of TLR4 signaling (117,151,165), affects the ability of transfected YFP-TLR4 to interact with Flag-MD2 under basal state and upon LPS stimulation, and examined cellular distribution of fluorescent WT vs. polymorphic YFP-TLR4 variants. Co-immunoprecipitation experiments revealed no differences in TLR4-MD2 interactions regardless of whether or not the D299G polymorphism was present, and confocal imaging showed that WT and mutant TLR4 species exhibit comparable cellular distributions. These data indicate that defective LPS-elicited signaling by D299G TLR4 could not be due either to its association with MD2 cellular localization.

LPS is recognized by TLR4 in conjunction with co-receptors, CD14 and MD2 (54-56,75-77). Five of the six fatty acid chains of hexa-acylated LPS bind to a hydrophobic pocket within MD2, imposing conformational changes of MD2 and re-orientation of the critical F126 residue that presents the remaining acyl chain of LPS to the neighboring TLR4-MD2 complex, promoting TLR4 homo-dimerization (53,55). Hydrophobic interactions of lipid A with TLR4 residues F440 and 463, at the core, and L444 at the periphery of the receptor, and interactions of the two phosphate groups of lipid A with positively charged K388, R264, K341, K362 residues within TLR4 are important for promoting receptor homo-dimerization (55). Since the D299G and T399I polymorphisms are located in a relatively close vicinity to the R264 and K341, K362 residues, I hypothesized that they could influence the ability of TLR4 to bind LPS. To study whether the D299G and T399I polymorphisms affect LPS binding to the TLR4/MD2/CD14 complex, I examined binding of biotinylated LPS to 293/CD14/MD2 transfectants expressing WT or mutant TLR4 variants, as revealed by FACS measurement of streptavidin-APC bound to cell-associated biotinylated LPS. These studies demonstrated similar LPS binding patterns in 293/CD14/MD2 cells expressing WT, D299G or T399I YFP-TLR4 species under conditions of comparable TLR4 expression. My data are consistent with a recent publication demonstrating comparable affinities of interactions of radioactively labeled LOS with WT, D299G or T399I TLR4 species (176). However, they cannot exclude a possibility that despite equal LPS binding to the TLR4/MD2/CD14 complex in cells expressing WT vs. mutant versions of TLR4, the presence of the D299G and T399I polymorphisms could affect intricate TLR4

interactions with MD2-presented LPS, resulting in altered TLR4 engagement and homo-dimerization. Future studies, using pull-down of LPS bound to MD2-associated WT or mutant TLR4 species followed by immunoblot analyses of LPS-bound receptor components, in combination with crystal structures of lipid A in complex with MD2-associated WT or mutant TLR4 species are required to precisely define the impact of the D299G and T399I polymorphisms on LPS interactions with MD2/TLR4.

Ligand recognition initiates TLR4 homo-dimerization that brings intracellular TIR domains into a close proximity, forming docking platforms to enable recruitment of adapter proteins Mal and MyD88 to the plasma membrane-associated TLR4, and TRIF and TRAM to TLR4 translocated to endosomes. These events initiate the MyD88- and TRIF-dependent signaling pathways responsible for expression of of pro-inflammatory cytokines, chemokines (MyD88-dependent signals) and type I IFNs and type I IFN-dependent genes (TRIF-dependent signals), respectively (30,97,120). One mechanism by which the polymorphic variants impair TLR4 signaling could be their interference with LPS-inducible TLR4 homodimerization that would underlie defective recruitment and activation of adapter proteins and kinases involved in activation of the MyD88- and TRIF-dependnent pathways. This intererence could be due to intricate differences in MD2-assisted LPS presentation events, or because of inherent conformational changes imposed by the mutations that would prevent proper protein-protein interactions between interacting TLR4-MD2 complexes, even though total LPS binding and TLR4-MD2 interactions are preserved. Because TLR4 dimerization initiates the TIR docking platform assembly that is required for

recrutiment of MyD88, I first analyzed LPS-mediated MyD88 recruitment to TLR4. Co-immunoprecipitation experiments revealed that in contrast to robust LPS-inducible recruitment of transfected AU1-MyD88 to WT YFP-TLR4, the D299G YFP-TLR4 variant was significantly compromised in promoting AU1-MyD88 recruitment in response to LPS stimulation. Since Mal serves as a "bridging" adapter required for MyD88 recruitment to TLR4 signalsome (122,123,190,201,215), these results suggest that defective LPS-inducible recruitment to the D299G TLR4 polymorph could be due inefficient interaction of Mal with the polymorph receptor. The inability of the D299G TLR4 to recruit MyD88 is likely to lead to deficient Myddosome assembly, including deficiency in IRAK kinase recruitment and activation, because IRAK kinases associate with TLR4-recruited MyD88 via death-death domain interactions (124-126).

MyD88 recruitment to TLR4 is a prerequisite for initiatng downstream MyD88-dependent LPS-driven pathways (97,99,120,141,205). Consistent with this notion, this study demonstrates that deficient recruitement of MyD88 to the D299G TLR4 species was associated with impaired LPS-mediated induction of IL-8 mRNA in 293T/CD14/MD2 cells expressing D299G or T399I YFP-TLR4s compared to robust responses seen in WT TLR4-expressing cells. Similarly, complementation of $TLR4^{-/-}$ iBMDMs with human WT YFP-TLR4 restored their ability to respond to LPS with up-regulation of TNF-α mRNA, while the introduction of the D299G YFP-TLR4 variant failed to confer LPS responsiveness. These results are consistent with reported deficiencies of the D299G and T399I TLR4 variants in mediating cell activation in response to F protein of RSV and chlamydial HSP60 (163,170), other

TLR4:MD2-dependent ligands (214). Deficiencies of the TLR4 polymorphs in mediating MyD88-dependent signaling that governs expression of inflammatory cytokines and chemokines (1,30,42,99) involved in Th polarization and B cell activation (42,58,65,69), and reported expression of TLR4 in T- and B-lymphocytes (40) suggest that the D299G and T399I TLR4 polymorphisms can utlimately hamper activation of the adaptive immune responses. Indeed, the TLR4 alleles rs2770150 and rs6478317 have been linked to lower titers of the pertussis toxin-specific immunoglobulin G after immunization of children with whole-cell pertussis vaccine (216,217). However, other studies failed to observe significant differences in T- and B-lymphocyte responses between individuals expressing WT *vs.* polymorphs of TLR4 (218-221) neccesitating further genome-wide association studies to unambigously dissect the impact of the TLR4 SNPs on adaptive immunity.

In addition to the MyD88-dependent pathway, TLR4 also triggers the TRIF-dependent signaling pathway from the endosomal compartment (120), leading to delayed activation of NF-κB and MAP kinases, production of type I IFNs and type I IFN-dependent genes (122,200). Studies on the effects of polymorphic mutations in *TLR4* reported altered TLR4-mediated activation of MyD88-dependent cytokines, *e.g.* TNF-α, IL-1β, IL-6, IL-8 (117,162,163,166,167), and MyD88/TRIF-dependent activation of MAP kinases and NF-κB (117,162,163). To the best of my knowledge, there have been no reports on how the carriage of the D299G or T399I mutations affects the ability of TLR4 to activate TRIF-dependent signaling, *e.g.* activation of TBK1, IRF-3 and expression of TRIF-dependent type I IFNs and type I IFN-dependent genes. To determine the impact of the D299G and T399I TLR4

polymorphisms on LPS-inducible activation of the TRIF-dependent pathway, I first examined phosphorylation of TBK1 kinase, a signaling event prerequisite for activation of TBK1 kinase activity (187,222). These experiments revealed a significant decrease in the LPS-inducible phosphoprylation of TBK1 in 293T/CD14/MD2 cells transfected with the D299G YFP-TLR4 variant compared to a robust response observed in cells complemented with WT YFP-TLR4. Consistent with the requirement for TBK1 kinase activity for IRF-3 phosphorylation and activation (121,223-225), my experiments showed compromised TLR4-elicited phosphorylation of IRF-3 in 293/CD14/MD2 cells expressing the D299G YFP-TLR4 version compared to WT YFP-TLR4-expressing cells. Impaired LPS-inducible phosphorylation of TBK1 and IRF-3 in 293/CD14/MD2 cells expressing the D299G TLR4 species was not due to decreased protein levels of these intermediates. Phosphorylated IRF3 translocates to the nucleus and, together with NF-κB, AP-1 and ATF-2 transcription factors, binds to the enhancer elements within the IFN-β gene (226-231). To study IRF3 transactivation potential, I determined LPS-mediated, IRF3-dependent activation of IFN-β promoter-driven luciferase reporter, and examined induction of IFN-β mRNA. These experiments showed significant deficiency of the D299G YFP-TLR4 to mediate IRF3 transactivation and induction of IFN-β mRNA upon its transfection into 293/CD14/MD2 transfectants. HEK293 transfectants are limited in their spectrum of TLR4-mediated responses, exhibiting NF-κB and IRF3 induction and expression of IL-8 and IFN-β genes but lacking many responses observed in macrophages (27,232). Therefore, mouse iBMDMs that behave similarly to primary macrophages (190-193) were used in this study to

confirm data in cells with the macrophage phenotype. Transfection-based complementation of TLR4$^{-/-}$ iBMDMs with human WT YFP-TLR4 resulted in a significant restoration of LPS-inducible IFN-β mRNA expression, whereas transfection of the D299G TLR4 species failed to activate this TRIF-dependent response. Overall, these data indicate that the D299G polymorphism compromizes the ability of TLR4 to trigger the TRIF-dependent signaling pathway.

Defective signaling by the D299G TLR4 polymorph could be accounted for by altered translocation of mutant TLR4 into the endosomal compartment. However, using confocal imaging, I did not observe apparent differences in the cellular localization of WT or mutant TLR4 in basal state or after LPS activation. Although these results argue against altered trafficking of polymorphic TLR4s as a possible explanation of their ability to activate TRIF-dependent signaling, more experiments are required to definitively rule out this possibility, using endosome-specific markers. MyD88 and TRIF compete for their binding to the common TIR interface (233) formed by dimerization-induced assembly of the TIR domains (234,235). Since compromised MyD88 recruitment to the D299G TLR4 was observed, it is likely that the D299G mutation alters recruitment of TRIF to TLR4-TRAM complexes in endosomes, events that are prerequsite for activation of the TRIF-dependent pathway (236). Future studies are necessary to dissect the exact molecular mechanism by which the D299G polymosphism mitigates the capacity of TLR4 to trigger TRIF-dependent signaling.

Activation of p38 MAP kinase and the transcription factor NF-κB is governed by the MyD88-dependent (early responses) and TRIF-dependent (delayed activation) pathways. I examined the impact of the D299G and T399I polymorphisms on TLR4-elicited activation of p38 and NF-κB, as defined by phosphorylation of p38, which correlates with activation of p38 kinase activity (188,189) and activation of NF-κB-driven pELAM-luciferase reporter, which is dependent on NF- κB transactivation potential (163). LPS stimulation of 293T/CD14/MD2 cells complemented with WT YFP-TLR4 led to robust TLR4-elicited phosphorylation of p38, whereas the transfected D299G YFP-TLR4 species exhibited significantly decreased responses, differences that were not due to variable levels of total p38. To the best of my knowledge, this is the first demonstration of compromised LPS-inducible activation of p38 MAP kinase as a consequence of the presence of the D299G mutation in TLR4. While my studies focused on the most signaling-deficient D299G TLR4 polymorph, it remains to be elucidated whether the T399I TLR4 associated with milder LPS deficiencies (117,153) could also impact LPS-mediated phosphorylation of p38 MAP kinase. In keeping with previous results (163,176), my results demonstrate that complementation of 293/CD14/MD2 transfectants with D299G and T399I YFP-TLR4 polymorphs reduced activation of the NF-κB reporter in response to LPS. This defect was specific for TLR4, as TNF-α elicited similar activation of NF-κB luciferase reporter regardless of whether WT or polymorph TLR4s were transfected. NF-κB activation is regulated at several levels: i) K48-linked ubiquitination and proteasomal degradation of IκB proteins, relieving NF-κB dimers to translocate to the nulceus; ii) post-translational modifications of NF-κB proteins;

iii) export of IκB and NF-κB from the nucleus; and iv) association of NF-κB proteins with other transcritpion factors to form enhanceosomes (237-240). Because I observed that the D299G polymorphism affects TLR4 signaling at the proximal levels, such as MyD88 recruitment and TBK1 activation, it is likely that deficient recruitment and activation of proximal adapter/kinase models is responsible for deficiencies in downstream signaling events. Consistent with defective transactivation of p38 MAP kinase and NF-κB, and in line with their critical roles in transcriptional activation of cytokine expression, I observed impaired production of pro-inflammatory cytokines and chemokines, such as TNF-α and IL-8.

In summary, data presented herein provide important insights for elucidating how the D299G and T399I TLR4 polymorphs affect TLR4 function. Within the context of the current model, total expression of transfected WT, D299G and T399I TLR4 species and their similar cellular localization were unaltered, suggesting that these TLR4 polymorphs are unlikely to affect chaperone-TLR4 interactions. LPS binding to the TLR4/MD2/CD14 complex and TLR4-MD2 interactions also remained unaffected regardless of the presence of absence of the D299G and T399I polymorphisms. My research has shown significant deficiencies of the TLR4 polymorphs in their capacities to activate the MyD88-dependent pathway, as evidenced by compromised LPS-inducible recruitment MyD88 to the D299G TLR4, and impaired inducibility of IL-8 and TNF-α genes in cells expressing the D299G and T399I TLR4 species. The ability of the D299G TLR4 mutant to trigger TRIF-dependent phosphorylation of TBK1 and IRF3, transactivation of IRF3 and expression of IFN-β mRNA was found to be also impaired . LPS-induced activation

of NF-κB and p38 that are dependent on both MyD88 and TRIF signaling pathways were also impacted by the D299G and T399I polymorphisms. The focus of future research will be on crystallographic studies to define the impact of the D299G and T399I polymorphisms on molecular interactions of lipid A with MD2 associated with WT *vs*. polymorphic TLR4 variants, TLR4 interactions with CD14 and other co-receptors (e.g., CD11b/CD18 and CD36), TLR4 cellular trafficking and homo-dimerization, recruitment of Mal, IRAK kinases, TRIF and TBK1 to TLR4. It will be important to confirm results obtained in overexpression systems in cells expressing endogenous polymorphic TLR4 alleles, and to carry out mechanisitic studies with knock-in mice expressing humanized version of TLR4 proteins to define the impact of these mutations on susceptibility to diseases. An improved understanding of the molecular mechanisms by which the D299G and T399I TLR4 polymorphic species impact TLR4 signal transduction is crucial for the development of new therapeutic strategies for improved treatment of inflammatory and infectious diseases associated with carriages of these TLR4 polymorphisms in susceptible human patients.

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