Genome-Wide Expression Profiling and Mutagenesis Studies Reveal that Lipopolysaccharide Responsiveness Appears To Be Absolutely Dependent on TLR4 and MD-2 Expression and Is Dependent upon Intermolecular Ionic Interactions

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Genome-Wide Expression Profiling and Mutagenesis Studies Reveal that Lipopolysaccharide Responsiveness Appears To Be Absolutely Dependent on TLR4 and MD-2 Expression and Is Dependent upon Intermolecular Ionic Interactions

Jianmin Meng,* Mei Gong,* Harry Björkbacka,† and Douglas T. Golenbock*

Lipid A (a hexaacylated 1,4′-bispophosphate) is a potent immune stimulant for TLR4/MD-2. Upon lipid A ligation, the TLR4/MD-2 complex dimerizes and initiates signal transduction. Historically, studies also suggested the existence of TLR4/MD-2–independent LPS signaling. In this article, we define the role of TLR4 and MD-2 in LPS signaling by using genome-wide expression profiling in TLR4- and MD-2–deficient macrophages after stimulation with peptidoglycan-free LPS and synthetic Escherichia coli lipid A. Of the 1396 genes significantly induced or repressed by any one of the treatments in the wild-type macrophages, none was present in the TLR4- or MD-2–deficient macrophages, confirming that the TLR4/MD-2 complex is the only receptor for endotoxin and that both are required for responses to LPS. Using a molecular genetics approach, we investigated the mechanism of TLR4/MD-2 activation by combining the known crystal structure of TLR4/MD-2 with computer modeling. According to our murine TLR4/MD-2–activation model, the two phosphates on lipid A were predicted to interact extensively with the two positively charged patches on mouse TLR4. When either positive patch was abolished by mutagenesis into Ala, the responses to LPS and lipid A were nearly abrogated. However, the MyD88-dependent and -independent pathways were impaired to the same extent, indicating that the adjuvant activity of monophosphorylated lipid A most likely arises from its decreased potential to induce an active receptor complex and not more downstream signaling events. Hence, we concluded that ionic interactions between lipid A and TLR4 are essential for optimal LPS receptor activation. The Journal of Immunology, 2011, 187: 3683–3693.

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Microarray data have been deposited to Gene Expression Omnibus with SuperSeries accession number GSE31078.

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Abbreviations used in this article: Ala, alanine; BM, bone marrow; BMDM, bone marrow-derived macrophage; co-IP, coimmunoprecipitation; EASE, Expression Analysis Systematic Explorer v2.0 software; GEDI, Gene Expression Dynamics Inspector v2.1; GO, gene ontology; hTLR4, human TLR4; IB, immunoblotting; IP, immunoprecipitation; MGH, Massachusetts General Hospital; mTLR4, mouse TLR4; Pam2, Pam2CSK4; WB, Western blotting; WT, wild-type; YFP, yellow fluorescent protein.

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MyD88-independent (TRIF) pathway and, hence, mediate the adjuvant activity of lipid A with minimal toxicity (27).

Although the role of TLR4/MD-2 as an LPS receptor has been unequivocally established, alternative LPS receptors have been suggested to exist. Prior to the discovery of TLRs, numerous “LPS receptors” had been reported to exist (28–34). Indeed, although no functional evidence was reported, close examination of one of these “pre-Toll receptor” papers suggests that the discovery of MD-2 as an LPS-binding protein might have preceded the reports of Miyake et al. (12, 30). More recently, Haziot et al. (35) reported that the lipid A component of Escherichia coli LPS activated some proinflammatory responses in CD14- or TLR4-deficient mice that resulted in increased bacterial clearance. Similarly, Miyake and coworkers (36) demonstrated that LPS-induced tyrosine phosphorylation of CD19 was RP105 dependent but TLR4 independent. Furthermore, Schwartz and coworkers (37) reported that TLR4-independent signaling was activated when a TLR4-deficient murine macrophage cell line was incubated with Bacteroides fragilis LPS. It was also reported that Neisseria meningitidis LPS is recognized independently from TLR4, MD-2, or CD14 in human meningeal cells (38).

To clarify the discrepancy and unambiguously identify any potential alternative LPS receptors, we carried out genome-wide microarray profiling of LPS responses in wild-type (WT) and TLR-4 and MD-2–deficient mouse macrophages. Synthetic lipid A was used for cell stimulation to exclude contaminants that could compromise data analysis. In addition, we used an LPS preparation that we had previously prepared that we knew to be free of lipoproteins and peptidoglycan (39). Under these conditions, we failed to unveil the existence of an alternative LPS receptor. With the knowledge that TLR4/MD-2 represent the only means that phagocytes have to respond to LPS, we refocused our approach to characterizing TLR4. To determine the functional relevance of the intermolecular ionic interactions on lipid A activation, we assessed five positively charged residues on mouse TLR4 (mTLR4): R264, K341, and K362 (positive patch 1) and K367 and R434 (positive patch 2). We chose these residues because our computer model predicted that the two negatively charged phosphates on lipid IVA would coordinate with these residues in the active TLR4/MD-2/ lipid IVA dimer:dimer (23). We found that when either positive patch was abolished by mutagenesis into Ala, the responses to lipid A or LPS were almost completely abrogated. However, the MyD88-dependent and -independent pathways were impaired to the same extent, indicating that decreased receptor activation, rather than selective pathway activation, is likely to account for the adjuvant activity of monophosphorylated lipid A. Hence, we concluded that TLR4 and MD2 are absolutely required for the proinflammatory responses to E. coli LPS in mouse macrophages, and the optimal activation of the TLR4/MD-2 complex depends on the formation of intermolecular ionic interactions between TLR4, MD-2, and LPS.

Materials and Methods

Materials

Synthetic lipid IVA and lipid A were gifts of Dr. Shoichi Kusumoto (Osaka University, Osaka, Japan). LPS from E. coli strain O111:B4 (Sigma) was repurified by a repeat phenol-chloroform extraction (40). Pam3CSK4 (Pam2) was obtained from EMC Microcollection (Tübingen, Germany). C57BL/6 WT mice were purchased from The Jackson Laboratory (Bar Harbor, ME). TLR4-deficient and MD-2–deficient mice were gifts from S. Akira (Osaka University, Osaka, Japan) and K. Miyake (University of Tokyo, Tokyo, Japan), respectively, and were subsequently backcrossed onto a C57BL/6 background for at least six generations.

Cell culture and stimulation

Bone marrow (BM) was harvested from the femurs and tibias of 6–8-wk-old mice. BM-derived macrophages were differentiated from BM cells cultured in DMEM medium (Mediatech, Herndon, VA), supplemented with 10% heat-inactivated FBS, 20% L929-conditioned media, and 10 μg/ml ciprofloxacin (Bayer, West Haven, CT). Ex vivo-cultured macrophages were collected at day 8. Cell purity was analyzed by flow cytometry using CD11b-PerCP-Cy5.5 and F4/80-PE and showed >95% double-positive cells. The next day, cells were treated with 10 ng/ml lipopolysaccharide- and peptidoglycan-free LPS, 100 ng/ml synthetic lipid A, or 10 nM Pam2 for 2 h before RNA isolation.

RNA isolation and quantitative RT-PCR

Total RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA). Quantitative RT-PCR analysis was performed on RNA samples using SuperScript III Two-Step qRT-PCR Kit with SYBR Green (Invitrogen, Carlsbad, CA) on the DNA engine opticon 2 cycler (MJ Research, Boston, MA). Gene-expression data were normalized to β-actin expression and are presented as a ratio of gene copy number per 100 copies of β-actin. Primer sequences are shown in Table I.

TLR4+/−-expression profile

BM cells from four C57BL/6 WT, TLR4-deficient mice were pooled and cultured as described above. Total RNA was isolated from cells stimulated for 2 h. RNA integrity was assessed using a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). RNA was then labeled and hybridized to four individual arrays, swapping the Cy3 and Cy5 dyes as subsequently detailed. Eight milligrams of RNA of both test and reference samples were reverse transcribed and differentially labeled with monoreactive Cy3 and Cy5 Dyes (Amersham, Piscataway, NJ) using the Atlas Powderscript Fluorescent Labeling Kit (Clontech, Mountain View, CA). Hybridized arrays were scanned using GenePix 400B microarray scanner and its accompanying software (GenePix 4000B microarray scanner; Axon Instruments, Union City, CA). The gene-expression profiles were compared with that of PBS-treated cells incubated for the same time. A comparison between WT and TLR4-deficient PBS-treated cells was used to control for gene-expression differences due to the genotype. The experiment was repeated once in its entirety and yielded nearly identical results.

MD-2+/−-expression profile

BM cells from C57BL/6 WT or MD-2-deficient mice were cultured and treated as described above. Total RNA isolated from the cells was reverse transcribed, labeled, and hybridized to two individual microarrays, swapping the Cy3 and Cy5 dyes. Cells from two individual mice of each genotype were used, and each mouse served as its own PBS-treated control. Control comparisons between WT and MD-2–deficient PBS-treated cells were also performed. RNA quality was assessed as described above. Microarrays were rehydrated with Cyscribe GFX Purification Kit (Amersham) and hybridized overnight using Genomic Solutions GeneTAC Hybridization Station (Perkin-Elmer, Boston, MA) to the PGA mouse v1.1 oligonucleotide array (Massachusetts General Hospital [MGH], Cambridge, MA). These arrays, including 19,549 reporters, were printed on an Omnimicrprint (Geneshemes, San Carlos, CA) from 384-well polyethylene V-bottom plates (Genetic, Charlestown, MA) to SureArray slides. Fluorescent images from the arrays were acquired using a microarray scanner and its accompanying software (GenePix 4000B microarray scanner; Axon Instruments, Union City, CA). The gene-expression profiles were compared with that of PBS-treated cells incubated for the same time. A comparison between WT and TLR4-deficient PBS-treated cells was used to control for gene-expression differences due to the genotype. The experiment was repeated once in its entirety and yielded nearly identical results.

Microarray analysis

All microarray data were stored, filtered, and normalized in the BioArray Software Environment (http://base.thep.lu.se) (41). The t test statistics with Benjamin–Hochberg correction for multiple testing were calculated in the R software environment for statistical computing (http://www.r-project.org/). Hierarchical and K-means clustering analysis was performed using Multidimensional Experiment Viewer software (http://www.tm4.org) (42). Self-organizing map clustering was performed with Gene Expression Dynamics Inspector v2.1 (GEDI; http://web1.ch.harvard.edu/research/ingber/GEDI/gedihome.htm)
veloping procedures were used for detection of respective proteins. Anti-mouse secondary Ab (Bio-Rad) and standard de-

ELISA

BM-derived macrophages (BMDMs) (day 7) were plated and cultured in 96-well plates (Becton Dickinson Labware, Franklin Lakes, NJ) at 5 × 10^4/well for 1 d prior to treatment. Cell-culture supernatants were collected 16 h after stimulation and analyzed for the presence of TNF-α and RANTES by ELISA, according to the manufacturer’s recommendations (R&D Systems, Minneapolis, MN).

Mutagenesis

All mutants were created by site-directed mutagenesis, according to the manu-

Luciferase assay

The effects of these mutations on LPS, lipid A, and lipid IVa signaling were tested in HEK293 cells by transient transfection using the transfection reagent GeneJuice (Invitrogen). HEK293 cells were plated in 96-well plates at a density of 20,000 cells/well. The next day, cells were transfected with one of the TLR4 mutants (1 ng/well), WT human TLR4 (hTLR4) construct on a PCDNA3 vector (23). The two other mutants, K263A_R337A_K360A and K263A_R337A_K360A_K367A_R434A, were created from WT hTLR4 construct (100 ng/well). The two mutants, R264A_K341A_K362A and R264A_K341A_K362A_E369K_Q436R, were created from WT human MD-2 (mMD-2) construct (100 ng/well), an NF-κB luciferase plasmid (40 ng/well) (45), and the Renilla luciferase was used to normalize for NF-κB-luciferase or IFN-β-luciferase. The effects of these mutations on LPS, lipid A, and lipid IVa signaling were tested in HEK293 cells by transient transfection using the transfection reagent GeneJuice (Invitrogen). HEK293 cells were plated in 96-well plates at a density of 20,000 cells/well. The next day, cells were transfected with one of the TLR4 mutants (1 ng/well), WT human TLR4 (hTLR4) construct on a PCDNA3 vector (23). The two other mutants, K263A_R337A_K360A and K263A_R337A_K360A_K367A_R434A, were created from WT hTLR4 construct on a PCDNA3 vector. All mutations were verified by DNA sequencing (Geneviz, South Plainfield, NJ).

Immunoprecipitation and immunoblotting analysis

HEK293T cells were plated on six-well plates at a density of 500,000 cells/well. The next day, cells were transfected with 1 μg TLR4 mutant and 1 μg MD-2 WT constructs/well using GeneJuice. After 2 d, supernatants were removed, and cells were lysed with 350 μl lysis buffer (1% Triton X-100 in PBS). Cell lysates were cleared by centrifugation at 16,000 × g for 10 min at 4°C on a tabletop centrifuge. Clear cell lysates were either subject to SDS-PAGE for direct immunoblotting (IB)/Western blotting (WB) analysis or immunoprecipitation (IP) followed by IB/WB analysis.

Four to twenty percent gradient SDS-PAGE gels were used for IB. Pre-

FIGURE 1. The induction of LPS-responsive genes is dependent on TLR4 signaling. BMDMs from WT or TLR4^-/- mice were stimulated with 10 ng/ml LPS, 100 ng/ml lipid A, 10 mM Pam2, or PBS control. A, TNF-α and RANTES protein level was measured in the supernatant by ELISA after 16 h of stimulation. B, TNF-α and RANTES RNA level was measured in the cells by quantitative PCR after 2 h of stimulation. Data in A and B are representative of one of three experiments. C, Number of genes significantly induced or repressed by LPS, lipid A, and Pam2 in WT or TLR4^-/- macrophages. D, Number of genes with altered basal expression between WT and TLR4^-/- macrophages.

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<td>glycerol. Twenty microliters of elution buffer (20 mM Tris-HCl [pH 8], 100 mM KCl, 100 mM imidazole, 10% glycerol) were added to the beads to elute His-tagged proteins. The eluates were then adjusted to SDS-PAGE and IB analysis. After blocking with 10% milk solution, the membranes were cut into two pieces at the molecular mass ∼60 kDa, according to prestained molecular mass marker. The upper blots were blotted against anti-GFP mAb for detection of YFP-tagged TLR4 proteins, and the lower blots were blotted against penta-his mAb for detection of His6-tagged MD-2 proteins.</td>
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For IP with anti-GFP pAb, 700 µl clear cell lysates from each mutant was incubated with 2 µg anti-GFP polyclonal Ab (Invitrogen) and 20 µl protein A activated Sepharose beads (Thermo Scientific) overnight at 4°C. Supernatants were then removed and pellets were washed three times with PBST buffer (0.05% Tween in PBS) and subjected to SDS-PAGE and IB analysis. After blocking in 10% milk solution, the nitrocellulose membranes were cut into two pieces at the molecular mass ∼60 kDa. The upper blots were blotted against anti-GFP mAb for detection of YFP-tagged TLR4.

FIGURE 2. Cluster analysis of TLR4 and MD-2 dependency. A, Five clusters of TLR4-dependent genes were sorted out by hierarchical and K-means cluster analysis based on the gene-expression profile in response to LPS, lipid A, or Pam2. Clusters 1–3 contain genes induced by all three treatments in WT cells to different degrees. Cluster 4 contains genes induced by LPS and lipid A but not by Pam2. The genes repressed by LPS stimulation were grouped together in cluster 5. B, Gene expression in response to LPS, lipid A, or Pam2 stimulation in WT and MD-2−/− macrophages was visualized by hierarchical clustering analysis. *No gene was significantly induced or repressed in MD-2−/− macrophages after stimulation with LPS or lipid A; †no gene was significantly different between LPS- and lipid A-stimulated WT cells; ‡no gene was significantly different between WT and MD-2−/− cells after Pam2 stimulation.
proteins, and the lower blots were blotted against penta-his mAb for detection of His6-tagged MD-2 proteins.

Two wells of clear-cell lysates (350 µl/well) from each mutant were pooled and incubated with 3 µg biotinylated LPS (InvivoGen) and 20 µl streptavidin agarose resin (Thermo Scientific) at 4°C with constant shaking for the affinity-precipitation experiments using biotinylated LPS. The next day, supernatants were removed, and the pellets were washed three times with PBST buffer (0.05% Tween in PBS) and subjected to SDS-PAGE and WB analysis. Prestained rainbow marker (GE) and Kaleidoscope marker (Bio-Rad) were loaded into the same gel for molecular mass estimation. After being blocked with blocking buffer for 1 h, the nitrocellulose membranes were cut into two pieces at the molecular mass 60 kDa. The upper blots were blotted against anti-GFP mAb for detection of YFP-tagged TLR4 proteins, and the lower blots were blotted against penta-his mAb for detection of Hist6-tagged MD-2 proteins.

Results

Microarray analysis of the role of TLR4 in LPS signaling

We first assessed the absolute requirement of TLR4 in LPS signaling by using TLR4-deficient BMDMs. Synthetic lipid A or ultrapure LPS (that has gone through a second phenol-chloroform extraction, peptidoglycan digestion with mutanolysin and gel-filtration chromatography) were used as the stimulants to avoid stimulatory effects from potential contaminants. BMDMs from WT C57BL/6 and the TLR2 ligand, Pam2, were used as controls. TNF-α and RANTES production, which represent the activation of MyD88-dependent and -independent pathways, respectively, were assayed at the protein level by ELISA or at the RNA level by quantitative PCR (primer sequences are shown in Table I).

Compared with WT BMDMs, TLR4-deficient BMDMs did not respond to LPS or lipid A stimulation, whether judged by TNF-α production, as an example of a gene product that is dependent on the MyD88-dependent–signaling pathway, or RANTES production, as an example of the MyD88-independent–signaling pathway (Fig. 1A). This suggested that TLR4 is required for the canonical LPS response. The fact that TLR4-deficient BMDMs responded normally to Pam2 stimulation indicated that the defect in TLR4 accounted for the lack of inflammatory responses to LPS or synthetic lipid A.

To examine the existence of any noncanonical LPS-signaling pathway, we performed microarray RNA profiling in WT and TLR4-deficient BMDMs. We chose 2 h of stimulation for RNA profiling because, at this time point, mRNA expression of TNF-α and RANTES was consistent with the ELISA results (Fig. 1B). RNA was extracted after 2 h of treatment with 10 ng/ml LPS, 100 ng/ml lipid A, 10 nM Pam2 or PBS. A response was defined as any gene that was induced or repressed >1.6-fold over PBS control with a statistically significant difference (fold change >1.6 and p < 0.05 with Benjamini–Hochberg correction for multiple testing). With this approach, a set of 1396 genes was selected and analyzed in more detail. About equal numbers of genes (~700–800 genes) were responsive to each ligand in the WT macrophages (Fig. 1C). In contrast, not a single gene was responsive to lipid A or LPS in the TLR4-deficient BMDMs, whereas the Pam2 response was largely preserved in these cells (Fig. 1C). The lack of genes induced by either LPS or lipid A in TLR4-deficient macrophages in this genome-wide expression analysis showed that TLR4 is absolutely required for the initiation of an LPS response.

A comparison of the PBS-treated WT and TLR4-deficient macrophages revealed that only four genes had significantly different basal levels of expression (Fig. 1D). These genes were CD274 Ag (PD-L1), occludin/ELL domain containing 1, proteasome 26S subunit non-ATPase 4, and RIKEN cDNA 9130017C17 of unknown function. However, at the chosen significance level

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*Number of genes found in the cluster out of the total number of genes with this annotation.

*The p value for overrepresentation of GO accession in the cluster compared with randomly selecting an equal number of genes. Bonferroni corrected for multiple testing.

Table II. GO analysis of LPS-responsive macrophage gene clusters

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\( p < 0.05 \), with Benjamini–Hochberg correction, \( \sim 70 \) genes would still be expected to be false positives, even after multiple testing corrections (at \( p < 0.05 \), 5% of 1396 genes may still be false positives). In fact, we subsequently tested LPS-induced gene expression of these four genes by RT-PCR and failed to induce message, confirming that these were false positives.

We next set out to group the set of 1396 responsive genes based on their expression profile. Five main gene clusters containing the most induced and repressed genes emerged by hierarchical and K-means clustering analysis (Fig. 2A). In cluster 1, 29 genes were highly induced by all treatments in WT cells, with expression increasing \( >30\)-fold upon LPS or lipid A treatment. Cluster 2 includes moderately LPS-induced genes (\( \sim 8–30\)-fold), and cluster 3 includes slightly LPS-induced genes (\( \sim 2–8\)-fold). The expression profile of clusters 1–3 was similar, and group association was dictated by the level of induction. In contrast, cluster 4 displayed a different profile and contained genes induced by LPS and lipid A treatment but not Pam2 stimulation. Cluster 5 includes LPS-repressed genes.

To investigate whether the grouping of genes into five main clusters also reflected differences in function among these groups, we analyzed the GO definitions assigned to these genes. Statistically overrepresented GO definitions (compared with a random selection) were identified with EASE software (Table II). To visualize how genes mapped to different GO definitions, we created a network with Cytoscape software (Fig. 3). Interestingly, genes from clusters 1–3 mapped to a continuous network, whereas clusters 4 and 5 were segregated to separate networks. Thus, clusters 1–3 with similar expression profiles also contain genes with similar functions, whereas clusters 4 and 5 may contain genes with separate biological function. Interestingly, cluster 4 contains many IFN-regulated genes, with a significant overrepresentation of genes with the GO definition “response to virus.” This may indicate that the gene programs in response to LPS, but not Pam2, are also important for viral host defenses, consistent with the presence of many IFN-regulated genes. The genes downregulated by LPS found in cluster 5 are associated with protein–DNA complex assembly genes, such as histones. Perhaps downregulation of these genes is required to allow the massive inflammatory gene program to be mobilized in response to a pathogen insult. Even if clusters 1–3 map to a continuous functional network (Fig. 3), some preferences exist. Cluster 1 includes classical inflammatory cytokines, such as TNF-\( \alpha \) and IL-1\( \beta \). In contrast, cluster 2 includes immune-response genes with attributed functions in the regulation of biological processes. Cluster 3 includes many well-known immune-response genes, but no further common function could be found from the overrepresentation of GO definitions.

\textbf{Microarray analysis of the role of MD-2 in LPS signaling}

The canonical LPS-signaling pathway through TLR4 depends on the coreceptor MD-2 (11, 12, 46). To test whether MD-2 is absolutely required for any LPS response in analogy with TLR4, we determined the gene-expression profile of WT and MD-2–deficient BMDMs after 2 h of stimulation with 10 ng/ml LPS, 100 ng/ml lipid A, 10 nM Pam2, or PBS control. Basal gene expression was compared in PBS-stimulated WT and MD-2–deficient BMDMs. No gene was significantly different in the PBS-treated WT versus MD-2–deficient macrophages.

In WT BMDMs, 912 genes were significantly induced or repressed (fold change \( >1; p < 0.05 \)) after stimulation with LPS, lipid A, or Pam2 (Fig. 2B). However, in the MD-2–deficient BMDMs, not a single gene was significantly stimulated by LPS or lipid A, showing that MD-2 expression is absolutely necessary for the induction of an LPS response. In comparison, the gene-expression profile for Pam2 stimulation remained the same for WT and MD-2–deficient BMDMs, confirming that MD-2 is indeed required for LPS signaling. Noticeably, similar genes were activated by LPS and

\[ \text{FIGURE 3. A functional network of the LPS response. The statistical overrepresentation of GO definitions in clusters 1–5 from Fig. 2A was analyzed by EASE software (Table II). Genes (boxes) were mapped to GO definitions (circles) and visualized with Cytoscape software.} \]
lipid A in WT BMDMs, indicating that lipid A is the active component in LPS that triggers the proinflammatory responses.

To compare the microarray experiments done in TLR4- and MD-2–deficient BMDMs, we first identified genes present on both microarray platforms (6013 genes) by ensuring the reporter sequences aligned to the same target sequence using the basic local alignment search tool algorithm. We found that 361 genes were induced or repressed 1.3-fold by LPS in the WT BMDMs of each experiment. A mosaic map was generated for each stimulant of each genotype by GEDI software that clusters genes with a self-organizing map algorithm (Fig. 4). This analysis showed that the global expression profile induced by LPS and lipid A is very similar and absolutely dependent on both TLR4 and MD-2, whereas the global expression profile induced by Pam2 is largely unaltered by the absence of TLR4 and MD-2.

In view of the absolute need to engage TLR4/MD-2 in responding to LPS, we set out to determine the mechanistic details of receptor activation. Our analysis was designed to take into account both of these essential receptor components and was consistent with the hypothesis, first enunciated by our group in 1990 (47), that by defining the details of the receptor that engages and responds to the lipid A precursor known as lipid IVa, we would simultaneously define how LPS functions.

Our previous studies suggested that ionic intermolecular actions between positively charged residues on TLR4/MD-2 and lipid IVa are likely to be critical elements in the activation of TLR4/MD-2. Thus, we more closely examined the role of ionic interactions in TLR4 activation. We previously showed that ionic interactions at the dimerization interface are essential for the species-specific activation of lipid IVa (23, 48). In our current analysis, we set out to define the role of ionic interactions in lipid A/LPS activation.

Two sets of ionic interactions were observed between the two phosphates of lipid IVa and two patches of positive charges on mouse TLR4 (mTLR4) in our dimeric mTLR4/mMD-2/lipid IVa model (Fig. 5A, 5B). One is between the 1PO3 and K263, R337, K360 (positive patch 1) on mTLR4 within the same TLR4-MD-2-LPS complex, and the other is between the 49PO3 and K367 and R434 (positive patch 2) at the dimerization interface. To examine the functional relevance of these ionic interactions on the general lipid A/LPS response, we mutated these positively charged residues to alanine (Ala) and examined their effects by reporter-driven luciferase assay and coimmunoprecipitation (co-IP).

An mTLR4 triple mutant in which K263, R337, and K360 were replaced with Ala is strikingly impaired in lipid A responsiveness

We first examined the triple mutations of K263A, R337A, and K360A on lipid A/LPS responsiveness. As shown in Fig. 6A, when these three positively charged residues were mutated to Ala simultaneously, NF-κB activation by lipid A, LPS, or lipid IVa was significantly impaired, indicating that ionic interactions between K263, R337, and K360 and the phosphates on lipid A are essential for the activation of the MyD88-dependent pathway by these
FIGURE 6. Ionic interactions between the positively charged residues (K263, R337, and K360) on mTLR4 and the two phosphates on lipid A/LPS are essential for LPS signaling. HEK293T cells were transfected with mMD-2, WT, or the mutant K263A_R337A_K360A (M) mTLR4 constructs, as well as reporter constructs. After overnight transfection, cells were stimulated with 1000, 100, 10, or 0 ng/ml of lipid A, LPS, or lipid IVa. The next day, supernatants were removed, and NF-κB luciferase (A) or IFN-β luciferase (B) activities were measured in cell lysates. Relative luciferase unit (RLU) was normalized by Renilla luciferase. C. For WB, HEK293T cells were transfected with WT/M mTLR4 (1 μg/well) and WT mMD-2 construct (1 μg/well). After 48 h of transfection, clear-cell lysates were subjected to SDS-PAGE for direct IB with anti-GFP mAb to detect hTLR4, penta-his Ab to detect hMD-2, and anti-β-actin Ab to detect β-actin. Clear-cell lysates were also subjected to IP with Ni-NTA agarose and IB, with anti–GFP/protein A-Sepharose and IB, and IP with biotinylated LPS/streptavidin Sepharose and IB. Polyclonal anti-GFP Ab was used for IP, and monoclonal anti-GFP Ab was used for IB. Data shown are representative of one of four experiments.

Likewise, IFN-β activation by lipid A and LPS was almost abrogated after these mutations (Fig. 6B), indicating that ionic interactions with these residues are also required for the MyD88-independent pathway. As shown in Fig. 6C, protein expression (cell lysates) and mMD-2–binding activity (IP: Ni-NTA, IP: anti-GFP) were not affected by these mutations. In addition, the LPS-binding activity of the mTLR4/mMD-2 complex was not affected by these mutations (IP: biotinylated LPS). Thus, the loss of function after the triple mutation results from impaired function but not disrupted protein expression or folding that would impair LPS binding.

Positively charged K367 and R434 at the dimerization interface are also required for mTLR4 activation

Using charge-reversal mutagenesis, we previously showed that ionic interactions between K367 and R434 on mTLR4 and the phosphate on lipid IVa at the dimerization interface are essential for the species-specific activation of lipid IVa (23, 48). To assess the role of this set of ionic interactions on the general lipid A response, we mutated K367 and R434 into Ala and examined the effect by inducible luciferase assay and co-IP.

As shown in Fig. 7A, NF-κB activation was greatly reduced by lipid A and LPS after the K367A and R434A mutations; similarly, IFN-β activation was significantly impaired after the combined mutations (Fig. 7B). Meanwhile, protein expression and MD-2/LPS–binding activity were not affected by these mutations (Fig. 7C). Thus, the loss of function after the charge abolishment at positive patch 2 arises from impaired activation rather than impaired protein folding or MD-2/LPS binding.

hTLR4 loses its LPS response when the three positively charged residues R264, K341, and K362 are simultaneously changed to Ala

A similarly positively charged patch was observed when we reviewed the crystal structure of hTLR4 (21). hTLR4 interacts extensively with the negatively charged phosphates on the lipid A portion of LPS in the cocrystal structure (Fig. 5C, 5D). To address the physiologic role of these positively charged residues in the activation of the human TLR4/MD-2 receptor, we generated the triple mutant, R264A_K341A_K362A, and assessed its effect by inducible luciferase activity and co-IP.

As shown in Fig. 8A, NF-κB activation was significantly impaired in TLR4 that had the combined mutations of R264A, K341A, and K362A. Similarly, the IFN-β–inducible activity was greatly reduced after these mutations (Fig. 8B). This diminished response was not due to diminished receptor expression caused by the triple mutations (Fig. 8C). These results suggested that ionic interactions between the positively charged residues on TLR4 in positive patch 1 and the two phosphates on lipid A/LPS are essential for both MyD88-dependent and -independent pathways of TLR4 activation.

Charge reversal at the dimerization interface conferred LPS responses to the hTLR4 R264A_K341A_K362A mutant

Unlike mTLR4, with its two positively charged residues at the dimerization interface (K367 and R434; Fig. 5B), hTLR4 has two negatively charged residues at these locations (E369 and Q436; Fig. 5D). We showed that the charge differences are responsible
for the role of TLR4 in the species-specific activation of lipid IVa (23, 48). Thus, we examined the effect of charge reversal at the dimerization interface on hTLR4 activation in the presence of the R264A_K341A_K362A mutations. Purified monomeric mMD-2 protein was used to complement transfected hTLR4; mMD-2 and hTLR4 will not form a functional receptor when cotransfected in HEK293 cells because of the extremely poor expression of mMD-2 under these circumstances.

Interestingly, the quintuple mutant retained a partial lipid A response, retained a complete response to LPS, and acquired a response to lipid IVa (Fig. 9A). Again, protein expression (Fig. 9B) and MD-2/LPS binding (Fig. 9C) were not affected by these mutations. This indicated that gain of ionic interactions at positive patch 2 outweighed the loss of ionic interactions at positive patch 1 for the hTLR4/mMD-2 combination with regard to LPS responsiveness, whereas ionic interactions at positive patch 1 are required for a full response to lipid A.

**Discussions**

The initiation of host defenses against Gram− bacterial invasion is primarily dependent upon LPS recognition by TLR4/MD-2. However, the presence of an alternative LPS receptor was suggested by studies from different groups (31–33, 35, 37, 38, 49, 50), and it remained an unanswered question whether alternative means of recognizing LPS existed, especially for so-called “noncanonical responses.” To examine this possibility, with the hopes of identifying “the alternative LPS receptor,” we carried out an unbiased genome-wide screen of the mRNA transcription profile in mouse macrophages after 2 h of LPS stimulation (49). Under these conditions, we found that the TLR4/MD-2 receptor complex was absolutely required for *E. coli* LPS signaling. Although we cannot exclude the possibility that LPS-induced TLR4/MD-2–independent signaling is detectable beyond the 2-h time point, or in another cell type, or at higher LPS doses, it seems likely that mammals lack any additional receptor systems to respond to LPS.

These experiments also helped to determine the role of MD-2 in the response to bacterial lipoprotein, an important alternative means of Gram+ recognition after the LPS response, as well as an important means that the innate immune system uses for detecting Gram+ bacteria. MD-2 was reported to play a role in TLR2-mediated responses of lipoproteins (51). However, in our screen, the control Pam2 stimulation failed to initiate a differential-expression profile in WT and MD-2–deficient macrophages. As such, our screen suggested that Pam2-initiated TLR2 signaling is completely independent of MD-2.

Upon ligand recognition, TLR4 activates both the MyD88-dependent and -independent pathways, whereas TLR2 activates only the MyD88-dependent pathway. In accordance with this observation, we found that 38 genes were activated by LPS but not by Pam2 (cluster 4; Fig. 2A). At least 10 genes in this cluster are IFN-related genes. Moreover, several Pam2-induced genes (e.g., Irg1, Icos1, Traf1, and Mlp) were MyD88 independent when LPS was used as the stimulating ligand (49). This suggested that the adaptor functions of MyD88/MAL are not identical for TLR2 versus TLR4 signaling.
LPS isolated from different bacterial species have different biological effects. Even the highly conserved lipid A component of LPS differs slightly between bacterial species (52, 53). For example, *E. coli* lipid A usually has six to eight acyl chains, whereas *Yersinia pestis* produces four or six acyl chains, depending on the growth conditions (54). The tetra-acylated LPS from *Y. pestis* is less virulent and is thought to be involved in the immune evasion of *Y. pestis* and the propagation of plague (53). The characteristic structural features of *E. coli* lipid A, especially its two phosphates, are required to trigger full TLR4/MD-2 activation in human cells (25). We propose that the reason why different acylation variants of lipid A have different biological activities is not primarily because they induce different degrees of MD-2 conformational changes but because they influence the position of the phosphates in lipid A because it sits upon the hydrophobic groove of MD-2.

Lipid IVa and monophosphorylated lipid A are two lipid A variants that have interesting biological activities. Monophosphorylated lipid A has reduced proinflammatory activity (24) and is being increasingly used as a vaccine adjuvant (55–57). Furthermore, monophosphorylated lipid A was shown to bias the MyD88-independent pathway (27). To understand the underlying mechanisms, we created a series of mutants that abolished interactions with either phosphate of lipid A and examined the stimulatory activity of lipid A. We found that the removal of charge interactions with either phosphate on lipid A, when present, substantially decreased its stimulatory activity. However, the MyD88-dependent and -independent pathways were impaired to the same extent. In other words, MyD88-independent signaling was not spared from mutagenesis that abolished ionic interactions with either phosphate, contrary to what would be predicted from the findings that monophosphorylated lipid A is a TRIF-biased agonist (27).

Lipid IVa is an LPS agonist in mouse cells but is an antagonist in human cells (57). We previously showed that in the presence of functional mMD-2 proteins, hTLR4 gained lipid IVa responsiveness after charge reversal mutagenesis at the dimerization interface (i.e., after the E369K_Q436R mutations) (23). In this article, we showed that in the presence of functional mMD-2 proteins, hTLR4 retained lipid IVa responsiveness after the combined mutations of E369K_Q436R at the dimerization interface and R264A_K341A_K362A at positive patch 1. Therefore, the loss of ionic interactions at positive patch 1 did not impair the ability of the mutant hTLR4/mMD-2 complex to respond to lipid IVa. The importance of these observations is that we can now state with some degree of confidence that ionic interactions at the dimerization interface are the ultimate driving force for the species-specific activation of lipid IVa.

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Disclosures
The authors have no financial conflicts of interest.

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