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Individual LPS Responsiveness Depends on the Variation of Toll-like Receptor (TLR) Expression Level

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Abstract An individual's immune response is critical for host protection from many different pathogens, and the responsiveness can be assessed by the amount of cytokine production upon stimulating bacterial components such as lipopolysaccharide (LPS). The difference between individuals in their peripheral blood mononuclear cells (PBMC) responsiveness to LPS, a Gram-negative endotoxin, was investigated from 27 healthy individuals. We observed a large variation in IFN γ production among different individuals. The PBMC of the consistently three highest and three lowest IFN γ producers were investigated. Since previous studies described that a single point mutation in the coding region of TLR2 and TLR4 is linked to the individual responsiveness to pathogenic bacterial infections, we first examined the known point mutations in the coding region of TLR2^{Pro681His}, TLR4^{Pro714His} located in the cytoplasmic regions of the Toll-like domain as well as TLR4^{Asp299Gly} located in the extracellular region. None of these mutations were associated with an individual's responsiveness to LPS, despite the presence of TLR4^{Asp299Gly} mutation. Further investigation revealed that the variation of PBMC responsiveness to LPS among healthy individuals was due to constitutive expression levels of TLR4 and TLR2. This result is consistent with an aging-related low expression of Toll-like receptors in the mouse model of LPS responsiveness. The present study therefore suggests that the constitutive expression levels of TLR2 and TLR4 may contribute to the individual response to LPS.

Keywords: Cytokine, lipopolysaccharide, Toll-like receptor

Toll-like receptors (TLR) were first identified in *Drosophila*. Soon after, mammalian homologs were characterized as

mediators of the immune response to microbial ligands [21, 37]. Although CD14 has been recognized as a co-receptor for lipopolysaccharide (LPS) [30], TLR2–9 have also been identified for the receptors of microbial and pharmaceutical products [1, 2, 8]. TLRs are pattern recognition molecules and it has been suggested that TLR can discriminate between Gram-negative and Gram-positive organisms [31]. Gram-positive bacteria and yeast cell wall components are ligands for TLR1, 2, and 6, whereas the predominant Gram-negative bacterial product LPS is a ligand for TLR4. Recent data examining LPS responses in TLR2-deficient mice indicate that TLR2 is not required for LPS signaling when TLR4 is present [7, 29, 31]. Numerous reports have indicated that LPS is not a ligand for TLR2, the TLR2-stimulating activity of commercial LPS being due to the presence of contamination with other microbial components triggering TLR2 signaling [9].

Upon stimulating TLRs, there is the recruitment of adaptor molecules in the cytoplasm, such as MyD88 [8, 12, 13] and Toll/IL-1R domain-containing adaptor protein (Triap) [4, 10] consequently activating IL-1R-associated kinases (IRAKs) by phosphorylation. Then, IRAK downstream signaling molecules activate tumor necrosis factor receptor associated factor-6 (TRAF-6) [20, 38], transforming growth factor- β -activated kinase-1 (TAK1) [19], I- κ B kinase, I- κ B degradation, and finally NF- κ B nuclear translocation, respectively. Bacterial components including LPS induce various cytokine productions in mouse macrophage cells mainly through the NF- κ B signal pathway [14, 18]. The measurement of cytokine productions is the easiest assessment for cell responsiveness, which is used for different *in vitro* models [15, 17, 28].

A point mutation within mice TLR4^{Pro712His} [24] and TLR2^{Pro681His} [31] underlines the bacterial pathogen hyporesponsiveness. Although there has not been reported a

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point mutation within human TLR4 and TLR2 in the Toll-like domain structural basis of human, TLR2 and TLR4 explain an importance of those residues for interacting with downstream signaling molecules [36]. However, recently, a point mutation in the extracellular domain of TLR4^{Asp299Gly} has been suggested to be responsible for the hyporesponsiveness of healthy individuals after LPS stimulation [23].

Although the variability of LPS responsiveness in many mice models has been identified by a positional cloning with a particular inbred strain of mice, little is known about the variability of LPS responsiveness in individuals except for a point mutation in the extracellular domain of TLR4, which may be a unique case. Thus, it is possible that a common causative factor may exist for the variability of LPS responsiveness in healthy individuals. In the present study, we demonstrate that the constitutive expression rates of TLR4 and TLR2 influence the variability of LPS responsiveness in healthy individuals.

MATERIALS AND METHODS

Reagents

Histopaque, LPS, and Tri-Reagent were obtained from Sigma (St. Louis, MO, U.S.A.). The PCR purification kit was purchased from QIAGEN (Valencia, CA, U.S.A.). Superscript II RT and DNA *Taq* polymerase were purchased from Invitrogen (Carlsbad, CA, U.S.A.). IL-12 was obtained from Peprotech (Rocky Hill, NJ, U.S.A.). The liquid-phase electrochemiluminescence (ECL) reagents were obtained from Bioveris (Gaithersburg, MD, U.S.A.).

Cytokine Assays

PBMC were isolated from healthy donors using Histopaque (Sigma). The combined Colorado Investigational Review Board approved the study. PBMC (1.5×10^7) were seeded in 96-well plates in 0.2 ml of RPMI containing 10% FCS. Cells were then stimulated with LPS (Sigma), phenol-extracted LPS (pLPS, excluding TLR2 activation was prepared as described before [26]), or IL-12 (Peprotech) plus IL-18, which was produced as described [16]. The plates were placed in a cell culture incubator for 24 h. The culture supernatant was collected for human IFN γ and IL-10 measurements. The liquid-phase ECL method was used to measure different cytokines in cell culture media (Bioveris). The amount of electrochemiluminescence was determined using an Origen Analyzer (Bioveris).

RT-PCR

Total RNA was isolated with Tri-Reagent (Sigma) from freshly isolated PBMC. Superscript II RT (Invitrogen) converted 2 μ g of total RNA to first-strand cDNA and then the PCR reaction was performed at 94°C for 45 sec, 70°C

for 2 min, and 59°C for 1 min for 30 cycles. A pair of sense and reverse primers was used for the RT-PCR; for TLR2, 5' TGT GGG TTG AAG CAC TGG ACA (sense) and 5' CTT AAA TAT GGG AAC CTA GGA C (reverse); for TLR4, 5' CAC GGA GGT GGT TCC TAA TAT (sense) and 5' CCT CTT CAG ATA GAT GTT GCT (reverse); for CD14, 5' GGT TCG GAA GAC TTA TCG ACC (sense) and 5' ATT CTG TCT TGG ATC TTA GGC (reverse); for GAPDH, 5' ACC ACA GTC CAT GCCATC AC (sense) and 5' TCC ACC ACC CTG TTG CTG TA (reverse). The PCR products were subjected to 1% agarose gel electrophoresis and photographed.

DNA Sequencing

For DNA sequencing, the PCR products of TLR2 and TLR4 were amplified with the following sense and reverse primers for specific regions of the known mutation sites of both TLR2/TLR4; for TLR2^{Pro681His}, AAT GTC ACA GGA CAG CAC TGG (sense) and 5' GCA GAA GCG CTG GGG AAT G (reverse); for TLR4^{Asp229Gly}, 5' TGG AGA CAA ATC TAG CAT CTC (sense) and 5' TCA ATA GTC ACA CTC ACC AGG (reverse); for TLR4^{Pro714His}, 5' GTC TAT AAG TTC TAT TTT CAC C (sense) and 5' TCC AGC AGG GCT TTT CTG AGT (reverse). These PCR products were cleaned with a PCR purification kit (QIAGEN) and then sequenced at the core facility in the University of Colorado Health Sciences Center. For DNA sequencing, the following primers were used: for TLR2^{Pro681His}, 5' CAG CAG GAA CAT CTG CTA TGA; for TLR4^{Asp229Gly}, 5' AGA AGT CCA TCG TTT GGT TC; for TLR4^{Pro714His}, 5' ATG AGG ACT GGG TAA GGA ATG.

RESULTS

Individual Variation in the Response to LPS

In order to investigate LPS responsiveness in individuals, human PBMC were freshly isolated from 27 healthy volunteers. Among these volunteers, three individuals were high producers (500 pg/ml) of IFN γ , and three individuals were low producers (50 pg/ml) of human IFN γ , whereas 21 volunteers remained between 50 and 500 pg/ml of IFN γ induction after 24 h by LPS (Fig. 1). Phenol-extracted LPS (pLPS)-induced IFN γ , excluding TLR2 activation, was similar to LPS-induced IFN γ . In parallel, PBMC from the same individuals were stimulated with IL-12 plus IL-18, which directly stimulated T-cells in PBMC. All 27 individuals showed pronounced induction of IFN γ compared with LPS or pLPS (Fig. 1). These results demonstrated that there was no defect in the T-cell response to IL-12 or IL-18.

We next examined other cytokines produced by three individuals who showed high response and low response to LPS, which may postulate an initial observation of

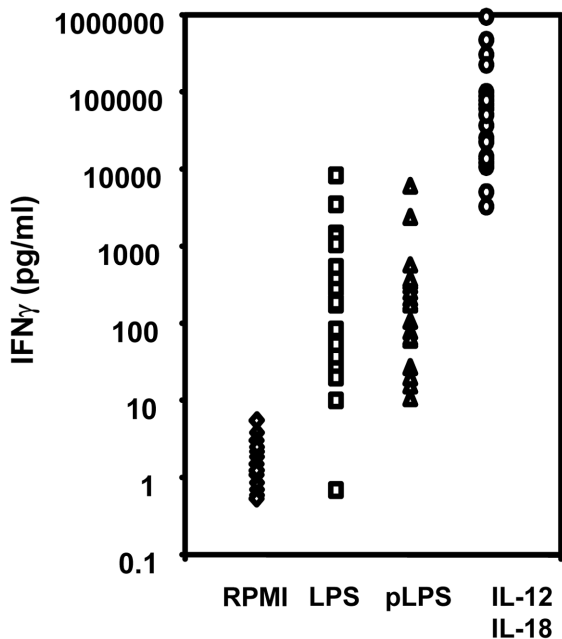


Fig. 1. Variability between individuals in the response to LPS by IFN γ induction.

Human peripheral blood mononuclear cells (PBMC) were isolated from 27 healthy donors and were stimulated as indicated [LPS (1 μ g/ml), pLPS (1 μ g/ml), or IL-12 (1 ng/ml) plus IL-18 (20 ng/ml)]. ECL measured the IFN γ in PBMC culture supernatant.

individual LPS responsiveness by IFN γ induction. The production of Th2 cytokine IL-10 (Fig. 2B) showed a contrary result to IFN γ induction (Fig. 2A). IL-10, known as a suppressor of Th1 cytokines, was highly induced in individuals with poor responsiveness to LPS in terms of IFN γ production.

Examination of Single Point Mutation in TLR2 and TLR4

We investigated whether the variation between high and low responders was due to the known TLR2 and TLR4

A TLR2 (Intracellular domain)

P681H

	Asp	Phe	Ile	Pro	Gly	Lys	Amino acid
TLR2	GAC	TTC	ATT	CCT	GGC	AAG	Wild type
V03	GAC	TTC	ATT	CCT	GGC	AAG	High
V08	GAC	TTC	ATT	CCT	GGC	AAG	Low
V11	GAC	TTC	ATT	CCT	GGC	AAG	High
V14	GAC	TTC	ATT	CCT	GGC	AAG	Low
V26	GAC	TTC	ATT	CCT	GGC	AAG	High
V43	GAC	TTC	ATT	CCT	GGC	AAG	Low

B TLR4 (Extracellular domain)

D299G

	Tyr	Leu	Asp	Asp	Ile	Amino acid
TLR4	TAC	CTC	GAT	GAT	ATT	Wild type
V03	TAC	CTC	GAT	GRT	ATT	High
V08	TAC	CTC	GAT	GRT	ATT	Low
V11	TAC	CTC	GAT	GRT	ATT	High
V14	TAC	CTC	GAT	GRT	ATT	Low
V26	TAC	CTC	GAT	GAT	ATT	High
V43	TAC	CTC	GAT	GAT	ATT	Low

C TLR4 (Intracellular domain)

P714H

	Asp	Phe	Ile	Pro	Gly	Val	Amino acid
TLR4	GAC	TTT	ATT	CCC	GGT	GTG	Wild type
V03	GAC	TTT	ATT	CCC	GGT	GTG	High
V08	GAC	TTT	ATT	CCC	GGT	GTG	Low
V11	GAC	TTT	ATT	CCC	GGT	GTG	High
V14	GAC	TTT	ATT	CCC	GGT	GTG	Low
V26	GAC	TTT	ATT	CCC	GGT	GTG	High
V43	GAC	TTT	ATT	CCC	GGT	GTG	Low

Fig. 3. DNA sequence alignment of six individuals, three high and three low responders.

The sequence alignments data are (A) the sequencing result of the intracellular domain in the region of amino acid residue Pro681 of TLR2; (B) extracellular domain in the region of amino acid residue Asp229 of TLR4; (C) intracellular domain in the region of amino acid residue Pro712 of TLR4. High or low responder is indicated on the right of the figure.

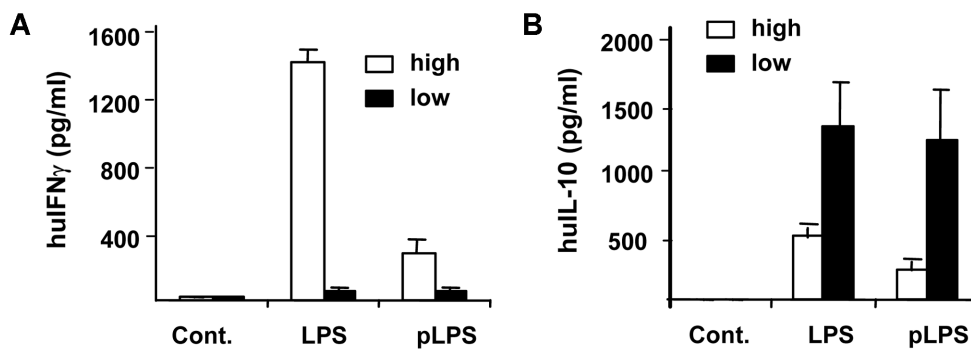


Fig. 2. Comparison of the three high or three low responders in cytokine inductions.

(A) LPS or pLPS-induced IFN γ and (B) LPS or pLPS-induced IL-10 in three high or low responders. The graphs represent the mean of the three individuals of high or the three individuals of low responders from Fig. 1.

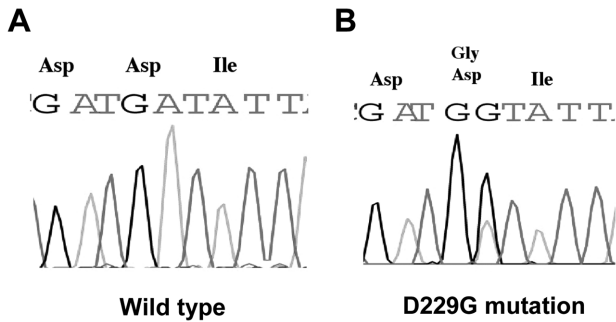


Fig. 4. DNA chromatography sequencing result of the RT-PCR product.

A. The wild-type volunteer shows no mutation at amino acid residue Asp299 in the extracellular domain of TLR4. The result represents one of two volunteers. **B.** The heterozygote volunteer shows a mixed A signal with R(G/A). The result represents one of four volunteers.

single point mutation in the cytoplasmic region of the Toll-like domain. The complete coding regions of TLR2 and TLR4 RT-PCR products were amplified with a specific sense and reverse primer in order to examine a possible point mutation in the Toll-like domain of TLR2 and TLR4. As shown in Figs. 3A and 3C, both the three high and three low responders exhibited no mutation in TLR2^{Pro681His} or in TLR4^{Pro714His}. Although the extracellular domain of TLR4^{Asp299Gly} had a point mutation in individuals as shown in Fig. 3B, there was no correlation between the mutation and LPS responsiveness in individuals. The DNA sequencing chromatography result of one high and low responder is shown in Fig. 4. The two volunteers (26 and 43) had no mutation as depicted in Fig. 4B, but the other four volunteers (3, 8, 11, and 14) had a heterozygote point mutation.

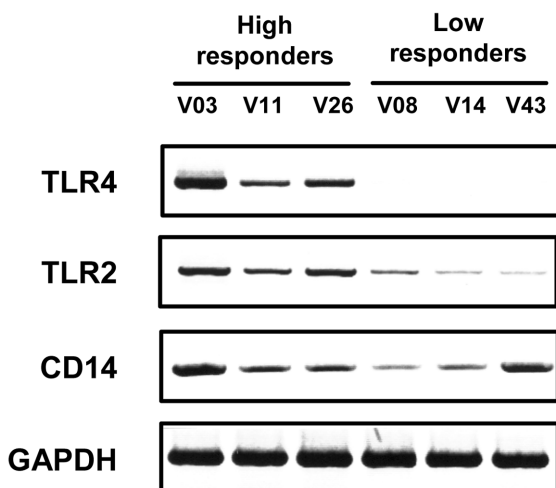


Fig. 5. The examination of TLR4, TLR2, and CD14 expression by RT-PCR.

The left three lanes are individual high responders to LPS and the next right three lanes are low responders, as indicated at top of the figure. The RT-PCR of TLR4 (top), of TLR2 (middle), and of CD14 (bottom) are shown.

Constitutive Steady-State Expression of TLR4 and TLR2

We further investigated individual LPS responses by performing semiquantitative RT-PCR for genes directly related to LPS activity. The total RNA samples from three high and three low responders were examined for the complete TLR4 coding region by RT-PCR. The results revealed that the expression level of TLR4 in the high responders was consistently greater than the level in low responders (Fig. 5). Similar differences in the level of TLR2 expression were also observed between high and low responders. However, there was no significant difference in the level of CD14 expression between high and low responders (Fig. 5).

DISCUSSION

In the present study, we demonstrate that the variability of LPS responsiveness among individuals is associated with differences in the level of constitutive expression of TLR2 and TLR4. TLR4 is a receptor for endotoxin, the glycolipid present on the outer wall of all Gram-negative bacteria [7, 31]. Patients with Gram-negative bacterial infection can exhibit a severe clinical condition called sepsis, which eventually leads to septic shock with multi-organ failure and a high mortality. Gram-negative bacterial cell wall is an important mediator of these symptoms, which is called endotoxin (LPS). LPS was identified as the main active component of the Gram-negative bacterial cell wall, which induces hyperactivation during Gram-negative sepsis, and its binding protein (LBP) structure was characterized [27].

Arbour *et al.* [3] reported a single nucleotide A896G substitution, resulting in replacement of the aspartic acid residue with glycine at amino acid residue 299 in the extracellular domain of human TLR4. They have shown that a single point mutation of Asp299Gly decreased LPS-induced NF- κ B gel shift by only 25%, which was compared with wild type in transfected THP-1 cells. Among 74 individuals, 9 were heterozygote and one was homozygote in this mutation. Although Arbour *et al.* [3] suggested a cause of LPS hyperresponsiveness by a single point mutation Asp299Gly, this finding does not account for the actual frequency of LPS responsiveness as well as a cause of LPS hyperresponders. In our study from freshly isolated human PBMC, the LPS responsiveness variability occurred more frequently. For example, three of 27 were hyporesponders and three of 27 were hyperresponders. Contrary to the result of a single point mutation, our finding of the variability of LPS responsiveness was tightly correlated to the level of the constitutive expression rate of TLR4, but not CD14. Soluble or glycosylphosphatidylinositol-anchored (GPA) membrane-bound CD14 is required for LPS signaling [35]. Interestingly, TLR2 expression was also low in individuals low responsive for LPS (Fig. 5).

Certain transcription factors involvement in the promoters of TLR2 [6, 23, 34] and TLR4 [11, 25] modulate the expression of TLR2 and TLR4, respectively. It is likely that the constitutive expression levels of TLRs contribute to the variability in the response to LPS. TLR2 and TLR4 promoters are distinct in their regulation of each putative 5'-proximal promoter activation without the typical TATA box. The enhance elements of NF- κ B and Sp1 in the 5' upstream region of the TLR2 gene are necessary for maximal transcription of murine TLR2 induced by *Mycobacterium avium* [34]. However, human TLR2 constitutive expression is controlled by two Sp1 sites, including the involvement of a PU1 site within -220 of the transcription initiation site of the TLR2 promoter [6]. In contrast to TLR2, a PU1 site in the proximal promoter region of TLR4 [25] tightly regulates TLR4 constitutive expression. It is possible that the difference in the constitutive expression levels in TLR4 and TLR2 contributing to individual LPS responsiveness in PBMC is dictated by PU1 or other transcription factors. The control of these transcription factors in high and low LPS responders remains unknown.

Recently, an age-related decline in immune function has been observed between young (2–3 months) and old (18–24 months) C57BL/C mice [26], due to the decrease of TLRs expression and function. In humans, the age-related decline of immune response is due to a decreasing signal sensitivity of p38 MAPK rather than the expression level of the Toll-like receptor [33]. However, the reduction of p38 MAPK was not significant in that report. This study illustrates the decline in immune response, which is a hallmark of ageing in individuals that may be correlated with the effectiveness of vaccination [5] as well as explain age-related change in the adaptive immune response [22]. Although the present study was carried out in healthy male and female volunteers with ages between 20 and 40 years, our data and Renshaw *et al.* [26] consent in the explaining that response to LPS tightly correlates to the TLRs expression. In addition to this, Van der Graaf *et al.* [32] recently described that no differences in either the production of the proinflammatory cytokine TNF α or the antiinflammatory cytokine interleukin-10 were observed between volunteers with the wild-type allele, volunteers heterozygous for the Asp299Gly allele, and one volunteer homozygous for the TLR4 variant.

The present study suggests that the constitutive TLRs gene expression contributes to LPS responsiveness in individuals. Although our finding and the Renshaw *et al.* [26] report are independent investigations, the two studies comply in that there is a correlation between TLRs gene expression rate and LPS responsiveness. The low or high expression rate of TLR2/TLR4 on the cell surface probably influences the downstream events of LPS signal triggering an endotoxin response. These data encourage further study in the regulation of constitutive TLR2 and TLR4 gene expression correlated to an individual's LPS responsiveness.

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