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Toll-Like Receptor 4, But Not Toll-Like Receptor 2, Is a Signaling Receptor for *Escherichia* and *Salmonella* Lipopolysaccharides

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Two members of the mammalian Toll-like receptor (TLR) family, TLR2 and TLR4, have been implicated as receptors mediating cellular activation in response to bacterial LPS. Through the use of mAbs raised against human TLR2 and TLR4, we have conducted studies in human cell lines and whole blood to ascertain the relative contribution of these receptors to LPS induced cytokine release. We show that the contribution of TLR2 and TLR4 to LPS-induced cellular activation correlates with the relative expression levels of these two TLRs in a given cell type. In addition, we have found that significant differences in cell stimulatory activity exist between various smooth and rough LPS types that cannot be ascribed to known LPS structural features. These results suggest that impurities in the LPS may be responsible for some of the activity and this would be in agreement with recently published results of others. Upon repurification, none of the commercial LPS preparations activate cells through TLR2, but continue to stimulate cells with comparable activity through TLR4. Our results confirm recent findings that TLR4, but not TLR2, mediates cellular activation in response to LPS derived from both *Escherichia coli* and *Salmonella minnesota*. Additionally, we show that TLR4 is the predominant signaling receptor for LPS in human whole blood. *The Journal of Immunology*, 2000, 165: 5780–5787.
human TLR4 acquire the ability to activate NF-κB in response to LPS (22, 23). Thus, genetic and biochemical evidence has identified TLR4 as a receptor that mediates cellular activation in response to LPS.

The idea of whether TLR2 or TLR4 represents the physiologically relevant LPS receptor has been the source of some debate especially as recently generated TLR2 knockout mice appear to have no significant defects in LPS responsiveness (24). In this study, we have studied the contribution of both TLR2 and TLR4 to LPS-induced production of TNF-α and IL-8 in both human cell lines and human whole blood. Through transfection studies and the use of blocking monoclonal anti-TLR2 and anti-TLR4 Abs, we have found that the contribution of each of the two receptors to LPS-induced inflammatory responses is dependent upon both the cell type used and the source of the LPS. Most importantly, we have observed that after repurification, commercial preparations of both *Escherichia coli* and *Salmonella minnesota* LPS no longer induce cellular activation through TLR2, indicating that impurities in these LPS preparations are responsible for the observed TLR2-mediated signaling. Our results show that TLR4, and not TLR2, is the predominant receptor mediating LPS-induced cellular activation in human whole blood.

### Materials and Methods

#### Reagents and Abs

Heat-killed *Staphylococcus aureus* (HKSA) was a kind gift of Dr. Colleen Fears (The Scripps Research Institute, La Jolla, CA). Soluble peptidoglycan (PGN) purified from *S. aureus* was a kind gift of Dr. Roman Dziarski (Indiana University School of Medicine, Gary, IN). All commercial LPS preparations were purchased from List Biological Laboratories (Cambell, CA). Polymyxin B was obtained from Sigma (St. Louis, MO). The anti-CD14 mAb 63D3 was obtained from the American Type Culture Collection (Manassas, VA). The anti-CD14 mAb 28C5 was a gift from Dr. A. Moriarty and Dr. D. Leturcq (R.W. Johnson Pharmaceutical Research Institute, La Jolla, CA). The anti-TLR2 mAbs mAb 2380 and mAb 2392 have been previously described (25). The mAbs HTA405, HTA414, and HTA1216, against human TLR4, were generated by immunizing mice with TLR4-expressing Baf3 cells (23). Spleen cells from immunized mice were fused with SP2/O myeloma cells and hybridomas were chosen that specifically stain TLR4-expressing Baf3 cells as described (23).

#### Repurification of LPS

Commercial LPS preparations were repurified in endotoxin-free water containing 0.2% triethylamine followed by vortexing. A portion of each commercial LPS preparation was repurified using a modified phenol-water extraction procedure followed by ethanol precipitation as described (26–28). Recovery of LPS was determined by colitose assay for smooth LPS types and 3-deoxy-D-manno-octulosonic acid assay for rough LPS types as described (29). LPS recoveries were typically 65–85% and were confirmed by Northern blot analysis.

#### Construction of TLR4 expressing THP-1 cell lines

The cDNA encoding full length TLR4 was amplified by PCR using the primers 5′-CCTTGAGAACGTCGACCTATCAGATAGATGTC-3′ and 5′-CCTTTGGATCCGTCGACCTATCAGATAGATGTC-3′. The resulting amplified DNA was cloned as a Xhol/BamHI fragment into the Xhol and BamHI sites of the retroviral vector pMSCV-blasto. Cells containing stably integrated vector sequences were selected for 6μg/ml blasticidin. Cells containing stably integrated vector sequences were selected for 5 μg/ml of medium containing 5 μg/ml blastidcin. Cells containing stably integrated vector sequences were selected for 3 wk in medium containing 5 μg/ml blastidcin.

#### Cell activation assays

THP-CD14 cells were grown in RPMI 1640 medium containing 10% FBS, 1% penicillin/streptomycin/glutamine, and 800 μg/ml G418, to maintain CD14 expression. On the day of the assay, the cells were washed three times in serum-free RPMI 1640 medium and resuspended in the same medium containing 2% human serum. About 10^5 cells were added to each well of a 96-well plate and activated with various agonists as indicated in the figures. Upon the addition of an agonist, the cells were placed in an incubator at 37°C and 5% carbon dioxide for 6 h. Where indicated, Abs were preincubated with cells for 30 min at a concentration of 10 μg/ml before agonist addition.

U373 cells, a human astrocytoma cell line, were grown in RPMI 1640 medium containing 10% FBS and 1% penicillin/streptomycin/glutamine. The day before the assay, the cells were trypsinized, transferred into 96-well plates, and allowed to recover overnight. The adherent cells were washed three times with serum-free RPMI 1640 medium. Cells were stimulated for 6 h in the presence of 2% human serum using Re595 or 0111B4 LPS as indicated in the figure legends. The amount of LPS used in the Ab blocking experiments reflects the fact that the average molecular mass of rough Re595 LPS is 10-fold less than that of smooth 0111B4 LPS. Where indicated, Abs were preincubated with cells for 30 min at a concentration of 10 μg/ml before agonist addition.

#### PCR analysis

Total RNA was isolated from U373 cells using TRizol reagent (Life Technologies, Rockville, MD) according to protocols supplied by the manufacturer. The synthesis of cdNA was performed, in the presence and absence of reverse transcriptase, using the Superscript Preamplification system (Life Technologies) and oligo(dT) as recommended by the manufacturer. PCR amplification of 500 ng cDNA was performed using *Taq* polymerase (Life Technologies) and TLR2- or TLR4-specific primers. The sequence of the primers for TLR2 were 5′-GGCCGCAAGAAATCCTGTGT-3′ and 5′-TTTCCCAACCATGAGCATC-3′. The sequences of the primers for TLR4 were 5′-TGAAGCAGTCGTGCTGTAAC-3′ and 5′-CAGGGGTTTCTT TACC-3′. PCR products were analyzed on gels composed of 2% NuSieve (FMC, Chicago, IL) and 1% agarose and the DNA was visualized with ethidium bromide.

#### Northern blot analysis

Total RNA was isolated from either THP-1 cells or HUVECs using TRizol reagent (Life Technologies) according to protocols supplied by the manufacturer. RNA samples, 10 μg per lane, were separated on 1% agarose-formaldehyde gels and transferred to nitrocellulose (BA85, Schleicher & Schuell, Keene, NH) in 20× SSC overnight (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate). The membranes were prehybridized for 6 h at 60°C in hybridization buffer (50 mM PIPES/50 mM sodium phosphate (pH 7), 100 mM NaCl, 0.1 mM EDTA, 5× SDS containing 20 μg/ml yeast RNA and 50 μg/ml salmon sperm DNA) then hybridized overnight in the same buffer containing 32P-labeled DNA probes for either TLR2, TLR4, or GAPDH. The filter was washed with 1× SSC followed by autoradiography. The probe for TLR2 was generated by random priming of the 1002-bp NdeI to EcoRl fragment of the TLR2 gene. The probe for TLR4 was generated by random priming of the 621-bp PstI to EcoRl fragment of the TLR4 gene. The probe for GAPDH was generated by hybridizing the two partially complementary oligonucleotides for GAPDH, 5′-ATGTTCCAGTGA TTGATCCACCCAGCGCAATGTCACGGCAGCTGAGTGC-3′ and 5′-ATTCGCGTCTGGAAGATGGTGATGGCCTTCCCAGGTCGTC-3′. PCR products were analyzed on gels composed of 2% NuSieve (FMC, Chicago, IL) and 1% agarose and the DNA was visualized with ethidium bromide.

#### Flow cytometry analysis

THP-CD14 cells were washed twice in ice cold FACS buffer (10 mM HEPES (pH 7.4), 150 mM NaCl, 1% BSA). Approximately 10^5 cells were incubated for 1 h on ice in FACS buffer containing 5% normal rabbit serum and 5 μg/ml primary Ab. The cells were washed and labeled in FACS buffer containing 5% normal rabbit serum and FITC sheep anti-mouse IgG (PharMingen, La Jolla, CA). After a final wash, the cells were resuspended in FACS buffer and analyzed by flow cytometry using a FACSscan flow cytometer (Becton Dickinson).

#### Whole blood activation assay

Whole blood from healthy donors was collected into tubes containing heparin the day of the assay. The blood was diluted 1:4 in RPMI 1640 medium...
and aliquoted into 96-well plates. Upon the addition of agonist, the plate was placed in an incubator at 37°C and 5% carbon dioxide for 4 h. Where indicated, Abs were preincubated in blood for 30 min at a concentration of 10 μg/ml before agonist addition.

ELISAs

After incubation, cell supernatants were removed and assayed for cytokine production by standard sandwich ELISA using 96-well Immunlon plates (Dynatech Laboratories, Chantilly, VA). The TNF-α ELISA was performed using mAbs 68B6A3 or 68B2B3 for capture and the biotinylated mAb 68B3C5 (BioSource International, Camarillo, CA) followed by streptavidin HRP for detection. The IL-8 ELISA was performed using the mAb MAB208 for capture and a biotinylated polyclonal rabbit anti-human IL-8 Ab (R&D Systems, Minneapolis, MN) followed by streptavidin HRP for detection. The IL-6 ELISA was performed using polyclonal goat anti-human IL-6 (R&D systems, Minneapolis, MN) for capture and polyclonal rabbit anti-human IL-6 (Endogen, Woburn, MA) followed by HRP-conjugated goat-anti-rabbit IgG for detection (BioSource International). All ELISAs were developed using o-phenylenediamine as a substrate, and OD was determined at a wavelength of 490 nm using a Spectramax plate reader and software (Molecular Devices, Sunnyvale, CA). All values were interpolated from either a log-log or a four-parameter fit of a curve generated from appropriate standards.

Results

Previous studies have shown that THP-1 cells, a human monocytic cell line, are responsive to low concentrations of LPS. The LPS responsiveness of these and other cells is dependent upon the presence of the cell surface LPS receptor CD14 (34–36). THP-1 cells can be induced to express CD14 either by differentiation or by stable transfection, as shown by generation of the THP1-CD14 cell line (33). To ascertain the role of TLR4 in mediating LPS-induced cellular activation and cytokine production, we generated THP1-CD14 cells that overexpress TLR4 by retroviral gene transfer (see Materials and Methods). In addition, empty vector control THP1-CD14 cells were similarly generated. These two cell lines were subsequently compared for their responsiveness to a variety of agonists as measured by production of TNF-α and IL-8. Overexpression of TLR4 had no effect on the magnitude or sensitivity of THP1-CD14 cells in response to PMA, HKSA, or PGN (Fig. 1). In contrast, THP1-CD14 cells that overexpress TLR4 exhibited increased sensitivity and responsiveness to both Re595 LPS and 0111B4 LPS. These effects on cellular activation were reflected in measurements of both TNF-α and IL-8 release. Taken together, these results show that TLR4 enhances the ability of THP1-CD14 monocytes to respond to LPS. It is noteworthy that the magnitude of the cytokine response of THP1-CD14 cells to 0111B4 LPS was at least 20-fold higher than the response to Re595 LPS.

Previous studies have implicated both TLR2 and TLR4 as receptors mediating LPS-induced cellular activation. Therefore, we used mAbs against these receptors to determine the contribution of TLR2 and TLR4 to LPS-induced cytokine release. The anti-TLR2 mAbs used were mAb 2380 and mAb 2392 and the anti-TLR4 mAbs used were HTA405, HTA414, and HTA1216. In addition, we tested the effects of two anti-CD14 mAbs, 63D3 and 28C5, on cellular activation. As shown in Fig. 2, none of the aforementioned Abs had any effect on PMA-mediated activation of THP1-CD14 cells as measured by TNF-α or IL-8 production. In addition, none of the anti-TLR4 Abs had any measurable effect on HKSA- or PGN-mediated cytokine release. However, cellular activation induced by either HKSA or PGN was almost completely blocked by mAb 2392, an antagonistic anti-TLR2 Ab.

Interestingly, the anti-TLR2 Abs had no effect, while two of the anti-TLR4 Abs inhibited over half of the Re595 LPS-induced cytokine release from THP1-CD14 cells. In sharp contrast, the anti-TLR2 Ab mAb 2392 inhibited almost 80% of cytokine release induced by 0111B4 LPS while the anti-TLR4 Abs were only marginally inhibitory. As observed in Fig. 1, cytokine release of THP1-CD14 cells in response to Re595 LPS was at least 20-fold weaker than to 0111B4 LPS. Polymyxin B, a cationic peptide that inhibits LPS-induced cellular activation, blocked at least 80% of both Re595- and 0111B4-mediated cytokine release. In addition, 28C5, an Ab that blocks binding of LPS to CD14, greatly inhibited cellular responses to both Re595 LPS and 0111B4 LPS. Taken together, these results indicate that TLR2 and TLR4 are the predominant receptors of THP1-CD14 cells that mediate activation in response to 0111B4 LPS and Re595 LPS, respectively. Thus, the relative contribution of TLR2 and TLR4 to LPS-induced monocyte activation is dependent upon the type or source of LPS used.

To ascertain the generality of the above results we also tested the effects of the anti-TLR2 and anti-TLR4 Abs on the LPS-induced activation of U373 cells. U373 cells are a cell line derived
from a human astrocytoma and release IL-6 in response to LPS in a CD14-dependent fashion (37). In contrast to THP1-CD14 cells, Re595 LPS and 0111B4 LPS induced roughly comparable levels of cytokine release in U373 cells (Fig. 3B). We also observed that the anti-TLR4 Ab HTA405 inhibited 50 and 20% of IL-6 release from U373 cells induced with Re595 LPS and 0111B4 LPS, respectively (Fig. 3C). As expected, activation mediated by either Re595 or 0111B4 LPS was almost completely abrogated both by the anti-CD14 Ab 28C5 and by polymyxin B. Surprisingly, the anti-TLR2 Abs had no effect on cellular activation mediated by 0111B4 LPS indicating that, in contrast to THP1-CD14 cells, TLR2 makes no contribution to this event in U373 cells. This observation prompted us to examine the expression of TLR2 and TLR4 in U373 cells. RT-PCR analysis clearly detected the presence of TLR4 message but did not detect any TLR2 message in U373 cells (Fig. 3A). This finding has been previously noted (38), and demonstrates that U373 cells do not express TLR2. In support of this, FACS analysis indicates that cell surface expression of TLR2 is much higher than that of TLR4 in THP1-CD14 cells (Fig. 4B). Taken together with the data obtained using U373 cells, these results suggest that the cell-type-specific contribution of TLR2 and TLR4 to 0111B4 LPS-induced cellular activation may simply reflect different TLR expression levels. In support of this we have shown that overexpression of TLR4 in THP1-CD14 cells renders them more responsive to LPS (Fig. 1) and this enhanced response is inhibited by anti-TLR4 Abs (data not shown).

Despite the significant expression level of TLR2 and the strong contribution of TLR2 to cytokine release induced by HKSA, PGN, or 0111B4 LPS, there is no measurable contribution of this receptor to cytokine release induced by Re595 LPS.

0111B4 is an E. coli-derived smooth-type LPS, possessing lipid A, inner core, outer core, and a large repeating oligosaccharide unit known as the O-Ag. In contrast, Re595 LPS is a deep rough type LPS, derived from an S. minnesota mutant, and missing part of the inner core as well as all of the outer core and O-Ag (40). We wanted to determine whether a structural difference between
0111B4 and Re595 allowed the former LPS, but not the latter LPS, to act as an activator of TLR2 signaling. To this end, we tested the effect of the anti-TLR Abs on a number of smooth (wild type, 0111B4), rough (Ra) or deep rough (Re595, D31m4) LPS types derived from either \textit{S. minnesota} or \textit{E. coli}. Using THP1-CD14 cells, we found that LPS types including wild type, Re595, and 055B5 weakly induced TNF release, while other LPS types including Ra, 0111B4, and D31m4 more strongly induced TNF release (Fig. 5). In general, we found that weakly activating LPSs were significantly inhibited by anti-TLR4 Abs and that strongly activating LPSs were greatly inhibited by the anti-TLR2 Ab (Fig. 5). Moreover, we found that the level of stimulation, or the effect of anti-TLR Abs on LPS activities, could not be predicted based on the known structure of the LPSs or the bacterial species from which they were derived.

Previous studies have shown that LPS preparations, repurified by a method that removes contaminating proteins, completely lack the ability to activate TLR4-defective C3H/HeJ macrophages (26–28). These findings, coupled with our inability to assign the aforementioned effects of the anti-TLR Abs to known LPS properties, prompted us to repurify our commercial LPSs using a published modified phenol-water repurification procedure (26–28). The recovery of the repurified LPSs was typically 65 to 85% as determined by a KDO or colitose assay and confirmed by a \textit{Limulus} amebocyte lysate assay (see Materials and Methods). Upon repurification, every LPS type now displayed a low and comparable level of activity for the stimulation of THP1-CD14 cells (Fig. 5). Moreover, while the anti-TLR4 Abs inhibited at least 50% of the TNF release induced by every repurified LPS, the anti-TLR2 Ab had no effect on the activity of any repurified LPS. Similar results were obtained using repurified LCD25 and K235 LPS from \textit{E. coli} as well as repurified Rb, Rc, and Rd LPS from \textit{S. minnesota} (data not shown). Taken together, these results demonstrate that upon repurification commercial LPS preparations lose the ability to mediate cellular activation through TLR2 suggesting that impurities in these LPS preparations are responsible for the TLR2-mediated signaling. In contrast, repurified LPS appears to retain the ability to activate cells through TLR4 indicating that this receptor is a signaling receptor for \textit{E. coli} and \textit{S. minnesota} LPS.

To ascertain the contribution of TLR2 and TLR4 to LPS-induced immune responses under more physiologic conditions, we tested the effect of the anti-TLR2 and anti-TLR4 Abs on LPS-mediated cytokine release in human whole blood. Fig. 6 shows that all the anti-TLR4 Abs significantly inhibited TNF-\(\alpha\) and IL-8 release from whole blood treated with either commercial Re595 or 0111B4 LPS. In contrast, the anti-TLR2 Abs had no effect on cytokine production in whole blood treated with Re595 LPS and only marginally, but reproducibly, inhibited cytokine production.
induced by commercial 0111B4 LPS. This low level of inhibition was only observed at higher LPS concentrations in agreement with the idea that the TLR2-mediated response is due to impurities in the commercial 0111B4 LPS. The anti-TLR2 Ab mAb 2392 specifically blocked cytokine release induced by PGN, demonstrating that this Ab is blocking the TLR2 receptor under the conditions of this whole blood assay. As expected, the anti-CD14 mAb 28C5 dramatically inhibited LPS-induced cytokine production in whole blood.

To extend these observations we assayed the previously tested series of smooth and rough LPS types from E. coli and S. minnesota in human whole blood. Levels of TNF release mediated by all of the LPS preparations in whole blood was comparable and inhibited by anti-TLR4 Abs regardless of whether the LPS was repurified or not (Fig. 7). The anti-TLR2 Ab only weakly inhibited whole blood TNF release mediated by commercial LPS preparations and this weak inhibition was not detected using repurified LPSs. Taken together, these results confirm that impurities in commercial LPS preparations activate cells through TLR2. In addition, these results demonstrate that in human whole blood, TLR4 is a predominant receptor mediating LPS-induced cellular activation.

**Discussion**

Our studies began by attempting to assess the relative contribution of TLR2 and TLR4 to LPS-induced cellular activation. We observed that while commercial preparations of both E. coli 0111B4 LPS and S. minnesota Re595 LPS activate cells through TLR4, only the former LPS stimulates cells through TLR2. The difference in activity between these two LPSs is most apparent in THP1-CD14 cells, a cell type that expresses significantly more TLR2 than TLR4; less apparent in whole blood, where isolated polymorphonuclear leukocytes and monocytes have been shown to express comparable levels of TLR2 and TLR4 (43); and nonexistent in U373 cells, a cell line that does not express any TLR2. The simplest interpretation of our results is that the relative contribution of TLR2 and TLR4 to LPS-induced signaling is a reflection of the relative cellular expression levels of these two TLRs. In support of this, we have found that overexpression of TLR4 in THP1-CD14 cells reduces the difference in signaling observed between commercial preparations of 0111B4 and Re595 LPS (Fig. 1).
To determine a structural basis for the difference in activity observed between 0111B4 and Re595 LPS we extended our experiments to a range of rough and smooth LPS types from both *E. coli* and *S. minnesota*. However, the differences in cellular activity that we observed were mediated by TLR2 could not be ascribed to differences in TLR2 expression. In this regard, LPS has proven difficult to completely purify and there exists a long history of contaminating molecules in LPS with cell stimulatory activity (reviewed in Refs. 26, 27). Therefore, we repurified the commercial LPS preparations using a modified phenol-water extraction method followed by ethanol precipitation, a procedure that has been shown to remove trace proteins (26, 27). Repurification of the commercial LPS preparations eliminated TLR2-mediated cell signaling but retained TLR4-mediated cell signaling, demonstrating that TLR4, but not TLR2, is a signaling receptor for pure *E. coli* and *S. minnesota*-derived LPS.

The idea that TLR4, and not TLR2, mediates LPS-induced cellular activation has been strongly argued in a recent review (44). Central to this argument is that a mutation in the gene encoding CD14 cells that express significantly greater levels of TLR2 compared with TLR4 are most sensitive to impure commercial LPS preparations. The contention that the observed activation is due to repurification of the commercial LPS preparations is inhibited by polymyxin B, a cationic peptide that has historically been used to block LPS-induced cellular stimulation. At present we do not know whether polymyxin B is inhibiting TLR2-dependent cellular activation by interacting with the impurity itself or by interacting with the LPS with which it is associated. Regardless, these results demonstrate a necessity to carefully assess the purity and potency of pathogen-derived components when assigning their actions to the function of individual TLRs.

**References**


