A missense mutation in the cytoplasmic domain of the Toll-like receptor-4 (TLR-4) has been identified as the defect responsible for lipopolysaccharide (LPS) hyporesponsiveness in C3H/HeJ mice. TLR-4 and TLR-2 have recently been implicated in LPS signaling in studies where these receptors were overexpressed in LPS nonresponsive 293 human embryonic kidney cells. However, the signaling role of TLR-4 or TLR-2 in human cells with natural LPS response remains largely undefined. Here we show that human dermal microvascular endothelial cells (HMEC) and human umbilical vein endothelial cells express predominantly TLR-4 but very weak TLR-2 and respond vigorously to LPS but not to Mycobacterium tuberculosis 19-kDa lipoprotein. Transient transfection of non-signaling mutant forms of TLR-4 and anti-TRL-4 monoclonal antibody inhibited LPS-induced NF-κB activation in HMEC, while a monoclonal antibody against TLR-2 was ineffective. In contrast to LPS responsiveness, the ability of HMEC to respond to 19-kDa lipoprotein correlated with the expression of TLR-2. Transfection of TLR-2 into HMEC conferred responsiveness to 19-kDa lipoprotein. These data indicate that TLR-4 is the LPS signaling receptor in HMEC and that human endothelial cells (EC) express predominantly TLR-4 and weak TLR-2, which may explain why they do not respond to 19-kDa lipoprotein. The differential expression of TLRs on human EC may have important implications in the participation of vascular EC in innate immune defense mechanisms against various infectious pathogens, which may use different TLRs to signal.

Lipopolysaccharide (LPS), or endotoxin, is the major component of the outer surface of Gram-negative bacteria. LPS is a potent activator of cells of the immune and inflammatory systems, including macrophages, monocytes, and endothelial cells, and contributes to the systemic changes seen in septic shock (1, 2). Sepsis and septic shock are increasing causes of mortality, with approximately 200,000 deaths annually in the United States (3). The basic paradigm of septic shock is that microbial antigens such as Gram-negative bacterial LPS, Gram-positive bacterial cell wall components, such as soluble peptidoglycan (PGN), lipoteichoic acid (LTA), and bacterial lipoproteins, initiate an uncontrolled network of host-derived proinflammatory mediators, which ultimately lead to cardiovascular shock and death (1, 2, 4). Vertebrates and invertebrates initiate a series of defense mechanisms following infection by various microorganisms by sensing the presence of conserved pathogen-associated molecular patterns, such as bacterial LPS (5). Host organisms have developed a set of receptors that can specifically recognize pathogen-associated molecular patterns and are referred to as pattern recognition receptors (6, 7). A family of such pattern recognition receptors involved in innate immune recognition and cellular activation in response to bacterial LPS, lipoproteins, PGN, and LTA has recently been discovered to be human toll-like receptors (TLRs) (8–17).

TLR family members are transmembrane proteins containing repeated leucine-rich motifs in their extracellular portions, similar to other pattern recognition proteins of the innate immune system (18, 19). TLRs also contain a cytoplasmic domain which is homologous to the signaling domain of the IL-1 receptor, and activation of TLRs results in activation of NF-κB and induction of cytokines and costimulatory molecules required for the activation of the adaptive immune response (18–20). The toll gene controls dorsoventral pattern formation during the early embryonic development of Drosophila melanogaster (21) and initiates a signaling pathway homologous to mammalian NF-κB activation cascade (19, 21). At least eight mammalian Toll-like receptor family members (TLR1–8) have been identified to date, but the function of each individual family member is uncertain.

The C3H/HeJ and C57BL10ScCr mouse strains are hyporesponsive to LPS, while they respond normally to Gram-positive bacterial LPS. This paper is available online at http://www.jbc.org.
to LPS maps to a single autosomal locus (lps) (22). Recently, this defect in the LPS response was found to be due to a missense mutation in the cytoplasmic domain of TLR-4 in C3H/HeJ (17, 23, 24) and to an independent mutation at the lps locus in the C57BL10ScCr strain resulting in the absence of TLR-4 message (17). These genetic studies strongly suggest that TLR-4 is the signal transducing component in the LPS receptor complex (15, 17, 23). Akira and colleagues (25) also showed that mice in which the TLR-4 gene has been deleted have the same defects as mice with mutations in lps.

Transient transfection experiments using LPS non-responsive human embryonic kidney cells (HEK 293) revealed that both TLR-2 (8, 9) and TLR-4 (11) overexpression can enable these cells to respond to LPS, suggesting that perhaps TLR-2 can also be considered a lipopolysaccharide receptor. However, more recent reports have shown that mice lacking TLR-2 can still respond to LPS in the same way as wild type animals (26). Several studies have identified TLR-2 as the receptor mediating the cellular responses to Gram-positive bacteria (27), PGN, LTA (10, 12), and more recent reports have shown that mice lacking TLR-2 can respond to LPS, suggesting that perhaps TLR-2 and TLR-4 (11) overexpression can enable these cells to respond to LPS, suggesting that perhaps TLR-2 can also be considered a lipopolysaccharide receptor. However, more recent reports have shown that mice lacking TLR-2 can still respond to LPS in the same way as wild type animals (26).

We have previously shown that the LPS-induced signal transduction pathway to activate NF-κB shares the IL-1 receptor signaling molecules, including MyD88, IL-1 receptor-associated kinase (IRAK), IRAK-2, and TNF receptor-associated factor-6 (28). Additionally, we have shown that a crucial signaling component in the LPS receptor complex in human dermal microvessel endothelial cells (HMEC) may belong to the IL-1 receptor/TLR superfamily (28). In the current study, we investigated the expression pattern of TLRs in LPS-responsive human cells is yet undefined. Monocytes and macrophages, dendritic cells, and endothelial cells (EC) play a pivotal role in the development of innate immune responses against invading microorganisms. We therefore investigated the mechanisms involved in bacterial LPS and lipopolysaccharide-induced activation of vascular EC.

We have previously shown that the LPS-induced signal transduction pathway to activate NF-κB shares the IL-1 receptor signaling molecules, including MyD88, IL-1 receptor-associated kinase (IRAK), IRAK-2, and TNF receptor-associated factor-6 (28).

**Expression Vectors and cDNA Constructs**

ELAM-NF-κB luciferase and pCMV-β-galactosidase vectors were used as described previously (28). C3H/HeJ and C3H/HeN TLR-4 cDNAs were obtained from Bruce Beutler (Scripps Institute, La Jolla, CA). A flag-tagged mutant human TLR-4 construct with a deletion in the intracellular domain (TLR-4 Dicd), lacking the 155 COOH-terminal amino acids of the wild type TLR-4 was obtained from Tularik (San Francisco, CA). Wild type human TLR-2 construct was a gift from Ruslan Medzhitov (Yale University, New Haven, CT). The plasmids were prepared with a endotoxin-free Plasmid Maxi kit (Qiagen, Valencia, CA).

**Transfection of Human Dermal Endothelial Cells**

HMEC were plated at a concentration of 50,000 cells/well in 24-well plates. Cells were co-transfected the following day with FuGene 6 Transfection Reagent (Roche Molecular Biochemicals) following manufacturer’s instructions. Reporter genes pCMV-β-galactosidase (0.5 μg) and ELAM-NF-κB-luciferase (0.5 μg), and pCMV empty vector (0.5 μg) or C3H/HeJ TLR-4 (0.5 μg), TLR-2 (0.5 μg) were co-transfected. After overnight transfection, cells were stimulated for 6 h with 50–100 ng/ml LPS or 10 ng/ml human TNF-α (R & D Systems). Cells were then lysed in 60 μl of reporter lysis buffer (Promega, Madison, WI), and luciferase activity was measured with a Promega kit and a luminometer. β-Galactosidase activity was determined by calorimetric method to normalize transfection efficiency as described previously (28). Data shown are mean ± S.D. of three or more independent experiments and are reported as a percentage of LPS-stimulated NF-κB promoter activity, or relative luciferase activity.

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis**

Total RNA was isolated from HMEC, HUVEC, and THP-1 cells using a Qiagen kit (Valencia, CA) following manufacturer’s instructions and treated with RNase-free Dnase I. For RT reaction, the MMLV preamplification system (Life Technologies, Inc.) was applied. PCR amplification was performed with Taq gold polymerase (Perkin Elmer, Foster City, CA) for 32 cycles at 95 °C for 45 s, 54 °C for 45 s, and 72 °C for 1 min for both TLR-2 and TLR-3, and 35 cycles at 95 °C for 45 s, 50 °C for 45 s, and 72 °C for 1 min for TLR-1, TLR-3, and TLR-5. The oligonucleotide primers used for RT-PCR were: TLR-1, 5'-CTATACACCAACTGTCGAC and 5'-GCTCTCACAATCTGAAGGTG; TLR-2, 5'-GCACAACTGTTCATTG and 5'-TTGAAAGTTCCAGCTCCTG; for TLR-3, 5'-GATCAGTCTCATAAAGTGCTG and 5'-GACAGATTCGAGATGGTGT; for TLR-4, 5'-TGGATACGTTTCCTTATAAG and 5'-GAATGCCAGGCACCCTCT; and for TLR-5, 5'-CTAGCTCTTACATCTGATG and 5'-CCATTGGAACTTGGCTG. Glyceraldehyde-3-phosphate dehydrogenase primers were obtained from CLONTECH (Palo Alto, CA).

**Northern Blot Analysis**

Total RNA was isolated by the guanidine isothiocyanate method with minor modifications. Total RNA was analyzed by electrophoresis through 1% agarose/formaldehyde gels, followed by northern blot transfer to Gene Screen Plus membranes (NEN Life Science Products). The plasmids containing human TLR-2, TLR-4, or actin cDNAs were labeled with [α-32P]dCTP (3,000 Ci/mmol; Amersham Pharmacia Biochem, Buckinghamshire, United Kingdom). Membranes were pre-treated and hybridized in 50% formamide (Merek, Rahway, NJ) with 10% dextran sulfate (Sigma) and washed twice with 2 × SSC (1 × SSC: 0.15 M NaCl, 0.015 M sodium citrate) and 1% sodium dodecyl sulfate (SDS) at 60 °C for 30 min, and finally washed twice with 0.1× SSC at room temperature for 30 min. Membranes were exposed for 4–48 h at −80 °C with intensifying screens.

**FACS Analysis and Immunofluorescent Staining**

Flow Cytometry—Surface expression of TLR-2 in HMEC and THP-1 cells was determined using mouse mAb to TLR-2 (2392). The mouse mAb was a generous gift from Patrick Brennan and John Belisle (Colorado State University, Fort Collins, CO, under NIH Contract NO1-AI-75520). Protein free Escherichia coli K235 LPS was obtained from Stefanie N. Vogel (Uniformed Services University, Bethesda, MD). Purified RdPAM was obtained from Nilofer Qureshi of Wisconsin, Madison, WI, and human recombinant soluble CD14 from Sanna Boyert (North Shore University Hospital, Manhasset, NY).
monoclonal TLR-2 antibody was detected with fluorescein isothiocyanate-conjugated goat anti-mouse antiserum (CalTag Laboratories, Burlingame, CA). Samples were acquired on Becton-Dickinson FACScan (Becton-Dickinson Immuno cytometry Systems, Inc., San Jose, CA).

Immunofluorescence—HMEC were cultured on eight chamber microscop e slides. Cells were fixed in paraformaldehyde solution (4%), incu bated with mouse anti-TLR-2 mAb in PBS buffer, or rabbit polyclonal anti-TLR-2 antisera, and incubated for 1 h. Primary antibodies were detected with fluorescein isothiocyanate-conjugated goat anti-mouse antiserum or, in the case of rabbit polyclonal antiserum, goat anti-rabbit antiserum (CalTag). Samples were viewed and photographed using a Zeiss Axioshot microscope.

RESULTS AND DISCUSSION

To investigate the involvement of TLR-2 and TLR-4 in LPS-responsive human vascular EC, we first analyzed the expression pattern of TLRs in HMEC, HUVEC, monocytes, or THP-1 cells by RT-PCR (TLR1–5) and by Northern blot, immunofluorescent staining, and FACS analysis (TLR-2 and TLR-4). HMEC expressed TLR-1, TLR-3, TLR-4, and TLR-5, but only a weak TLR-2 transcript by RT-PCR (Fig. 1A). THP-1 cells expressed all five TLRs, including TLR-2 (Fig. 1A). The weak expression of TLR-2 by RT-PCR in HMEC is consistent with our previous observation (28) and was also observed in primary HUVEC (Fig. 1A). Northern blot analysis detected only the 3.4-kilobase pair band consistent with the TLR-4 expression in HMEC and HUVEC, and no TLR-2 message was observed in these two types of EC, while both TLR-2 and TLR-4 mRNA were detected in monocytes (Fig. 1B). Furthermore, immunofluorescent staining and flow cytometric analysis of HMEC with anti-TLR-2 mAb 2392 showed no TLR-2 expression in HMEC, while THP-1 cells showed positive staining (Fig. 1, C and D). We have previously observed that both TLR-4 and TLR-2 were expressed in HMEC by immunostaining using a non-affinity-purified polyclonal antiserum against TLR-2 and TLR-4 (28). However, the availability of a mAb against TLR-2 now allows us to show that there is no TLR-2 expression in HMEC and that the polyclonal anti-TLR-2 antiserum most likely cross-reacts, possibly with another TLR expressed in HMEC (data not shown).

In C3H/HeJ mice, the missense mutation in the intracytoplasmic domain of TLR-4 acts as a dominant negative molecule, possibly by sequestering the downstream adapter and signaling molecules (e.g. MyD88 and IRAK1), and prevents LPS-induced signaling and NF-κB activation (17, 24). To assess the functional role of TLR-4 in LPS-activated NF-κB activation in HMEC, we transiently transfected these cells with C3H/HeJ TLR-4 cDNA and measured LPS-mediated NF-κB activation in response to LPS (50 ng/ml) for 6 h, and NF-κB activation was measured by ELAM reporter construct luciferase activity. 19-kDa lipoprotein was unable to induce NF-κB activation in HMEC, even at 1,000 ng/ml (Fig. 4). However, expression of wild type TLR-2 cDNA in HMEC conferred responsiveness to 19-kDa lipoprotein (Fig. 4), whereas transfection of vector alone did not. In HMEC transfected with TLR-2, 19-kDa lipoprotein-induced NF-κB activation was inhibited by pre-incubation of the cells with anti-TLR-2 mAb (10 μg/ml) but not with control IgG1 Ab (Fig. 3) or with anti-TLR-4 mAb (data not shown). HUVEC were stimulated with LPS (50 ng/ml) or 19-kDa lipoprotein (50 ng/ml) for 6 h and the supernatants were collected for IL-6 measurement (enzyme-linked immunosorbent assay, BIOSOURCE International, Camarillo, CA). 19-kDa lipoprotein did not induce any IL-6 release from HUVEC while LPS induced a mean of 4-fold increase in IL-6 production compared with resting cells in triplicate experiments (data not shown).

LPS is a complex glycolipid consisting of a proximal hydrophobic lipid A moiety, a distal hydrophobic O-antigen polysaccharide region, and a core oligosaccharide that joins the lipid A and O-antigen structures. The lipid A portion of the molecule is the biologically active portion responsible for LPS responses, and removal of the fatty acid side chains from the lipid A moiety inactivates LPS (36). A special nontoxic pentaacyl diphosphoryl lipid A derived from the LPS of Rhodobacter sphaeroides (RsDPLA) is an LPS antagonist both in vivo and in vitro experimental conditions (37). Based on LPS binding studies, RsDPLA is believed to competitively antagonize LPS activity at its cell surface receptor, leading to inhibition of transmembrane signal transduction (37). Therefore, we next investigated whether RsDPLA blocks LPS- or 19-kDa lipoprotein-induced signaling in native or TLR-2-transfected HMEC, respectively. Pretreatment of HMEC with RsDPLA blocked
LPS-induced NF-κB activation but had no effect on 19-kDa lipoprotein-induced activation of TLR-2-transfected HMEC (Fig. 5). Furthermore, RsDPLA (1 μg/ml) did not affect IL-1- or TNF-α-induced NF-κB gene activation in HMECs (data not shown), indicating that the antagonistic activity of RsDPLA is closely linked to and specific to cell-surface components utilized.
by LPS. Since we showed that TLR-4 is the most likely receptor on HMEC that accounts for LPS responsiveness, our observations suggest that RsDPLA may selectively inhibit LPS-TLR-4 interaction.

We have previously shown that in contrast to monocytes and macrophages, EC do not express membrane CD14, and that LPS-induced EC activation is absolutely dependent on the presence of soluble form of the CD14 receptor (sCD14) present in serum (38). To determine the role of sCD14 in LPS-TLR-4 signaling for EC activation, we tested the response of HMEC to LPS in serum-free media, and serum-free media supplemented with recombinant human sCD14. LPS-induced NF-κB activation in HMEC was serum- and soluble CD14-dependent. LPS (100–1,000 ng/ml) was unable to induce NF-κB activation in serum-free media, and addition of sCD14 (1 μg/ml) was able to restore LPS-induced cellular activation (Fig. 6). However, 19-kDa lipoprotein induced similar levels of NF-κB in TLR-2-expressing HMEC cultured in both serum-containing and in...
serum-free media (Fig. 6). These findings suggest that, in contrast to LPS, which absolutely requires sCD14 to trigger TLR-4 and activate HMEC, 19-kDa lipoprotein is able to activate TLR-2-transfected HMEC in the absence of serum or sCD14.

Our data demonstrate that human microvascular ECs, which are one of the first lines of defense against invading microbial agents, including Gram-negative bacteria, actively participate in innate immune responses, and that LPS-induced responses in EC are specifically mediated via TLR-4. TLR-4 in human vascular EC participates in sensing LPS by a mechanism that is soluble CD14-dependent. After detecting the LPS molecules, TLR-4 transmits the information across the cell membrane, leading to the activation of NF-κB and regulation of several responsive genes. Similar to mice where mutations of several responsive genes. Similar to mice where mutations of TLRs to recognize different groups of microbial pathogens, humans who develop severe Gram-negative infections may have TLR-4 mutations at a higher rate.

Our results also indicate a differential expression of TLR-2 and TLR-4 in human vascular endothelial cells. This results in brisk EC responses to Gram-negative LPS but no EC responses to bacterial lipoproteins such as M. tuberculosis 19-kDa lipoprotein or to Gram-positive bacterial cell wall components such as PGN and LTA, which require TLR-2 to signal. The biological relevance of this observation is unclear and needs to be further investigated. Because the mammalian TLR family has many members, it is possible that the innate immune system and its individual cell types use different combinations of TLRs to recognize different groups of microbial pathogens. Finally, our findings indicate that TLR-4 may be a potential target in the development of novel treatment strategies for Gram-negative sepsis and endotoxic shock.

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REFERENCES


M. Arditi, E. Faure, O. Equils, L. Thomas, and F. X. Zhang, unpublished data.
Bacterial Lipopolysaccharide Activates NF-κB through Toll-like Receptor 4 (TLR-4) in Cultured Human Dermal Endothelial Cells: DIFFERENTIAL EXPRESSION OF TLR-4 AND TLR-2 IN ENDOTHELIAL CELLS

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