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*J Immunol* 2004; 173:1166-1170; doi: 10.4049/jimmunol.173.2.1166

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TLR4 Is the Signaling but Not the Lipopolysaccharide Uptake Receptor\textsuperscript{1,2}

Stefan Dunzendorfer, Hyun-Ku Lee, Katrin Soldau, and Peter S. Tobias\textsuperscript{3}

TLR4 is the primary recognition molecule for inflammatory responses initiated by bacterial LPS (endotoxin). Internalization of endotoxin by various cell types is an important step for its removal and detoxification. Because of its role as an LPS-signaling receptor, TLR4 has been suggested to be involved in cellular LPS uptake as well. LPS uptake was investigated in primary monocytes and endothelial cells derived from TLR4 and CD14 knockout C57BL/6 mice using tritiated and fluorescein-labeled LPS. Intracellular LPS distribution was investigated by deconvolution confocal microscopy. We could not observe any difference in LPS uptake and intracellular LPS distribution in either monocytes or endothelial cells between TLR4\textsuperscript{−/−} and wild-type cells. As expected, CD14\textsuperscript{−/−} monocytes showed a highly impaired LPS uptake, confirming CD14-dependent uptake in monocytes. Upon longer incubation periods, the CD14-deficient monocytes mimicked the LPS uptake pattern of endothelial cells. Endothelial cell LPS uptake is slower than monocyte uptake, LBP rather than CD14 dependent, and sensitive to polyanionic polymers, which have been shown to block scavenger receptor-dependent uptake mechanisms. We conclude that TLR4 is not involved in cellular LPS uptake mechanisms. In membrane CD14-positive cells, LPS is predominantly taken up via CD14-mediated pathways, whereas in the CD14-negative endothelial cells, there is a role for scavenger receptor-dependent pathways. The Journal of Immunology, 2004, 173: 1166–1170.

The genome-encoded innate immune system targets structurally conserved pathogen-associated microbial products, thereby allowing immediate responses to limit or eradicate invading microbes. So far, 10 human TLRs have been cloned, and some of them have been shown to mediate cellular responses to microbial products (1). Of the Toll family, TLR4 is expressed in a wide variety of human cells and serves as a receptor for endogenous as well as exogenous ligands (2–4). This transmembrane receptor is the primary recognition molecule for LPS from Gram-negative bacteria (5–7).

LPS binding protein (LBP)\textsuperscript{4} catalyzes the transfer of LPS to membrane or soluble CD14 (8), which in turn mediates the recognition of LPS through TLR4. The TLR4 signaling complex also requires the small glycosylated protein MD-2 for optimal function (9, 10). The central roles of CD14 and TLR4 in LPS signaling are emphasized by the fact that deficient mice are highly resistant to endotoxic shock (7, 11). In the course of sepsis, physical neutralization (12) and internalization of LPS by immune cells are important for its detoxification; after endocytosis, LPS is biologically deactivated by specific enzymes (13). Various cell surface molecules can bind LPS. In membrane CD14 (mCD14)-positive cells, LPS is internalized via CD14-dependent mechanisms (14–16), whereas in mCD14-negative cells, like endothelial and epithelial cells, scavenger receptors have been implicated in the clearance of LPS (17–19). Some recent publications suggest TLR4-dependent LPS internalization and intracellular LPS transport (20–22). The work by Latz et al. (22) confirmed CD14-dependent LPS uptake as observed earlier (14–16) and suggests these intracellular TLR4 movements might be transported on lipid rafts along with internalized CD14 (23).

It has been appreciated that TLR4 is a signaling receptor for LPS (7, 24). Although TLR4 is a pattern recognition receptor, like scavenger and mannose receptors (25), its LPS clearance function is unknown. By using primary cells (not transfected cell lines) from TLR4 knockout mice, our data exclude any role for TLR4 as an LPS uptake receptor.

Materials and Methods

Mice

TLR4\textsuperscript{−/−} mice in the C57BL/6 background were obtained from S. Akira (Osaka University, Osaka, Japan). CD14\textsuperscript{−/−} mice in the C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME). All animals were maintained under pathogen-free conditions in a barrier facility (Immunology Vivarium, The Scripps Research Institute), weighed between 20 and 35 g, and were between 6 and 10 wk of age at the time of use.

Mouse monocytes

Halothane-anesthetized C57BL/6 mice were sacrificed and bled by heart puncture. Peripheral blood monocytes were prepared using magnetic cell sorting (MACS; Miltenyi Biotec, Auburn, CA) after isolation of PBMC by density gradient centrifugation over FicoLite-LM (Atlanta Biologicals, Norcross, GA). In brief, mononuclear cells were incubated with MACS paramagnetic beads conjugated with monoclonal anti-mouse CD11b, and monocytes were isolated using MS separation columns according to the manufacturer’s protocol (Miltenyi Biotec). Purity of the preparations usually exceeded 95% as determined by FACS analyses after staining for mouse CD14.
Mouse endothelial cells

Mouse microvascular endothelial cells (MEC) were isolated from lung tissue. Anesthetized animals were sacrificed, and the lungs were explanted under sterile conditions, minced, and digested for 1 h at 37°C in 1 mg/ml collagenase type II in HBSS (Invitrogen Life Technologies, Carlsbad, CA). Cells were separated from debris and incompletely digested tissue with cell strainers, washed twice, and thereafter stained with rat anti-mouse CD31 (10 μg/ml; BD Pharmingen, San Diego, CA). Goat anti-rat IgG conjugated to paramagnetic beads (Miltenyi Biotec) was used for further purification by MACS. Endothelial cells were then cultured and maintained on fibronectin-coated tissue culture plates in mouse brain microvascular growth medium (Cell Application, San Diego, CA) supplemented with 15 ng/ml basic fibroblast growth factor (Sigma-Aldrich, St. Louis, MO). Purity of endothelial cells was determined by staining for mouse CD31, mouse CD106, or mouse CD144, and by uptake of Alexa Fluor 488-labeled acetylated low-density lipoprotein (Molecular Probes, Eugene, OR). Cells used in experiments were from passages 1–3.

LPS uptake experiments

Monocytes (1 × 10^6 cells/ml) were incubated with 100 ng/ml FITC-labeled LPS (Salmonella minnesota Re595; List Biological Laboratories, Campell, CA) (8) in RPMI 1640 for various time intervals at 37°C. When rLBP was added, this is stated in the figures. MEC were grown to confluence in six-well plates and incubated with 100 ng/ml tritiated LPS ([3H]LPS) (Escherichia coli K12 LCD25; specific activity, 2.08 × 10^6 dpm/μg; List Biological Laboratories) for 2 h (except in time course experiments) at 37°C along with Abs and human rLBP (26, 27). Anti-LBP (mAb 2B5) and anti-CD14 (mAb 28C5) were produced in our laboratory (28) and have previously been shown to react with bovine LBP and CD14, respectively (26). After incubation, cells were washed twice with PBS and further treated with 250 μg/ml proteinase K (Sigma-Aldrich) in HBSS for 30 min at room temperature to remove cell surface proteins/receptors and surface-bound LPS (29). The remaining LPS was considered to be intracellular. FITC-LPS in monocytes was measured by FACS (given as FL-1 mean channel fluorescence), and counts of [3H]LPS in MEC were assayed in scintillation fluid after lysis of the cells in 300 μl of 2% SDS/50 mM EDTA. To evaluate the participation of scavenger receptors in LPS uptake, cells were pretreated with dextran sulfate (DEX; molecular mass, 8000; 50 μg/ml), polynosinic acid (PNA; 20 μg/ml), polyadenylic acid (PAA; 20 μg/ml), or polylysine in LPS uptake as described above.

Cell stimulation experiments

Activation of mouse monocytes was measured by MIP-2 secretion. A total of 5 × 10^5 cells of one well of a 24-well tissue culture plate was stimulated for 4 h at 37°C in RPMI 1640/2% FCS with LPS (E. coli O55:B5; List Biological Laboratories) or the TLR2 agonist macrophage-activating lipopeptide-2 (MALP-2; Alexis Biochemicals, San Diego, CA). Thereafter, supernatants were harvested and assayed for MIP-2 using a commercially available ELISA kit (R&D Systems, Minneapolis, MN).

Immunofluorescence and deconvolution confocal microscopy

Monocytes or MEC derived from TLR4-deficient or control animals were incubated with 1 μg/ml FITC-LPS for 30 min or for 4 h at 37°C. Thereafter, cells were fixed and permeabilized using the Cytofix/Cytoperm kit from BD Pharmingen. mCD14 in monocytes was visualized using anti-CD14 (10 μg/ml; clone mCD14-1; BD Pharmingen) and a matching isotype control (clone RM58A6; BD Pharmingen) or with the TLR2 agonist macrophage-activating lipopeptide-2 (MALP-2; Alexis Biochemicals, San Diego, CA). Thereafter, supernatants were harvested and assayed for MIP-2 using a commercially available ELISA kit (R&D Systems, Minneapolis, MN).
collects especially low-intensity signals with a linear response to light intensity, the data are then deconvoluted using DeltaVision software SoftWoRx 2.5 (Applied Precision) based on the Agard/Sadat inverse matrix algorithm. This method can combine optical sections (0.15 μm) acquired by a confocal microscope to three-dimensional image stacks. Following computational deconvolution, it can provide high resolution three-dimensional images of cells.

**Results**

**TLR4 is not involved in LPS uptake in monocytes and MEC**

LPS uptake was investigated in monocytes and MEC derived from wild-type (wt), TLR4-deficient, or CD14-deficient mice. As expected, CD14-negative monocytes showed a highly impaired LPS uptake with almost no LPS ingestion within 60 min after starting the experiments. In contrast, wt cells did not differ in terms of LPS uptake compared with TLR4-deficient cells (Fig. 1A). In MEC wt cells, LPS uptake was minimal but could be enhanced 10-fold with human LBP (Fig. 1B); this effect was clearly LBP dose dependent (C). No differences in the LPS uptake were found in either genotype of MEC (wt, TLR4+/−, CD14+/−). LBP-stimulated uptake in MEC was sensitive to anti-LBP (mAb 2B5) but not to anti-CD14 (mAb 28C5) (Fig. 1, C and D).

**LPS uptake in CD14-deficient monocytes and in MEC is mediated by scavenger receptors**

Monocytes or MEC were pretreated with polyanionic or polycationic substances before LPS uptake was assayed in the presence or absence of human LBP. In the monocytes, LBP-stimulated LPS uptake was inhibited by DEX or PIA only in CD14−/− cells; no effect of these scavenger receptor blockers was seen in wt cells. In MEC, dextran and PIA inhibited LBP-stimulated LPS uptake in either genotype tested (wt, TLR4−/−). The polycations PAA and PCA were not inhibitory in either MEC or monocytes (Fig. 2).

**wt and TLR4-deficient cells show equal intracellular LPS distribution**

TLR4-deficient or wt MEC or monocytes were incubated with FITC-LPS (1 μg/ml) and thereafter investigated by deconvolution microscopy. Neither of the knockout cells showed an altered cellular distribution of LPS when compared with wt cells. In monocytes, FITC-LPS was found both bound to the membrane and intracellularly after 30 min and mostly intracellularly after 4 h (Fig. 3A). In MEC, no membrane localization could be observed and the FITC-LPS was paranuclear by 30 min (Fig. 3B). In both cell types,
three-dimensional images clearly revealed intracellular localization of FITC-LPS (not shown).

**TLR4 is necessary for LPS activation**

To show full functionality of the cells and to confirm the unique role of TLR4 in LPS signaling, mouse monocytes were stimulated with LPS (E. coli O55:B5; 10 ng/ml) or MALP-2 (1 ng/ml) before MIP-2 levels in the supernatants were measured. As expected, TLR4-negative cells were completely unresponsive to LPS. Responsiveness to MALP-2 was independent of genotype (data not shown).

**Discussion**

TLR4 is a primary recognition molecule for LPS and is the principal transmembrane LPS signaling receptor. TLR4’s ability to bind LPS and initiate signaling made us wonder whether it was also involved in LPS uptake. As best as we can determine, there are three papers that have approached this question, but none that directly resolved it. Tieleman et al. (20) studied LPS uptake in peritoneal macrophages from normal and two strains of Lps" mice, the TLR4-deficient (C57BL/10ScN) and the TLR4-signaling-inactive (C3H/HeJ) mice (7). They observed normal LPS binding to the membranes from all three strains of mice, but observed abnormal intracellular trafficking in the Lps" macrophages. Normal macrophage LPS uptake in these mice (carrying a TLR4 mutation) does not exclude TLR4 from involvement in LPS uptake, because the defect may only influence TLR4 signaling. C57BL/10ScCr mice are homozygous for a mutant Lps allele (7), resulting in lack of TLR4 mRNA. In connection with LPS, they are therefore very close to TLR4 knock-out mice. C57BL/10ScCr dendritic cells and macrophages phagocytose bacteria similarly to control cells, indicating that TLR4 is not compulsory for bacterial uptake (30).

Latz et al. (22) studied uptake of LPS and LPS/CD14 complexes in TLR4-transfected HEK293 cells, but seem not to have studied uptake in nontransfected cells. TLR4 transfection only minimally led to LPS uptake, whereas CD14 cotransfection dramatically improved the ability of those cells to ingest LPS (22). Their results confirmed that mCD14 is an LPS uptake receptor, as known from previous work (14–16), but their data do not show whether TLR4 has a role in LPS uptake. Transfection of TLR4 into HEK293 cell line (TLR4 negative) might not reveal a role for TLR4 in LPS uptake, because those cells might lack cofactors involved in uptake.

Suzuki et al. (21) showed a correlation between TLR4 expression and LPS uptake in intestinal epithelial cell lines, but did not directly establish a mechanistic connection. Their conclusions are probably based on the observations that IFN-γ enhanced TLR4 expression and LPS uptake by Colo205 cells, and that SW480 cells can take up LPS, although they are negative for surface CD14 but positive for surface TLR4. However, TLR4 was not detectable on the surface of Colo205 cells. Compared with these surface CD14-positive cells, SW480 LPS uptake was less and slower in time. According to our results, this would be typical for a scavenger receptor-mediated LPS uptake.

In contrast, our data seem quite clear. Fig. 1 shows that uptake of LPS is quantitatively identical in TLR4 wt and TLR4-/- mice as well as endothelial cells. Furthermore, as shown in Fig. 3, the TLR4 genotype has no bearing on the intracellular distribution of FITC-LPS. These data leave little room to argue that TLR4 is playing some critical role, either by virtue of acting as a transport protein or by acting as a signal initiating receptor, in either uptake or trafficking of LPS. Moreover, if endothelial cells would express CD14 on their surface, as suggested recently (31), the LPS uptake would follow the pattern observed in wt monocytes; but MEC LPS uptake clearly mimics that seen in CD14-/- mice.

There is evidence that scavenger receptors can bind not only lipopolysaccharides but also negatively charged phospholipids. Some of these pattern recognition receptors have been demonstrated to directly bind LPS and lipid A (17, 19, 32). Binding of LPS to scavenger receptors can be competed by anionic substances (18). This was also observed in our experiments where LPS uptake in CD14-deficient monocytes and in MEC was sensitive to polyanionic polymers. In terms of LPS uptake, there was no obvious difference between CD14-deficient monocytes and endothelial cells. In both cell types, LPS uptake was slow, stimulated by LBP, and blocked by polyanionic polymers. These findings strongly support the hypothesis that LPS is taken up as an LBP-LPS complex via scavenger receptor-dependent mechanisms in the MEC studied here. Jack et al. (33) have shown that, in LBP-/- mice, LPS clearance from the circulation is not affected. This may suggest that MEC uptake of LPS is not quantitatively important in vivo.

In summary, these data support the conclusions that 1) TLR4 plays no role in cellular LPS uptake by monocytes or endothelial cells regardless of their expression of mCD14, 2) these cell types have a scavenger receptor pathway to take up LPS as an LBP complex, and 3) in mCD14-expressing monocytes, there is an additional LPS uptake pathway that is more efficient than the scavenger receptor pathway.

**References**


