Lipopolysaccharide Could Be Internalized into Human Peripheral Blood Mononuclear Cells and Elicits TNF-α Release, but not *via* the Pathway of Toll-Like Receptor 4 on the Cell Surface

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Lipopolysaccharide (LPS), the principal component of the outer membrane of gram-negative bacteria, stimulate various cell types to release numerous proinflammatory mediators such as TNF-α, IL-6 and IL-12, which may damage cells and lead to organ injury, even sepsis and septic shock. Toll-like receptor 4 (TLR4) has been identified as the receptor involved in the recognition of LPS, but the role of LPS uptake in activating signal transduction remains controversial. In the present study, TNF-α was used as a marker of macrophages/ monocytes activated by LPS, and CQ was used as an inhibitor of endosome mature in order to definitude what stage the signal transduction elicited by LPS was interrupted. We found that there indeed existed internalization of LPS and internalization partially participated in LPS signaling since CQ inhibited cytokine release, and decreased accumulation of FITC-LPS in hPBMC. In contrast, anti-hTLR4 antibody could decrease cytokines' release, but no inhibition on accumulation of FITC-LPS. This result revealed that inhibition of cytokine release was related to reduction of FITC-LPS accumulation in the cells. But TLR4 on the cell surface didn't possibly participated in internalization of LPS. Thus, LPS signaling and internalization cannot be viewed as mutually independent processes. *Cellular & Molecular Immunology*. 2004;1(5):373-377.

Key Words: LPS, TNF-α, TLR4, internalization, CQ

Introduction

Lipopolysaccharide (LPS), the principal component of the outer membrane of gram-negative bacteria, stimulate various cells to release numerous proinflammatory mediators such as TNF-α, IL-6 and IL-12, which may damage cells and lead to organ injury, even sepsis and septic shock (1, 2). Monocytes/macrophages are prominent target cells which are activated by LPS to release various pro- and anti-inflammatory mediators.

Toll-like receptor 4 (TLR4) has been identified as the receptor involved in the recognition of LPS (3). In addition, recognition of LPS requires other molecules, such as LPS-binding protein (LBP), CD14, and MD-2 (4). TLR4 normally resides on the cell surface and recognition of LPS occurs on the cell surface. However, several reports have indicated that LPS may be recognized in the cytoplasm as well as on the cell surface after LPS is rapidly delivered into the cytoplasm after binding to the cell surface (5-8), which suggests that LPS also could be recognized independently of TLR4. This intracellular movement appears to be necessary for certain cellular responses, since agents that block vesicular transport such as wortmannin or cytochalasin D block the integrin-mediated adhesion of neutrophils in response to LPS (9), but the role of LPS uptake in activating signal transduction remains con troversial (10, 11).

Therefore, we wonder whether there exists internalization of LPS in hPBMC, and whether TLR4 on the cell surface is specifically involved in LPS uptake. With these doubts in mind, we undertook the current study. In the present experiments, TNF-α was used as a marker of macrophages/monocytes activated by LPS, and CQ was used as an inhibitor of endosome mature in order to definitude what stage the signal transduction elicited by

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Abbreviations: LPS, lipopolysaccharide; CpG ODN, cytosine guanine-containing oligodeoxynucleotides; TLR, Toll-like receptor; TNF, tumor necrosis factor; CQ, chloroquine; FITC-LPS, fluorescein isothiocyanate-labeled LPS; hPBMC, human peripheral blood mononuclear cell.

LPS was interrupted.

Materials and Methods

Reagents

Chloroquine (CQ), LPS and FITC-LPS from *Escherichia coli* O111:B4 were purchased from Sigma Chemicals (St Louis, MO, USA). PE-labeled rabbit anti-human TLR4 antibody was purchased from eBioscience. Human TNF-α and IL-6 ELISA kits were purchased from Biosource International (Camarillo, CA, USA). Lymphoprep™ was purchased from Axis-Shield PoC AS (Oslo, Norway).

Isolation of human peripheral blood mononuclear cells (hPBMC)

Human PBMC were isolated from four healthy male volunteers as described previously (12). Briefly, after obtaining informed consent from the donors, blood was collected by venipuncture and anticoagulated with 5 U pyrogen-free heparin per milliliter of blood, and then centrifuged at 500 g for 15 min. Leukocyte-rich buffer coats were then subjected to Lymphoprep™. After centrifugation, PBMC were collected from the light density fraction, and washed three times with ice-cold PBS (0.1 mM, pH 7.2) before being counted by hemocytometer. The cells were resuspended at the desired density (1×10^6 cells/ml) in RPMI 1640 medium supplemented with 10% low endotoxin fetal calf serum (Hyclone, Logan, UT, USA), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in a 37°C humid atmosphere with 5% CO_2.

Cell stimulation

Human PBMC (1×10^6 cells/ml) were stimulated with LPS as above. After further cultured for 2 h, the cells and supernatants were collected, respectively. The levels of TNF-α and IL-6 in the supernatants were analyzed.

LPS uptake

Human PBMC (1×10^6 cells/ml) were treated as above. After the supernatants were collected for TNF-α assay, the cells were analyzed with a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). The fluorescence intensity (FI) of cells was analyzed with CELLQuest software (Becton Dickinson).

LPS localization within living hPBMC

Human PBMC (1×10^6 cells/ml), grown on glass coverslips over-night, were incubated with 50 ng/ml of FITC-LPS for indicated time. Then the cells were washed three times with warm PBS, and examined with a confocal scanning microscope (ZEISS LSM 510 META, Jena, Germany).

Statistics and presentation of data

Cytokine concentrations are expressed as mean ± SD. Each experiment was repeated at least triplicate and each data point represented the mean of at least three parallel samples. A Student's *t*-test was used to examine the differences in cytokine concentrations in the cell supernatants. A *p* value of less than 0.05 was considered significant, and a value less than 0.01 was considered very significant.

Table 1. *TNF-α release from hPBMC induced by LPS.*

Treatment	TNF-α (pg/ml)
LPS	2056.8 ± 92.0
LPS + CQ	69.8 ± 8.7[a]
LPS + anti-hTLR4 antibody for 10 min	1618.8 ± 128.9[a]
LPS + anti-hTLR4 antibody for 1 h	1694.2 ± 48.8[a, b]
Medium	26.7 ± 5.3

PBMC (1×10^6 cells/ml) were pre-incubated with 50 μg/ml CQ for 2 h, or 0.5 μg/ml of anti-human TLR4 antibody for 10 min and 1 h. Cells were stimulated with 1 mg/ml of FITC-LPS. After further cultured for 2 h, the supernatants were collected. The levels of TNF-α in the supernatants were analyzed. [a]$p < 0.01$ compared with LPS, [b]$p > 0.05$ compared with LPS + anti-hTLR4 antibody for 10 min.

Results

CQ decrease TNF-α and IL-6 release induced by LPS

The releases of various cytokines, such as TNF-α, IL-6 and IL-12 are downstream events in macrophages/monocytes activated by LPS. TNF-α was thought as an early cytokine, whereas both IL-6 and IL-12 were considered as later cytokines.

The data showed 100 ng/ml LPS could induce hPBMC to release large amount of TNF-α and IL-6 release. CQ potently inhibited both cytokines' release induced by LPS in a dose- and time-dependent manner (Figure 1, 2). The CQ dose as low as to 5 μg/ml could effectively both cytokines' release. CQ was effective no matter it was added before or after LPS, but pretreatment of CQ could produce much stronger inhibition on cytokine release.

Anti-hTLR4 antibody partially block TNF-α release induced by LPS

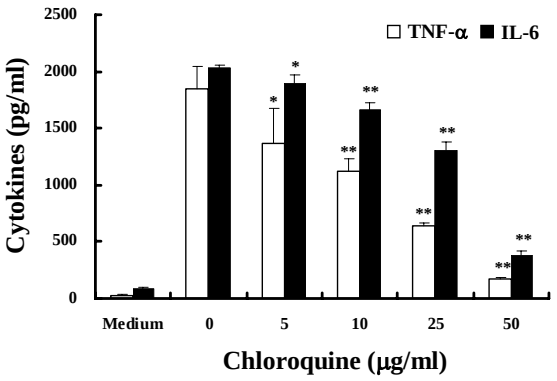

Figure 1. CQ inhibited TNF-α and IL-6 releases from hPBMC induced by LPS in a dose-dependent manner. Human PBMC (1×10^6 cells/ml) were pretreated for 2 h with indicated CQ, and then incubated with 100 ng/ml LPS for another 20 h. Cell-free supernatants were collected. The relative concentrations of TNF-α and IL-6 in the supernatants were determined by quantitative ELISA assay. Cytokine levels were expressed as mean ± SD in pg/ml. Student's *t*-test was used to examine the differences. *$p < 0.05$ compared with LPS, **$p < 0.01$ compared with LPS.

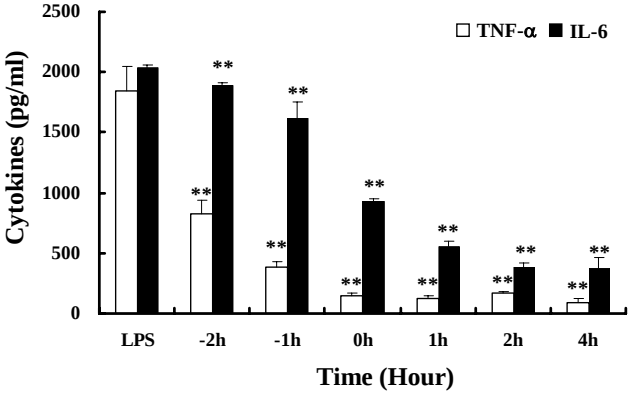

Figure 2. CQ inhibited TNF-α and IL-6 release from hPBMC induced by LPS in a time-dependent manner. Human PBMC (1×10^6 cells/ml) were pretreated for indicated time with 50 μg/ml CQ, and then incubated with 100 ng/ml LPS for another 20 h. Cell-free supernatants were collected. The relative concentrations of TNF-α and IL-6 in the supernatants were determined by quantitative ELISA assay. Cytokine levels were expressed as mean ± SD in pg/ml. Student's *t*-test was used to examine the differences. ** $p < 0.01$ compared with LPS.

Our results showed anti-hTLR4 antibody inhibited TNF-α release (Table 1); but compared with CQ, anti-hTLR4 antibody had weaker inhibition on TNF-α release ($p < 0.01$). These results suggested that blockade of cell-surface TLR4 only partially blocked TNF-α release elicited by LPS.

CQ decreases accumulation of LPS in hPBMC

To make sure whether there exists internalization of LPS, we observed the accumulation of LPS with or without CQ. Because the fluorescence label rate and fluorescence intensity are rather lower, the dose of FITC-LPS used in the experiments was 1 mg/ml. In the cells incubated with FITC-LPS, the fluorescence could be tested (20.9 ± 7.7) (Table 2). CQ decreased the FI of LPS (7.6 ± 0.5). Since CQ is an inhibitor of endosome mature, only FITC-LPS internalized into cells could be affected. Based on the supposition, we supposed that FITC-LPS might be internalized into hPBMC, and CQ probably decreased the accumulation or enhanced the degradation of LPS.

Confocal microscopy revealed that no fluorescence was observed within cells incubated with no fluorescence labeled LPS, but fluorescence of FITC-LPS adhered to and entered living cells if the cells incubated with FITC-LPS for 30 min (Figure 3B). The fluorescence was not evenly distributed. The fluorescence mainly distributed around the cell membrane, and the middle part had less fluorescence, CQ decreased the fluorescence intensity within the cells (Figure 3C). Over time, the FI transferred to middle part of the cells (Figure 3D). However, the fluorescence dissipated more rapidly in the present of CQ (Figure 3E, 3F).

Taken together, these results indicated that CQ quickened vanishment of the fluorescent LPS. The exact mechanism is not clear.

In association with CQ's inhibition on TNF-α release (Table 1), we thought the decreased fluorescence of LPS was positively related to the decreased TNF-α release.

Table 2. *Accumulation of FITC-LPS in hPBMC.*

Treatment	FI
LPS	20.9 ± 7.7
LPS + CQ	7.6 ± 0.5 [a]
LPS + anti-hTLR4 antibody for 10 min	18.4 ± 2.0 [b]
LPS + anti-hTLR4 antibody for 1 h	20.8 ± 4.2 [c]

Human PBMC were treated as described in Table 1. After the supernatants were collected for TNF-α assay, cells were collected and the fluorescence intensity (FI) and labeling rate were analyzed by flow cytometry. [a] $p < 0.01$ compared with LPS, [b, c] $p > 0.05$ compared with LPS.

Anti-hTLR4 antibodies has no effect on LPS accumulation

In order to clarify whether the cell-surface TLR4 is involved in the internalization of LPS, we investigated accumulation of FITC-LPS in the cells blocked by anti-hTLR4 antibody. The present data showed blockade of cell-surface TLR4 using specific antibody didn't affect accumulation of FITC-LPS no matter the antibody pre-incubated for 10 min or 1 h (Table 2) although CQ strongly inhibited the accumulation of FITC-LPS. No difference was observed between pretreatments with anti-hTLR4 antibody for 10 min and 1 h. Above results suggested TLR4 on the surface was not involved in the internalization of LPS.

Discussion

To the best of our knowledge, this is the first report showing that LPS could be internalized into hPBMC, and TLR4 on the cell surface isn't involved in internalization of LPS.

Although TLR4 is the principal signaling receptor, three other extracellular accessory proteins are required for sensing LPS (4). First, a 60 kD serum LPS-binding protein (LBP) is essential for the rapid induction of an inflammatory response to LPS or Gram-negative bacteria. LBP binds Gram-negative bacteria and acts as an opsonine. CD14 is the second accessory molecule needed for LPS sensing. It is present both as a glycosylphosphatidylinositol (GPI)-anchored protein on the surface of monocytes, macrophages and polymorphonuclear leukocytes, and as a soluble protein in the blood. The third protein that TLR4 requires to interact functionally with LPS is myeloid differentiation-2 (MD-2). The TLR4-MD-2 heterodimer is thought to form the complete recognition site for LPS.

TLR4 is a type I transmembrane protein, the cytoplasmic domain of which has a signaling domain called Toll-interleukin (IL)-1 receptor (TIR) domain. The TIR domain is shared by all TLRs and by members of the IL-1 receptor family. MyD88, which consists of a TIR domain and a death domain, works downstream of TLRs and the IL-1 receptor. TIR domain-containing adaptor protein (TIRAP) is another adaptor molecule that is required for a link between TLR4 and MyD88. The death domain of

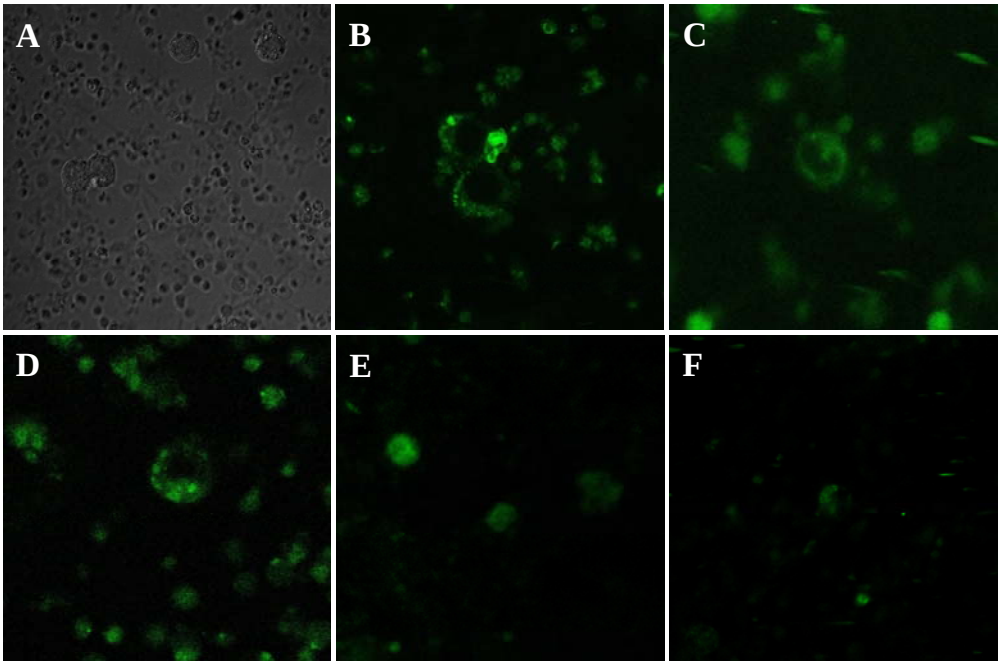

Figure 3. Localization of FITC-LPS in living cells. Human PBMC (8×10^5 cells/ml), grown on glass coverslips, were pre-incubated with 50 μg/ml CQ for 2 h. Then cells were stimulated with 50 ng/ml of FITC-LPS for 30 min to 2 h, washed and visualized under a ZEISS LSM 510 META laser confocal scanning microscope. (A) Nothing was added; (B) At the absent of CQ, FITC-LPS for 30 min; (C) At the absent of CQ, FITC-LPS for 1 h; (D) At the absent of CQ, FITC-LPS for 2 h; (E) At the present of CQ, FITC-LPS for 1 h; (F) At the present of CQ, FITC-LPS for 2 h. The pictures shown are representative of typical fields.

MyD88 recruits members of the IL-1 receptor-associated kinases (IRAKs), IRAK1 and IRAK4. IRAK4 is able to phosphorylate IRAK1, which together interact with TRAF6 (TNF receptor-associated factor 6). This then leads to the activation of mitogen-activated protein kinases (MAPKs), such as p38s, ERKs (extracellular signal-regulated kinases) and JNK (c-Jun N-terminal kinase). TRAF6 can also lead to the activation of the IκBα kinase complex (IKK), the phosphorylation and subsequent degradation of IκBα, and finally the activation of NF-κBs, resulting in the production of pro-inflammatory cytokines and the progression to adaptive immunity (4).

TLR4 is usually considered to reside and recognize LPS on the cell surface not *via* endocytosis. Internalization of LPS by various cells is an important step for its removal and detoxification, not accompanying with cytokine release (13). However, two CD14-dependent pathways of internalization have been demonstrated (5). Monomeric LPS complexed to soluble CD14 (sCD14) is delivered to the Golgi apparatus in HeLa cells, neutrophils, and cardiomyocytes (6, 7, 14); and LPS recognition may occur in the Golgi apparatus and require LPS internalization in intestinal epithelial cells (6). Prevention of LPS internalization impairs LPS response in intestinal epithelial cells but not in macrophages. (15).

Here, we showed that there indeed existed of LPS and internalization partially participated in LPS signaling since CQ inhibited cytokine release and decreased accumulation of FITC-LPS in hPBMC. In contrast, anti-hTLR4 antibody could decrease cytokines' release, but no inhibition on accumulation of FITC-LPS in hPBMC. This result revealed that inhibition of cytokines' release was related to reduction of FITC-LPS accumulation in the cells. But TLR4 on the cell surface didn't possibly participated in internalization of LPS, which was accordant with recent report by Dunzendorfer using primary monocytes and endothelial cells derived from TLR4 and CD14 knockout C57BL/6 mice (16). Thus, LPS signaling and internalization can not be viewed as mutually independent processes.

Taken together, above results suggested LPS binding to cell-surface TLR4 or internalized into cells was related to the downstream cytokines release, but the cell-surface TLR4 didn't participate in the accumulation of LPS. There was association between signaling and internalization of LPS.

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