LPS-induced depolymerization of cytoskeleton and its role in TNF-α production by rat pneumocytes

NORITAKA ISOWA, ALEXANDRE M. XAVIER, EWA DZIAK, MICHAL OPAS, DONNA I. McRITCHIE, ARTHUR S. SLUTSKY, SHAF H. KESHAVJEE, and MINGYAO LIU. LPS-induced depolymerization of cytoskeleton and its role in TNF-α production by rat pneumocytes. Am. J. Physiol. 277 (Lung Cell. Mol. Physiol. 21): L606–L615, 1999.—Lipopolysaccharide (LPS) polymerizes microfilaments and microtubules in macrophages and monocytes. Disrupting microfilaments or microtubules with cytochalasin D (CytoD) or colchicine can suppress LPS-induced tumor necrosis factor-α (TNF-α) gene expression and protein production from these cells. We have recently demonstrated that primary cultured rat alveolar epithelial cells can produce TNF-α on LPS stimulation. In the present study, we found that the LPS-induced increase in TNF-α mRNA level and protein production in alveolar epithelial cells was not inhibited by CytoD or colchicine (1 nM to 10 µM). In fact, LPS-induced TNF-α production was further enhanced by CytoD (1–10 µM) and inhibited by jasplakinolide, a polymerizing agent for microfilaments. Immunofluorescent staining and confocal microscopy showed that LPS (10 µg/ml) depolymerized microfilaments and microtubules within 15 min, which was prolonged until 24 h for microfilaments. These results suggest that the effects of LPS on the cytoskeleton and the role of the cytoskeleton in mediating TNF-α production in alveolar epithelial cells are opposite to those in immune cells. This disparity may reflect the different roles between nonimmune and immune cells in host defense.

Tumor necrosis factor-α; cytokines; lipopolysaccharide; microfilament; microtubule

LIPOLYSACCHARIDE (LPS) is the principal shock-inducing factor extracted from the outer membrane of gram-negative bacteria. Proinflammatory cytokines including tumor necrosis factor-α (TNF-α) derived from LPS-stimulated cells are responsible for the lethal effect of LPS (5). TNF-α, an early-response proinflammatory cytokine, is produced by many types of cells in response to LPS stimulation (4). Immune cells such as alveolar macrophages are major sources of TNF-α in the lung (31). It has recently been recognized, however, that nonimmune cells such as epithelial cells may also play an important role in host defense by producing cytokines and chemokines (15). McRitchie et al. (22) recently found that isolated rat alveolar epithelial cells can produce TNF-α in response to LPS stimulation.

TNF-α produced in alveolar epithelial cells may function as an intermediate signal to amplify LPS-induced cellular responses by further triggering the production of chemokines such as macrophage inflammatory protein-2 (32). TNF-α may play a crucial role in mediating acute lung injury. Nash et al. (24) used immunohistochemistry staining to examine the distribution of TNF-α in lung tissue from patients dying with adult respiratory distress syndrome. They found that TNF-α protein was located within epithelial cells resembling type II pneumocytes. There were relatively few TNF-α-positive cells in the early stages of adult respiratory distress syndrome, and TNF-α-positive cells were found throughout the epithelium in the later stages (24). It has also been demonstrated that ventilator-induced lung injury increased TNF-α mRNA expression in lung epithelial cells with in situ hybridization (30).

The mechanisms by which LPS stimulates TNF-α production from macrophages, monocytes, and other inflammatory cells have been studied extensively; however, the regulatory mechanisms acting in LPS stimulation of alveolar epithelial cells are unknown. The cytoskeleton plays an important role in mediating cytokine production by immune cells in response to LPS stimulation. Treatment of macrophages or monocytes with LPS increases polymerization of microfilaments (14, 28) and microtubules (3). Cytochalasin D (CytoD) is an inhibitor of actin polymerization, acting largely by binding to the barbed end of the actin filament (10). Colchicine is a broadly used anti-inflammatory drug inhibiting intracellular microtubule polymerization (1). CytoD (27) or colchicine (19, 25) blocks LPS-induced TNF-α gene expression and/or protein synthesis in macrophages. The purpose of the present study was to determine the effects of polymerization status of the cytoskeleton on LPS-induced TNF-α production in alveolar epithelial cells. We found that neither CytoD nor colchicine inhibited TNF-α gene expression and protein production. On the contrary, jasplakinolide, a novel microfilament-polymerizing agent (7), attenuated LPS-induced TNF-α production from alveolar epithelial cells but enhanced this effect from macrophages. Furthermore, LPS-induced depolymerization of both microfilaments and microtubules in alveolar epithelial cells. These data suggest that although alveolar epithelial cells produce TNF-α in response to LPS stimulation, the role of the cytoskeleton in mediating LPS-induced TNF-α production is different from that acting in immune cells such as macrophages and monocytes.
MATERIALS AND METHODS

Reagents, LPS (Escherichia coli and Salmonella typhosa), CytD, colchicine, rat IgG, mouse monoclonal anti-actin antibody, and rabbit skeletal muscle actin were purchased from Sigma (St. Louis, MO). DMEM, fetal bovine serum (FBS), and gentamicin were purchased from Gibco BRL (Life Technologies, Mississauga, ON). Porcine pancreatic elastase was purchased from Worthington Biochemical (Freehold, NJ). Pentobarbital sodium was purchased from Bimeda-MTC Pharmaceuticals (Cambridge, ON). Rat anti-mamalian α-tubulin antibody and FITC-conjugated rabbit anti-rat IgG were purchased from Serotec (Oxford, UK). J aspilakinolide, rhodamine-phalloidin, and FITC-DNase 1 were purchased from Molecular Probes (Eugene, OR). Peroxidase-linked sheep anti-mouse IgG was purchased from Amersham Life Science (Oakville, ON).

Rat alveolar epithelial cells isolation and culture. Alveolar type II cells were obtained with the method of Dobbs (13) as previously described (20, 22). Briefly, male adult Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing −250 g were anesthetized by an intraperitoneal injection of pentobarbital sodium (100 mg/kg body wt) and killed by transection of the descending aorta and inferior vena cava. Alveolar epithelial cells were separated from the alveolar basement membrane by incubation of the isolated lung tissue with porcine pancreati elastase was purchased from Sigma (St. Louis, MO) and killed by transection of the aorta. The viability of the fresh alveolar epithelial cells was determined by incubation of the isolated lung tissue with porcine pancreatic elastase. Contaminating alveolar macrophages were removed by differential adherence to rat IgG precoated petri dishes. The number and viability of fresh cell suspensions were counted after the cells were stained with crystal violet and trypan blue exclusion. The viability of the fresh alveolar epithelial cell suspensions was >95%.

The cells were cultured in DMEM containing 10% (vol/vol) FBS and 12.5 µg/ml of gentamicin. In most experiments, 1 ml of cell suspension (10^6 cells/ml) was seeded in 24-well culture plates (Corning Glass Works, Corning, NY) and maintained at 37°C in 5% CO2. The cells were cultured in 6-well plates to determine the relative amount of filamentous actin (F-actin) and to study TNF-α gene expression or in 96-well plates for cytotoxicity assay. For immunofluorescent staining, the cells were seeded on four-well plastic Lab-Tek chamber slides (Nunc, Naperville, IL). To reduce the contamination of alveolar macrophages in the primary culture, the culture medium was changed daily for 2 days before LPS treatment. As McRitchie et al. (22) have recently reported, this maneuver reduced the number of macrophages to undetectable levels by cell surface ectoenzyme-alkaline phosphatase staining or by immunofluorescent staining with a monoclonal antibody for CD45, a surface marker for macrophages and leukocytes. The purity of alveolar epithelial cells in the culture system was confirmed with phase-contrast microscopy and immunofluorescent staining with anti-cytokeratin and anti-surfactant proprotein C antibodies (specific markers for epithelial cells and type II pneumocytes, respectively; data not shown).

Table 1. Optimized conditions for semiquantitative RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>Size of PCR Fragments, bp</th>
<th>Thermocycle Conditions</th>
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| β-Actin  | Forward 5’-GTCGCGCTTACGCAAGACA-3’  
Reverse 5’-CTCTCTCCCTTCGAGGATCTC-3’ | 539                       | 95°C for 5 min for 1 cycle (94°C for 1 min) → 56°C for 1 min → 72°C for 1 min for 30 cycles → 72°C for 10 min for 1 cycle |
| TNF-α    | Forward 5’-GGATCTCATCTTCAAAAATCCTG-3’ 
Reverse 5’-TACACAGGAGACATTGACTCGAATCC-3’ | 419                       | 95°C for 5 min for 1 cycle (94°C for 1 min) → 56°C for 30 s → 72°C for 30 s for 40 cycles → 72°C for 10 min for 1 cycle |

TNF-α, tumor necrosis factor-α.
staining, the first antibody was replaced with nonspecific rabbit IgG (Sigma) or omitted from the staining procedure. Confocal laser scanning was performed with a confocal laser scanning microscope (MRC-600, Bio-Rad, Mississauga, ON) equipped with a Kr/Ar laser. In each experiment, control and LPS-treated cells were cultured on the same chamber slide and processed simultaneously for comparison.

Measurement of TNF-α. TNF-α concentrations in the culture medium were measured with ELISA kits (BioSource, Camarillo, CA) following the manufacturer’s instructions. According to the manufacturer, the kit for rat TNF-α has no cross-reactivity to many human and mouse cytokines and no cross-reactivity with rat interferon-γ and macrophage inflammatory protein-2. It has 0.15% cross-reactivity to human TNF-α and 100% cross-reactivity to mouse TNF-α. The optical density of each well was read at 450 nm with a NIM600 microplate reader (Dynatech Laboratories, Chantilly, VA). The final concentration was calculated by converting the optical density readings against a standard curve.

Extraction, gel electrophoresis, and immunoblotting analysis of F-actin. Cells were cultured in six-well plates (4 × 10⁶ cells/well, 3 wells/group) in 10% FBS-DMEM. Forty-eight hours after isolation, the cells were treated with and without LPS (10 µg/ml) in 10% FBS-DMEM for 24 h. After being washed twice with ice-cold PBS, the cells were lysed by adding 200 µl of a 1% Triton X-100 solution containing 1 mM EGTA, 50 mM Tris (pH 7.2), 1 mM benzamidine, 0.1 mM Na₃VO₄, 250 µg/ml of leupeptin, 25 µg/ml of aprotinin, and 0.1 mM phenylmethylsulfonyl fluoride to each well and holding on ice for 20 min. The cell lysates from each group were pooled and centrifuged at 14,000 rpm for 5 min. The supernatants (600 µl in total) were collected as the Triton-soluble fraction. The Triton-insoluble pellets were washed with cold PBS, centrifuged again, and resuspended in 40 µl of SDS sample buffer containing 60 mM Tris (pH 8.0), 5% (vol/vol) β-mercaptoethanol, 2% (wt/vol) SDS, 0.0025% (wt/vol) bromophenol blue, and 10% (vol/vol) glycerol. A fraction of the Triton-soluble supernatants (15 µl) was mixed with 5 µl of 4× SDS sample buffer. All samples were boiled for 10 min, and 15 µl of each sample was subjected to SDS-PAGE (10% polyacrylamide gel) (23). The gels were stained with Coomassie blue and destained in methanol-water-acetic acid (2:7:1 by volume). Actin protein was identified by its molecular mass (43 kDa) and by comparison with purified actin from rabbit skeletal muscle as a positive control. For immunoblotting, after electrophoresis, the proteins were electrophoretically transferred onto nitrocellulose membranes. Nonspecific binding was blocked by incubation with 8% (wt/vol) nonfat milk in PBS for 60 min at room temperature. The blots were washed with PBS and incubated overnight at 4°C with a mouse monoclonal anti-actin antibody (1:500 dilution). The blots were incubated for 60 min at room temperature with peroxidase-linked sheep anti-mouse IgG (1:10,000 dilution). After being washed, the blots were developed with SuperSignal Chemiluminescent Substrate (Pierce, Rockford, IL). The actin bands were scanned with a GS-690 imaging densitometer and quantified with a computer program, Molecular Analyst version 1.5 (Bio-Rad, Hercules, CA).

Fig. 1. Effects of cytochalasin D (CytoD) on lipopolysaccharide (LPS)-stimulated tumor necrosis factor (TNF-α) release from alveolar epithelial cells. A: cells (1 × 10⁶/ml) were cultured with 10% fetal bovine serum (FBS)-DMEM, treated with various concentrations of CytoD at 37°C for 2 h, and then incubated with and without (-) LPS for 4 h in presence of various concentrations of CytoD. Results are means ± SE of a representative experiment in triplicate. Significant difference among groups, *P < 0.0001 by 2-way ANOVA. +P < 0.05 vs. group treated with 1 nM CytoD plus LPS by Student-Newman-Keuls test. B: cells (1 × 10⁶/ml) were cultured with 10% FBS-DMEM, treated with and without CytoD at 37°C for 2 h, and then stimulated with various concentrations of LPS for 4 h in presence and absence of CytoD. Results are means ± SE of 3 experiments performed in triplicate. TNF-α production increased in an LPS dose-dependent manner (P < 0.001) that was significantly enhanced by CytoD (P = 0.0194 by 2-way ANOVA).
manufacturer's instructions. RT products from 0.2 µg of RNA were used for PCR. PCR primers for β-actin and TNF-α were synthesized by ACGT Corporation (Toronto, ON). The sequences of primers for β-actin and TNF-α are listed in Table 1. The PCR mixture was set up in a total volume of 30 µl containing 3 µl of 10× PCR buffer (200 mM Tris-Cl, pH 8.4, and 500 mM KCl), 1 µl of 50 mM MgCl2, 0.5 µl of 10 mM deoxynucleotide triphosphate mixture, 0.5 µl of each PCR primer (10 µM), and 0.3 µl of Taq polymerase (GIBCO BRL). PCR was performed with a programmable thermal cycler (PTC-100, MJ Research, Watertown, MA). The optimized PCR conditions are described in Table 1. Ten microliters of PCR product were electrophoresed on a 1% agarose gel, with ethidium bromide staining for visualization, and the gels were photographed and quantified with a gel-documentation system (Gel Doc 1000, Bio-Rad). To ensure comparability, RT-PCR was performed simultaneously on all samples collected from each experiment. PCR products were analyzed on the same gel. The optical density of the PCR product bands was quantified with integrated image-analysis software (Molecular Analyst version 1.5, Bio-Rad). With optimized PCR conditions, all data were collected without saturation or missing bands. The background of the optical density reading for each band was subtracted locally. RT-PCR was conducted at least two times for each sample to ensure reproducibility.

Statistical analysis. All experiments were carried out with materials collected from at least two to three separate cell cultures in duplicate or triplicate. All data are expressed as means ± SE from separate measurements and were analyzed with a personal computer with SigmaStat for Windows version 1.0 (Jandel, San Rafael, CA). Comparison of two groups was analyzed with Student's t-test. Comparison of more than two groups was carried out with one-way or two-way ANOVA followed by Student-Newman-Keuls test, with significance defined as P < 0.05.

RESULTS

CytoD enhanced LPS-induced TNF-α release from alveolar epithelial cells. McRitchie et al. (22) have recently shown that 10 µg/ml of LPS from E. coli maximally stimulated TNF-α production by alveolar epithelial cells within 4 h. In the present study, we found that treatment with LPS from S. typhosa (10 µg/ml) had a similar stimulatory effect on TNF-α production in the same preparation (data not shown).

Fig. 2. Jasplakinolide, a microfilament-polymerizing agent, inhibited LPS-stimulated TNF-α release from alveolar epithelial cells. A: cells (1 x 10^6/ml) were cultured with 10% FBS-DMEM, treated without and with various concentrations of jasplakinolide at 37°C for 2 h, and then incubated with LPS (10 µg/ml) for 4 h in absence and presence of jasplakinolide. Results are expressed as percent ratio to LPS-only group. Values are means ± SE of 3 experiments in duplicate or triplicate. Significant difference among groups, P < 0.0001 by 1-way ANOVA. *P < 0.05 vs. group treated with LPS only by Student-Newman-Keuls test.

Fig. 3. Colchicine did not inhibit LPS-stimulated TNF-α release from alveolar epithelial cells. A: cells (1 x 10^6/ml) were cultured with 10% FBS-DMEM, treated with increasing concentrations of colchicine at 37°C for 2 h, and then stimulated with and without LPS for 4 h. Results are means ± SE of a representative experiment in triplicate. B: cells (1 x 10^6/ml) were cultured with 10% FBS-DMEM, treated with and without colchicine at 37°C for 2 h, and then stimulated with various concentrations of LPS for 4 h in presence and absence of colchicine. Results are means ± SE of 4 experiments in duplicate or triplicate. TNF-α production increased in LPS dose-dependent manner (P < 0.001 by 2-way ANOVA), which was not affected by colchicine treatment.
LPS from E. coli was used in subsequent experiments except when otherwise specified. CytoD has been shown to inhibit LPS-induced TNF-α production from macrophages, monocytes, or neutrophils (27). We first examined whether CytoD has similar inhibitory effects in alveolar epithelial cells. Treatment of alveolar epithelial cells with various concentrations (1 nM to 10 µM) of CytoD for 6 h did not change the basal levels of TNF-α in the culture medium. The cells were pretreated with various concentrations of CytoD for 2 h, then challenged with LPS (10 µg/ml) for 4 h in presence of CytoD. TNF-α production was not affected by CytoD at concentrations equal to or below 0.1 µM. With higher concentrations (1–10 µM), CytoD enhanced LPS-induced TNF-α production (Fig. 1A).

Jasplakinolide inhibited LPS-induced TNF-α release from alveolar epithelial cells. To further clarify the role of microfilament polymerization in TNF-α production, cells were pretreated with various concentrations of jasplakinolide (1–100 nM) for 2 h and then challenged with LPS (10 µg/ml) for 4 h in the presence of jasplakinolide. A control group was handled similarly in the absence of jasplakinolide. TNF-α production was inhibited by jasplakinolide at all concentrations tested (Fig. 2). Treatment of alveolar epithelial cells with jasplakinolide (1–100 nM) alone for 6 h did not change the basal levels of TNF-α in the culture medium (data not shown).

Colchicine had no inhibitory effect on TNF-α release in alveolar epithelial cells. Microtubules, another major component of the cytoskeleton, are also involved in mediating LPS-induced TNF-α production in immune cells.
The effect of microtubule disruption on alveolar epithelial cells was investigated. When the cells were incubated with various concentrations of colchicine, neither the basal level nor the LPS stimulation-induced TNF-α production was affected by colchicine at all concentrations tested (1 nM to 10 µM; Fig. 3A). When the cells were stimulated with various concentrations of LPS (1 ng/ml to 10 µg/ml), TNF-α production was not inhibited by colchicine (1 µM) at any dose of LPS tested (Fig. 3B).

CytoD and colchicine inhibited and jasplakinolide enhanced LPS-induced TNF-α release from macrophage cells. To determine the efficiency of our CytoD, jasplakinolide, and colchicine treatment protocols, we used a murine macrophage cell line (RAW 264.7). RAW 264.7 cells were pretreated with CytoD (1 µM), jasplakinolide (100 nM), or colchicine (1 µM) for 2 h and then incubated with LPS and CytoD, jasplakinolide, or colchicine for 4 h. With an optimized dose of LPS (10 ng/ml), RAW 264.7 cells exhibited a marked increase in TNF-α release, an effect that was significantly inhibited by CytoD or colchicine and enhanced by jasplakinolide (Fig. 4).

CytoD and colchicine did not block LPS-induced TNF-α mRNA expression in alveolar epithelial cells. In macrophages, the inhibitory effects of CytoD (28) or colchicine (19) on LPS-stimulated TNF-α production was associated with inhibition of TNF-α mRNA expression. To determine whether the treatment with CytoD or colchicine had any effect on LPS-induced TNF-α gene expression by alveolar epithelial cells, the cells were incubated with CytoD (1 µM) or colchicine (1 µM) before and during LPS (10 µg/ml) stimulation. Total RNA was extracted, and steady-state levels of TNF-α mRNA were determined with semiquantitative RT-PCR. Because β-actin gene transcription was not affected by either LPS or any inhibitors tested, the ratio of densitometry units between TNF-α and β-actin mRNA was used to represent the steady-state levels of TNF-α mRNA. Similar to our previous observation, LPS induced an increase in TNF-α mRNA. Treatment of cells with CytoD or colchicine had no effect on LPS-induced TNF-α mRNA expression. A representative blot is shown in Fig. 5A. Densitometry data combined from three separate experiments are presented in Fig. 5B.

LPS induced depolymerization of microfilaments in rat alveolar epithelial cells. The opposite effects of CytoD and jasplakinolide on LPS-induced TNF-α production by alveolar epithelial cells compared with that by macrophages suggest that the effects of LPS on cells (1, 19, 25). The effect of microtubule disruption on alveolar epithelial cells was investigated. When the cells were incubated with various concentrations of colchicine, neither the basal level nor the LPS stimulation-induced TNF-α production was affected by colchicine at all concentrations tested (1 nM to 10 µM; Fig. 3A). When the cells were stimulated with various concentrations of LPS (1 ng/ml to 10 µg/ml), TNF-α production was not inhibited by colchicine (1 µM) at any dose of LPS tested (Fig. 3B).

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microfilaments of alveolar epithelial cells are different from those on macrophages. In most cell types, actin exists in both a G-actin and an F-actin form, which is regulated through a dynamic polymerization and depolymerization process (8). We examined these two kinds of actin with fluorescent staining and confocal microscopy. The intensity of F-actin staining was decreased in primary cultured alveolar epithelial cells by a short exposure (15 min) to LPS (10 µg/ml; Fig. 6B) compared with the untreated control cells (Fig. 6A). The staining of G-actin in alveolar epithelial cells (Fig. 6C) was not affected by short periods of LPS stimulation (Fig. 6D). We also examined the effect of LPS on microfilaments after longer periods of treatment. When alveolar epithelial cells were incubated with LPS for 24 h, the staining of F-actin was further reduced (Fig. 7, A and B), which was associated with a clearly increased G-actin staining (Fig. 7, C and D). This LPS-induced depolymerization was opposite to that described in macrophages (28) and monocytes (14).

To further confirm LPS-induced depolymerization of actin filaments, we isolated F-actin from alveolar epithelial cells by Triton extraction (23). After high-speed centrifugation, G-actin can be dissolved in the Triton-soluble fraction, whereas F-actin is mainly present in the Triton-insoluble pellets. Because actin is very abundant in the cell lysates, it can be separated by gel electrophoresis and displayed by Coomassie blue staining as a single band (Fig. 8A). This band was further confirmed to be actin by immunoblotting analysis (Fig. 8B). Densitometric quantification was performed, and the data were standardized by the cell number. Interestingly, the amount of actin in the Triton-insoluble fraction only accounted for a small percentage of the total actin in the cells (Fig. 8C). LPS stimulation significantly reduced the amount of Triton-insoluble actin to ~60% (Fig. 8C). Similar results were obtained when the cells were stimulated with LPS from S. typhosa (Fig. 8A).

Effects of LPS on microtubules in rat alveolar epithelial cells. Similarly, we investigated the effect of LPS on microtubules in alveolar epithelial cells using immunofluorescent staining and confocal microscopy. Unstimulated alveolar epithelial cells demonstrated a diffuse, homogeneous pattern of microtubules. The intensity of microtubule staining after a short incubation period (15 min; Fig. 9A) was stronger than that after a longer incubation period (24 h; Fig. 9B), which may be due to the change in cell morphology during the culture. After a short period of LPS stimulation (15 min), the intensity of microtubule staining decreased (Fig. 9C) compared with that in control cells (Fig. 9A). The reduced polymerization of microtubules was observed as soon as 1 min after LPS stimulation and lasted at least 4 h (data not shown). However, no difference in the microtubule pattern and staining intensity was observed between control and LPS-stimulated cells after a 24-h incubation (Fig. 9, B and D). When the first antibody was replaced with rabbit nonimmune IgG or omitted from the staining protocol, no staining was detected (data not shown). The LPS-induced transient depolymerization of microfilaments in rat alveolar epithelial cells is decreased by 10.2% (Fig. 8B, A and B).

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lization of microtubules in alveolar epithelial cells was also opposite to that observed in human monocytes (3).

**DISCUSSION**

In the present study, we observed that LPS stimulation induces TNF-α gene expression and protein production in alveolar epithelial cells, which is consistent with the previous study by McRitchie et al. (22). In contrast to macrophages and monocytes, LPS-induced TNF-α production by alveolar epithelial cells was enhanced by CytoD and was not suppressed by colchicine. Jasplakinolide, a microfilament-stabilizing agent, showed opposite effects on TNF-α production between alveolar epithelial cells and macrophages. Furthermore, we found that LPS induced depolymerization of microfilaments and microtubules in alveolar epithelial cells, which is also opposite to that reported in macrophages and monocytes. Taken together, these data indicate that although alveolar epithelial cells can produce TNF-α in response to LPS stimulation, the effect of LPS on the cytoskeleton and the role of the cytoskeleton in LPS-induced TNF-α production are substantially different from those of macrophages and monocytes.

The cytoskeleton is composed of three constituents: microfilaments, microtubules, and intermediate filaments. Both microfilaments and microtubules are involved in various activities of monocytes, macrophages, and neutrophils. LPS is known to affect the cytoskeleton system in immune cells (2, 3, 14, 16, 19, 27, 28). LPS induced a rapid reorganization of F-actin assembly in macrophages (28) and increased stiffness and F-actin assembly in monocytes (14) as well as enhanced formyl-methionyl-leucyl-phenylalanine-induced actin polymerization in neutrophils (16). Microfilaments are related to the trafficking of LPS after its internalization in neutrophils (11). In mononuclear phagocytes, LPS increased the number, length, and stability of microtubules after either 30 min or 18 h of incubation (3). Incubation of monocytes with LPS for 18 h increased the quantities of α-tubulin, β-tubulin, and tyrosinated α-tubulin as well as microtubule-associated protein-2 (2). Macrophages or monocytes must change their shape to migrate to inflammatory sites in response to stimuli such as LPS (15, 29). Increased polymerization of the cytoskeleton may be related to these functions.

In contrast, alveolar epithelial cells play an important role in maintaining the integrity of the epithelial structure and in the recruitment of immune cells. The decrease in polymerization of microfilaments and microtubules in alveolar epithelial cells after LPS stimulation may facilitate the migration of inflammatory cells across the epithelium to enter the alveolar spaces. Both gram-negative and gram-positive bacteria can enter nonphagocytic cells such as epithelial cells (9, 18). Inhibition of microfilament (9) and microtubule (18) polymerization of host cells may reduce the invasion of bacteria. The depolymerization of the cytoskeleton induced by LPS may have a similar protective effect on alveolar epithelial cells.

In the present study, the enhancement by CytoD on TNF-α production from alveolar epithelial cells was weak, whereas the inhibitory effect of jasplakinolide was dramatic. By contrast, the inhibitory effect of CytoD on TNF-α production from RAW 264.7 cells was remarkable, whereas the enhanced effect of jasplakinolide was small. These results suggest that when the effect of CytoD or jasplakinolide is opposite to the effect of LPS on microfilament polymerization, it blocks LPS-induced TNF-α production. On the other hand, when LPS increases or decreases microfilament polymerization in one particular cell type, further enhancement of this effect has less influence on TNF-α production.

LPS-induced depolymerization of microfilaments may enhance secretion activity in alveolar epithelial cells. It has been noted that CytoD can stimulate alveolar epithelial cells to release lung surfactant (26). Depolymerization from F-actin to G-actin is involved in terbutaline-induced transport and exocytosis of lamellar bodies, the storage form of lung surfactant, from type II cells (6). Intratracheal instillation of LPS to rats in-
increased secretion of surfactant proteins A and D (21). These observations suggest that a microfilament depolymerization-dependent secretion exist in type II cells. F-actin depolymerization induced by LPS-stimulation may facilitate TNF-α secretion through this mechanism.

The inhibitory effect of Cytod on LPS-induced TNF-α production in macrophages was observed at 0.2 μM (27), a concentration that is incapable of affecting F-actin by itself (33). At this low concentration, Cytod blocked LPS-induced elevation of TNF-α mRNA levels. The role of LPS-induced depolymerization of microfilaments and microtubules in macrophages by colchicine was also associated with inhibition of LPS-induced TNF-α gene expression (1, 19, 25). LPS stimulation rapidly activated mitogen-activated protein kinase associated with microtubules in macrophages (12). Therefore, in macrophages, polymerization of microfilaments and microtubules may be involved in LPS signaling leading to TNF-α gene expression and subsequently its synthesis. In the present study, agents affecting microfilaments and microtubules had no effects on LPS-induced elevation in TNF-α mRNA levels. The role of LPS-induced depolymerization of microfilaments and microtubules in alveolar epithelial cells may be involved in LPS-induced TNF-α production at the posttranscriptional level.

Alveolar epithelial cells are continually exposed to external pathogens and thus may play an important role in recruiting inflammatory cells by producing various kinds of chemoattractants, including the proinflammatory cytokines such as TNF-α. The different effects of LPS on the cytoskeleton between alveolar epithelial cells and macrophages shown in this study may be due to their different roles in host defense. The underlying molecular mechanisms need to be further studied.

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N. Isowa is a recipient of a fellowship from the Department of Surgery and Faculty of Medicine, University of Toronto (Toronto, Ontario, Canada). M. Liu is a Scholar of the Medical Research Council of Canada.

Address for reprint requests and other correspondence: M. Liu, Thoracic Surgery Research Laboratory, Toronto General Hospital, University Health Network, Room CCRW 1-821, 200 Elizabeth St., Toronto, Ontario, Canada M5G 2C4 (E-mail: mingyao.liu@utoronto.ca).

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