CD14-dependent Endotoxin Internalization via a Macropinocytic Pathway

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Gram-negative bacterial endotoxin (a lipopolysaccharide (LPS)) specifically binds to CD14, a glycosylphosphatidyl inositol (GPI)-anchored surface myeloid glycoprotein. This interaction leads to cell activation, but it also promotes LPS internalization and detoxification. In this work, we investigated the route of LPS and CD14 internalization and the relevance of CD14 GPI anchor in the endocytic pathway. In promonocytic THP-1 cells transfected with a GPI or a chimeric integral form of CD14, we showed by differential buoyancy in sucrose density gradients that these two forms of CD14 were sorted to different plasma membrane subdomains. However, both forms of CD14 associated preferentially with the same surface microfilament-enriched microvilli or ruffles. Electron microscopic studies indicated that CD14 internalized via macropinocytosis, a process resembling that of phagocytosis, different from “classical” receptor-mediated endocytic pathways, such as clathrin-coated pits or caveolae. With cell warming, the CD14-enriched ruffles fused and formed large vesicles. Later, these vacuoles made stacks and condensed into phagolysosomes. CD14 was specifically associated with all of these structures. Radiolabeled LPS internalization paralleled CD14 internalization. Confocal microscopic studies confirmed the co-localization of LPS and CD14 both at the cell surface and in endosomal compartments. The microfilament-disrupting, macropinocytosis blocking agent cytochalasin D inhibited LPS and CD14 internalization but did not prevent LPS-dependent activation, indicating that these two processes are dissociated.

Clearance of bacteria and bacterial molecules, predominantly performed by macrophages, is an important feature of bacterial immunity and requires specific recognition and endocytic pathways (1, 2). A prototypic example of a bacterial molecule with profound effects on mammalian cells is the Gram-negative lipopolysaccharide (LPS),1 or endotoxin (3). Various macrophage cell surface molecules can bind LPS (4), including scavenger receptors (5) and CD14 (6). However, in CD14-positive cells, LPS internalization is almost completely dependent on the presence of CD14 and is not inhibited by scavenger receptor ligands (7). CD14, a 55-kDa glycoprotein, has been recognized as a surface myeloid differentiation marker (8) and the LPS receptor (9). Engagement of CD14 by complexes of LPS and LPS-binding protein, as well as other bacterial ligands leads to cell activation (9, 10). The mechanism of transmembrane LPS signal transduction remains to be unraveled (11). CD14 also exists in a soluble form, and complexes of LPS and soluble CD14 activate CD14-negative cells, such as endothelial and epithelial cells (12).

Apart from cell activation, membrane-bound CD14 plays other important roles: it mediates LPS internalization (7, 13, 14), as well as Gram-negative bacteria and mycobacteria phagocytosis (15, 16). LPS internalization by myeloid cells is a critical step for its detoxification. After endocytosis, LPS is biologically deactivated by a specific enzyme (acyloxyacyl hydrolase) present in phagocytic granules (17).

Glycosylphosphatidyl-inositol (GPI)-linked molecules are sorted to plasma membrane subdomains, rich in sphingomyelin, cholesterol, receptors, and other signaling molecules (18–20). In epithelial cells, these plasma membrane domains correspond morphologically to noncoated pits and vesicles, known as caveolae (19). Caveolae have been implicated in GPI-linked receptor-mediated endocytosis (19, 21, 22). CD14 is naturally attached to macrophage and polymorphonuclear neutrophil plasma membranes via a GPI moiety (23, 24). CD14 association with particular membrane subdomains, routes of LPS and CD14 internalization remain to be elucidated.

In this study, we found that LPS and CD14 internalized via a macropinocytosis independently of “classical” receptor-mediated endocytic pathways, such as clathrin-coated pits or caveolae. The modification of the GPI-anchored CD14 into an integral protein did not significantly affect rates and pathways of receptor-ligand internalization, and CD14 endocytosis did not seem to be regulated by its ligand, LPS. Finally, cytochalasin D inhibited LPS endocytosis but did not prevent LPS-dependent cell activation.

MATERIALS AND METHODS

Cells—Wild type CD14 cDNA or a chimeric cDNA construct of CD14 attached to the transmembrane portion of human tissue factor were used to transfect a human promonocytic, CD14-negative, THP-1 cell line, as described elsewhere (15, 25). Stable transfecteds expressed either a GPI-anchored CD14 (wtCD14-THP1 cells) or a transmembrane form of CD14 (tmCD14-THP1 cells). Cells transfected with the empty vector (pRC/RcSV) served as control cells (25). Transfectants were cultured in suspension in RPMI 1640 medium containing penicillin and gentamicin antibiotics, 10% fetal bovine serum, and 0.5 mg/ml G418 (Geneticin®), all from Life Technologies, Inc. CD14 expression in wtCD14-THP1 and tmCD14-THP1 cells was 2 x 106 and 2 x 105 molecules/cell, respectively (25).

Membrane Targeting of CD14—Membrane targeting of CD14 was assayed using sucrose density gradient according to published methods.

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1 The abbreviations used are: LPS, lipopolysaccharide; GPI, glycosylphosphatidylinositol; FITC, fluorescein isothiocyanate; wt, wild type; tm, transmembrane; IL, interleukin.

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Briefly, 2 × 10⁵ wtCD14-THP1 and tmCD14-THP1 cells were lysed for 20 min at 4 °C in a lysis buffer containing anti-proteases (TNE (25 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA) plus 1 mM phenylmethylsulfonyl fluoride, 2 µg/ml leupeptin, 200,000 IU/ml aprotinin), and 1% Triton X-100 or 1% Triton X-100 plus 60 µM octylglucoside (26). Cell lysates were then homogenized (8 strokes, Dounce homogenizer), and 2 ml of an 80% sucrose solution was added to 2 ml of cell lysates. A 7.5-ml 5–30% linear sucrose gradient was layered onto the 40% sucrose/cell lysate mixture, and tubes were ultracentrifuged (Beckman SW41 rotor) at 39,000 rpm at 4 °C for 18 h. One-mL fractions were collected from the bottom of the tubes. Proteins in each fractions were precipitated in 10% trichloroacetic acid, resuspended in 2% SDS-polyacrylamide gel electrophoresis sample buffer containing 5% β-mercaptoethanol, boiled for 5 min, and separated using a 12% acrylamide SDS gel. Proteins were then electrotransferred onto a nitrocellulose membrane. CD14 was detected by Western blot using a goat anti-human CD14 antibody (from R. J. Ulevitch, The Scripps Research Institute, La Jolla), protein G-horseradish peroxidase (Bio-Rad), and ECL (Amersham Pharmacia Biotech). In some experiments, cell membrane proteins were detected by dot-blot (27); proteins contained in 2–20 µl of the sucrose density gradient fractions (non-trichloroacetic acid-precipitated) were diluted in 200-µl TNE buffer and transferred onto nitrocellulose by filtration (Bio-Rad DotBlot™ apparatus). Membrane proteins were detected by Western blot using specific monoclonal antibodies, a secondary goat anti-mouse IgG-HRP conjugate (Santa Cruz Biotechnology, Santa Cruz, CA), and the ECL detection system (27). Murine monoclonal antibodies to human membrane proteins used in this dot-blot assay were 28C5 (anti-CD14, a gift from D. Leturcq, RW Johnson Pharmaceutical Institute, San Diego), MEM 154 (anti-CD16), MEM 48 (anti-CD18), MEM 102 (anti-CD48), MEM 118 (anti-CD55), and MEM 43/5 (anti-CD59). Antibodies of the MEM series were characterized and donated by V. Horejsi, Prague, Czech Republic (28). In other experiments, GPI-rich membrane subdomains were isolated without detergent, using a combination of mechanical homogenization of cells, membrane sonication, successive Percoll™ (Amersham Pharmacia Biotech) and Optiprep™ (Nycomed Pharma, Oslo, Norway) gradients, as described elsewhere (29), and assayed by dot-blot for CD14 (see above).

Immunoelectron Microscopy—THP-1 transfectants were sequentially incubated for 2 h at 4 °C with 0.3 mg/ml of biotinylated 63D3 monoclonal antibody (a noninhibitory anti-CD14 monoclonal antibody, not interfering with LPS binding site) (ATCC, Rockville, MD) and for 1 h at 4 °C with a 1:10 dilution of streptavidin coupled to 10 nm colloidal gold particles (ANAWA, Wageningen, The Netherlands) in phosphate-buffered saline/bovine serum albumin 1% buffer. After two washes in 4 °C phosphate-buffered saline, cells were warmed and kept 5 or 20 min in a 37 °C water bath in the presence or in the absence of 2 µg/ml Escherichia coli K12 L25D25 LPS (List Biological Laboratories Inc., Campbell, CA)/10% fetal bovine serum. Cells were then fixed for 30 min at 20 °C with 2.5% glutaraldehyde diluted in pH 7.4 phosphate buffer, dehydrated, and processed for electron microscopy as described elsewhere (30). Thin sections were examined in a Philips electron microscope 301, and gold particles were quantitatively analyzed. For each time point studied, 450–800 gold particles from more than 50 cells were analyzed from cells expressing either wt- or tm-CD14. Gold particles were scored in terms of their association with cellular structures, such as microvilli or membrane ruffles, the inner leaflet of macropinosomes (vesicular structures), endocytic vesicles (clathrin-coated pits/vesicles), and lysosome-like organelles. The percentage of membrane surface represented by ruffles or microvilli was determined on randomly photographed pictures of wtCD14-THP1 cells (231.4 ± 11 µm of plasma membrane length on each micrography; n = 57) and tmCD14-THP1 cells (256.7 ± 12.6 µm; n = 50) at an initial magnification of ×7200 as described previously (31).

LPS Internalization—One hundred ng/ml [3H]LPS (biosynthetically tritiated E. coli K12 L25D25 LPS, List Biological Laboratories) (32), preincubated for 15 min at 37 °C with fetal bovine serum, were added to the cells for various times. In some experiments, the fluorescently labeled anti-CD14 antibody (Caltag, San Diego, CA) (1 µM) were added to the cells 30 min prior to the addition of LPS (33, 34). After various times of LPSt incubation, cells were washed and treated at 4 °C with 200 µg/ml Pronase (Boehringer Mannheim) for 1 h (35, 36). Supernatants and cells were separated by centrifugation and transferred into scintillation liquid. Radioactivity was measured in a scintillation counter to determine cell-bound and internalized (Pronase-resistant, cell-associated) counts. Internalization was defined as the percentage of counts resistant to Pronase relative to the total counts (cell-associated counts before the Pronase treatment). Cell viability during the experiment was tested using the Live/Dead™ Eukolight viability kit (Molecular Probes, Leiden, The Netherlands), according to the manufacturer’s protocol.

Confocal Laser Microscopy—wtCD14- and tmCD14-THP1 cells preincubated for 30 min in the presence or in the absence of 3 µM cytochalasin D (Sigma) were labeled with 60 µg/ml biotinylated 63D3 anti-CD14 monoclonal antibody and 30 µg/ml streptavidin-Texas red (Molecular Probes) at 4 °C for 45 min. After washing, 1 µg/ml fluorescein (FITC) LPS (donated by P. S. Tobias, The Scripps Research Institute, La Jolla) was added for 30 min at 4 °C and washed. Cells were then fixed in 2% formalin and mounted on slides.

Table I

| TABLE I | Association of wt- and tm-CD14 with plasma membrane structures using morphometrical studies of electron microscopy and immunogold labeling of CD14 |
|---------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Membrane surface found as microvilli or ruffles | Gold particles associated with microvilli or ruffles | CD14 enrichment on microvilli or rufflesa |
| wtCD14-THP1 cells | 54.9 ± 0.8 | 14.8 ± 0.07 |
| tmCD14-THP1 cells | 54.9 ± 0.8 | 14.8 ± 0.07 |

a CD14 enrichment on microvilli or ruffles was calculated as the ratio of the percentage of gold particles associated with microvilli or ruffles over the percentage of membrane surface found as microvilli or ruffles.
Institute, La Jolla, CA) in 10% normal human serum was added, and cells were put at 37 °C for 1 or 30 min. Cells were then chilled, washed, and fixed in Tris/glycerol/polyvinyl alcohol mounting solution. Specimens were examined with a Zeiss confocal laser fluorescence inverted microscope (LSM 410, Carl Zeiss, Oberkochen, Germany) using simultaneous lasers with excitation wavelengths of 543 and 488 nm for red and green, respectively, and detection using red and green narrow band filters. Cells were observed through an oil plan-neofluar 363/1.3 objective. The best plan of section was optimized through optical sectioning.

**Cell Activation**—Cytoplasmic protein tyrosine phosphorylation by LPS (200 ng/ml) in the presence or absence of 3 mM cytochalasin D (1 h pretreatment) was assayed using a 4G10 phosphotyrosine Western blot (Upstate Biotechnology), as described elsewhere (25). In similar experiments, LPS-induced nuclear factor-κB activation was assessed using classical electromobility shift assay of nuclear proteins (25). Interleukin-8 was measured by enzyme-linked immunosorbent assay (Endogen, Cambridge, MA) in conditioned supernatants from THP-1 transfectants treated for 7 h with various concentrations of LPS, with or without 3 mM cytochalasin D (25).

**RESULTS**

**Membrane Targeting of CD14 and Surface Events**—Using sucrose density fractionation of Triton X-100 cell extracts, we observed that the GPI form of CD14 (wtCD14) and the transmembrane CD14 chimera (tmCD14) were targeted to different membrane subdomains, as indicated by their differential buoyancy in the sucrose gradient. The Triton X-100-extracted wtCD14 floated in low sucrose density fractions, previously recognized as GPI-rich domains, whereas tmCD14 remained in the 40% sucrose solution (Fig. 1A). Octylglucoside known to disrupt GPI-rich domains solubilized wtCD14, which was recovered in the 40% sucrose density fractions (Fig. 1A), whereas the buoyancy of tmCD14 was not modified by this detergent. In Triton X-100 lysis buffer, wtCD14 co-localized in low sucrose density fractions with other GPI-anchored proteins (CD16, CD48, CD55, and CD59), but tmCD14 remained in the high sucrose density fractions, along with the transmembrane CD18 protein (Fig. 1B). Nondetergent isolation of low-density GPI-rich domains (29) showed that CD14 was enriched at least 100 times in these domains as compared with tmCD14-THP1 cells, as determined by serial dilutions of GPI-rich domains of wt- and tmCD14-THP1 cells in the dot-blot assay (data not shown). This latter experiment indicated that the separation by buoyancy of wt- and tmCD14 was not an artifact due to the presence of detergents. Taken together, these experiments demonstrated that the GPI anchoring was responsible for targeting CD14 to GPI-rich, lipid-rich plasma membrane subdomains, whereas tmCD14 was sorted into other membrane domains, where most integral proteins are found. LPS treatment did not modify the distribution pattern of wt- or tmCD14 in the sucrose density fractions (not shown).

CD14 from 1,25-(OH)₂ vitamin D₃-differentiated THP-1 cells (CD14-positive THP-1 cells) (6), human monocytes, and epithelial cells (COS and HepG2) transfected with GPI-CD14 was
found in the same sucrose density fractions as wtCD14 originating from transfected THP-1 cells (not shown), indicating that GPI-CD14 targeting to lipid-rich domains was independent of the cell type tested.

We next determined wt- and tmCD14 association with plasma membrane structures using electron microscopy and immunogold labeling of CD14. The THP-1 cell plasma membrane was found to be very convoluted, with 37% of the membrane surface found as microvilli or ruffles (Table I). CD14 immunogold labeling at 4 °C indicated that wtCD14, and to a lesser extent tmCD14, associated preferentially with membrane microvilli or ruffles, with enrichment factors of CD14 on these structures of 1.48 ± 3 and 1.22 ± 3, respectively (Table I and Fig. 2). Very little, if any, wt- or tmCD14 localized in clathrin-coated pits. We did not observe clear caveolar structures in THP-1 cells. No immunogold CD14 labeling was observed in RSV-control THP1 cells.

LPS and CD14 Internalization—Co-localization of LPS and CD14 at the plasma membrane level was assessed by confocal laser microscopy and dual LPS and CD14 fluorescent labeling. After 1 min of incubation at 37 °C, Texas red-immunolabeled CD14 and FITC-LPS co-localized exclusively at the membrane surface in both THP-1 transfectants (Fig. 3). No fluorescent labeling was detectable in control RSV cells. We next addressed whether LPS and CD14 co-localized during the process of endocytosis using confocal laser microscopy. After 30 min of cell warming, both LPS and CD14 internalized and were found to be co-localized in intracellular endocytic compartments (Fig. 3).

LPS internalization patterns and rates were assessed using a classical internalization protocol. Cells were put at 37 °C in the presence of [3H]LPS and serum for various times and then chilled and treated with Pronase. Cell-associated counts after Pronase treatment were considered internalized. Association of [3H]LPS with THP1 transfectants was entirely dependent on the presence of CD14, because RSV control cells did not show significant [3H]LPS binding. In addition, in experiment not shown here, anti-CD14 monoclonal antibodies specifically blocking [3H]LPS binding to CD14 (28C5 and 3C10, ATCC) prevented [3H]LPS binding and internalization. In wt- and tmCD14-THP1 cells, [3H]LPS binding (defined as 3H counts released by Pronase) was rapid and plateaued after 5–10 min (not shown). [3H]LPS was internalized with similar rates (~2% per min) in both THP1 transfectants. A plateau of internalization (maximal internalization of ~45%) was reached after 30–60 min in wtCD14-THP1 cells (Fig. 4). In tmCD14-THP1 cells, although initial internalization rates were quite similar to those measured in wtCD14, internalization did not quite reach a plateau after 120 min (~70% internalization at 120 min; Fig. 4).

Rates and routes of wt- and tmCD14 internalization were studied using immunoelectron microscopy. After immunolabeling of CD14 at 4 °C, THP1 transfectants were warmed at 37 °C for various times. Gold-labeled CD14, mainly present at the surface of microvilli or ruffles, localized in clathrin-coated pits. We did not observe clear caveolar structures in THP-1 cells. No immunogold CD14 labeling was observed in RSV-control THP1 cells.

Effect of Macropinocytosis Inhibitors on LPS and CD14 Internalization—Co-localization of LPS and CD14 at the plasma membrane level was assessed by confocal laser microscopy and dual LPS and CD14 fluorescent labeling. After 1 min of incubation at 37 °C, Texas red-immunolabeled CD14 and FITC-LPS co-localized exclusively at the membrane surface in both THP-1 transfectants (Fig. 3). No fluorescent labeling was detectable in control RSV cells. We next addressed whether LPS and CD14 co-localized during the process of endocytosis using confocal laser microscopy. After 30 min of cell warming, both LPS and CD14 internalized and were found to be co-localized in intracellular endocytic compartments (Fig. 3). LPS internalization patterns and rates were assessed using a classical internalization protocol. Cells were put at 37 °C in the presence of [3H]LPS and serum for various times and then chilled and treated with Pronase. Cell-associated counts after Pronase treatment were considered internalized. Association of [3H]LPS with THP1 transfectants was entirely dependent on the presence of CD14, because RSV control cells did not show significant [3H]LPS binding. In addition, in experiment not shown here, anti-CD14 monoclonal antibodies specifically blocking [3H]LPS binding to CD14 (28C5 and 3C10, ATCC) prevented [3H]LPS binding and internalization. In wt- and tmCD14-THP1 cells, [3H]LPS binding (defined as 3H counts released by Pronase) was rapid and plateaued after 5–10 min (not shown). [3H]LPS was internalized with similar rates (~2% per min) in both THP1 transfectants. A plateau of internalization (maximal internalization of ~45%) was reached after 30–60 min in wtCD14-THP1 cells (Fig. 4). In tmCD14-THP1 cells, although initial internalization rates were quite similar to those measured in wtCD14, internalization did not quite reach a plateau after 120 min (~70% internalization at 120 min; Fig. 4).

Rates and routes of wt- and tmCD14 internalization were studied using immunoelectron microscopy. After immunolabeling of CD14 at 4 °C, THP1 transfectants were warmed at 37 °C for various times. Gold-labeled CD14, mainly present at the surface of microvilli or ruffles, localized in macropinocytic vesicles after 5 min, forming large endocytosed vacuoles organizing in stacks (Figs. 2 and 5). After 20 min, CD14 was found deeper in the cytoplasm in denser endosome-like or lysosome-like structures (Figs. 2 and 5). These pictures are typical of macropinocytosis. CD14 internalization route was identical in wt- and tmCD14-THP1 cells. Very similar rates of CD14 internalization were found in both THP-1 transfectants (Figs. 4 and 5). Only tmCD14 internalized more and was found more associated with endosome-like or lysosome-like structures than wtCD14-THP1 cells (75% versus 45% internalization at 20 min, respectively) (Figs. 4 and 5). These findings are in accordance with what was found in the LPS internalization assay, with similar rates of LPS and CD14 internalization in both assays (Fig. 4). All of this strongly suggested a common pathway of LPS and CD14 internalization. In experiments not shown here, LPS treatment of THP-1 transfectants did not significantly influence rates or routes of CD14 internalization.
ternalization—The actin filament disrupting agent cytochalasin D markedly decreased [3H]LPS internalization in both wtCD14- and tmCD14-THP1 cells, with a maximum effect observed at 3 μM cytochalasin D concentration (Fig. 4). In confocal microscopy, cytochalasin D clearly prevented FITC-LPS and Texas red-CD14 internalization (Fig. 3). Phosphoinositide-3 kinase has been implicated in macropinocytosis (34). In experiments not shown here, the phosphoinositide-3 kinase inhibitor wortmannin decreased [3H]LPS internalization by 30%. Importantly, these inhibitors had no effect on cell viability at the concentrations tested.

LPS Activation of Cells—As previously shown (25), LPS treatment of wt- and tmCD14-THP1 cells induced rapid phosphorylation of p38 mitogen-activated protein kinase, NF-κB activation, and IL-8 secretion (Fig. 6). Although cytochalasin D had a profound inhibitory effect on LPS internalization, it modified neither LPS-induced p38 mitogen-activated protein kinase phosphorylation nor NF-κB activation. Importantly, LPS internalization and activation was assayed within the same time frame, i.e. 30–45 min (Fig. 6). IL-8 secretion occurring 7 h after LPS treatment was markedly increased by the treatment of cytochalasin D. These results strongly suggest that LPS internalization (blocked by cytochalasin D) and LPS activation (unaffected or increased by cytochalasin D) of cells are separate events.

DISCUSSION

Herein, we show that LPS is internalized via a CD14-dependent macropinocytic process resembling that of phagocytosis. This endocytic pathway has been proposed as an efficient route for nonselective endocytosis of solute macromolecules, as well as for invasion by some pathogenic bacteria, such as Salmonella thyphimurium (37, 38). Macropinocytosis has also been described for receptor-mediated endocytosis (22, 38), such as complement receptors CR1 (30) and CR3 (39), involved in phagocytosis by human neutrophils. Macropinocytosis requires the development of cytoplasmic projections or membrane ruffles in a microfilament-dependent mechanism (40). After ruffles form, they merge and close into large intracellular vesicles, termed macropinosomes. These endocytic vesicles then organize in stacks, interact with other intracytoplasmic compartments, and evolve into dense phagolysosomal-like structures. In our work, immunogold-labeled CD14 was preferentially localized on membrane ruffles or microvilli. Upon cell warming, CD14 was found specifically associated with all the macropinosomal and the phagolysosomal structures described above. Interestingly, before CD14 was recognized as the LPS receptor, Kang et al. (41) described in electron microscopic studies that LPS endocytosed through a phagocytic, pinocytotic mechanism in human monocytes, very similar to the process described in the present work. Similar findings were reported with murine macrophages (42). These authors showed that LPS-gold particles were found in deep labyrinthic invaginations of the plasma membrane and with coated pits budding off...
LPS and CD14 Internalization

Fig. 6. Effect of cytochalasin D on LPS-induced p38 mitogen-activated protein kinase phosphorylation, NF-κB activation, and IL-8 secretion. A, upper gel, phosphothreonine Western blot of cytoplasmic lysates of THP-1 transfectants treated with LPS, cytochalasin D (Cyto D) or both for 30 min. The phosphorylated p38 band is indicated by an arrow. Lower gel, electromobility shift assay of nuclear extract from THP-1 transfectants treated as above for 45 min. The complex of activated NF-κB protein and the 32P-radiolabeled NF-κB DNA oligoprobe is indicated by an arrow; n.s., nonspecific band. B, IL-8 secretion by wtCD14-THP1 cells (circles), tmCD14-THP1 cells (squares), and RSV cells (triangles). Cells were treated with LPS in the presence (filled symbols) or in the absence (open symbols) of cytochalasin D for 7 h. Data shown here are a representative experiment, and points are means ± S.D. of triplicates.

These invaginations. We did not find CD14-gold particles in coated pits or vesicles. To reconcile these data, one might argue that CD14 passes LPS onto a second molecule internalized via coated pits. Interestingly, cross-linked CD55, another GPI-anchored receptor, internalized via a similar pathway, and was found to be associated with the same endocytic compartments (35). LPS internalization has also been studied by confocal microscopy by two different groups: in monocytes (13) and in neutrophils (43). These authors found that fluorescent LPS was rapidly internalized (within 5 min), in a CD14-dependent manner. In our work, we showed that FITC-LPS and Texas red-CD14 co-localized both at the cell membrane and in endosomal compartments.

Macropinocytosis requires actin polymerization, and this process is sensitive to cytochalasins (44). In confocal microscopy experiments, we found that both LPS and CD14 endocytosis were abrogated by cytochalasin treatment of cells (Fig. 3). In a protease-based internalization assay, cytochalasin D markedly decreased LPS internalization (Fig. 4). The incomplete inhibition of LPS internalization by cytochalasin D in our Pronase assay could possibly indicate a transfer of LPS into a protease-resistant compartment (insertion of LPS into the plasma membrane, for example). This could explain why we did not observe LPS internalization in cytochalasin D-treated cells by confocal microscopy, yet some LPS remained cell associated after protease application. Wortmannin, a phosphoinositide-3 kinase inhibitor known to inhibit macropinocytosis (33, 34) and CD14-dependent phagocytosis of E. coli (15) decreased [3H]LPS internalization by 30% but had a less pronounced effect than cytochalasin D. LPS treatment of CD14-positive cells did not influence the rate or the route of CD14 endocytosis, as if this CD14-dependent uptake was not regulated by the ligand.

Cytochalasin D had a profound inhibitory effect on LPS internalization, probably by blocking the microfilament formation necessary for macropinocytosis. In contrast, this substance did not block LPS activation of cells (p38 mitogen-activated protein kinase phosphorylation and NF-κB activation) within the same time frame, indicating a dissociation between LPS internalization and activation, as suggested by others (5, 7, 14). Cytochalasin D enhanced LPS-induced IL-8 production by THP-1 transfectants. This could be explained either by a sensitization of cells to the cellular deformation induced by a microfilament disrupting agent (45), by a posttranslational effect of the drug, or by a prolonged time for ligand-receptor interactions at the surface of the cell. In the latter situation, our findings may indicate that LPS internalization could be a process aimed at down-regulating the activating effects of LPS.

We postulate that CD14 molecules present on macrophage membrane projections sense the extracellular milieu and bind LPS when they encounter it. Part of the LPS may be transferred to a protease-resistant membrane molecule/structure or simply be inserted into the lipid bilayer. Bound LPS and CD14 are then internalized in a ligand unregulated manner by macropinocytosis, a process not related to cell activation.

It also makes sense that the LPS internalization pathway resembles that of bacterial phagocytosis, because LPS is the most abundant ligand at the surface of Gram-negative bacteria. Cells of the innate immunity system may well have evolved with identical mechanisms for engulfment of bacteria and bacterial products (15). It was recently recognized that CD14 was the receptor for bacterial ligands other than LPS, originating from Gram-positive bacteria, mycobacteria, and yeasts (10). It was proposed that CD14 was a polyspecific receptor for nonself, conserved bacterial molecules, mediating innate immune responses upon ligand binding (10). Whether or not these non-LPS ligands internalize in a CD14-dependent manner, and by routes similar to that of LPS, remains to be determined.

The role of CD14 GPI anchoring has always puzzled investigators. In the present work we showed that GPI anchoring sorted CD14 to lipid-rich plasma membrane subdomains, where other GPI-anchored receptors are found. Modifying the anchoring system into a transmembrane-type protein sorted CD14 into “integral protein subdomains.” It has been postulated that GPI-anchored molecules specifically associated with caveoleae, or non-clathrin-coated pits (19). We did not find caveolar structures in promonocytic THP-1 cells, and clearly, gold-labeled CD14 was not associated with such structures. This is congruent with a previous report by Wang et al. (46).

These authors found that tritiated LPS associated with low-density, lipid-rich THP-1 membrane fragments in the absence of visualized caveoleae (46). Similar findings were reported for lymphocytes (47). This strongly suggests that GPI lipid-rich membrane domains are entities distinct from the morphological structure known as caveoleae (46, 47). A transmembrane chimeric CD14 construct was found to be targeted to different subdomains, as assessed by detergent solubility and differential buoyancy of sonicated membrane extracts. Neither the different anchoring systems nor their membrane localization affected the rate and the route of the ligand-receptor complex interactions at the surface of the cell. In the latter situation, our findings may indicate that LPS internalization could be a process aimed at down-regulating the activating effects of LPS.
endocytosis. No difference in rates of LPS-binding and LPS-dependent function was found in wt- versus tmCD14 transfectants (Fig. 6) (25, 48). The absence of association of tmCD14 with clathrin-coated pits and vesicles during the process of LPS internalization was somewhat surprising. This might suggest that tmCD14 associated early in its biosynthesis with another molecule responsible to keep it outside of coated pits. This contrasts with another system where a transmembrane form of the natural GPI-anchored folate receptor was targeted to clathrin-coated pits (21). The only difference observed between the two CD14 anchoring systems was the calcium mobilization induced by anti-CD14 cross-linking, present only in GPI but not in transmembrane CD14 cells (25), findings of unknown relevance.

In conclusion, our data demonstrate that in monocyctic cells, LPS and its receptor CD14 internalize through macropinocytosis, a pathway independent of coated pits or caveolae. Macropinocytosis resembles that of phagocytosis in several aspects, in particular in its requirement for intact microfilaments and formation of large vesicles. The natural GPI anchoring system in particular in its requirement for intact microfilaments and ropinocytosis resembles that of phagocytosis in several aspects, with clathrin-coated pits and vesicles during the process of LPS internalization.

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Note Added in Proof—Data published by Kitchens and Munford (49) after this manuscript was submitted indicate that LPS is internalized by a constitutive mechanism, compatible with our findings of a macropinocytic pathway.

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