Sepsis results from bacteria and their products entering the bloodstream and causing an overwhelming inflammatory response. Bacterial infections, as well as antibiotic therapy, cause the release of bacterial cell wall components including endotoxin (lipopolysaccharide (LPS)), lipoteichoic acid and peptidogycan (1); see Refs. 2 for review). Sepsis because of a Gram-negative bacterium is classically associated with endotoxemia, an acute phase reaction, and high mortality because of disseminated intravascular coagulation, multiple organ failure, and shock (3). LPS induces a broad spectrum of biological effects associated with the activation of immune and inflammatory cells, such as macrophages, monocytes, and endothelial cells. Systemic LPS-related activation of macrophages leads to overproduction of inflammatory mediators, such as leukocyte adhesion molecules, soluble cytokines and chemokines. LPS-activated phagocytes secrete tumor necrosis factor-α and IL-1β, which contributes to microcapillary damage, plasma leakage into tissue, hypotension and organ failure, the major manifestations of septic shock (for review see Refs. 4 and 5).

The endotoxic activity of LPS, as well as its cellular uptake and metabolism, appear to be mediated by an interaction with specific cell surface receptor(s). Activation of LPS-competent cells is initiated by LPS-binding protein, which transfers LPS from the bacterial wall to membrane-associated CD14. LPS-CD14 complexes signal via Toll-like receptor 4 to activate NF-κB, as well as the c-Jun NH2-terminal kinase, and p38 mitogen-activated protein kinases (6–8). The activation induces expression of genes encoding for tumor necrosis factor-α, IL-1β, IL-6, IL-8, leukocyte adhesion molecules (such as vascular cell adhesion molecular-1 and intracellular adhesion molecule-1), and chemotactic factors (such as monocyte chemotactant protein-1), which are believed to be involved with the development and progression of septic shock.

A large part of the host defense to septic shock involves the neutralization of LPS by its binding to high density lipoproteins (HDL), which ultimately results in the clearance of LPS by the liver (9, 10). When incubated with human plasma, ~90% of LPS is associated with lipoproteins, the majority (60%) of which are associated with HDL (11, 12). A smaller portion of LPS is associated with other plasma proteins including human serum albumin, LPS-binding protein, and soluble CD14 (11). This non-lipoprotein-associated LPS is highly inflammatory, and it appears to represent the major form of LPS-con

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During part of this study the author was a guest scientist at the Food and Drug Administration, Center for Biologics Evaluation and Research, Division of Cellular and Gene Therapies. To whom correspondence should be addressed.
contributing to sepsis. Infusion of HDL has been shown to significantly reduce the endotoxin-induced release of tumor necrosis factor, IL-6, and IL-8 in a murine model of endotoxemia (13, 14) and has been proposed as a potential therapy.

Bacterial LPS has been demonstrated to exist in high molecular weight (up to 1000 kDa) aggregates (cell wall debris) and in a monomerized state when it forms complexes with human serum albumin, CD14, LBP, low-density lipoproteins (LDL), or HDL. Aggregated LPS has been demonstrated to be rapidly taken up by the liver, lung, and spleen, organs with large reticuloendothelial cell populations, which abundantly express scavenger receptor class A (15, 16). Upon intravenous injection of iodinated LPS preparations that contain both partially monomerized LPS and aggregated LPS, the uptake of aggregated LPS by the reticuloendothelial system through scavenger receptor class A masks the participation of other receptors involved with the uptake of monomerized LPS in vivo. It has been reported that infusion of iodinated LPS monomerized by association with HDL results in an altered tissue uptake in mice (17). Of significance, the association with steroid-producing tissues, such as adrenal gland and ovary was increased. These observations raise the possibility that LPS tissue targeting may also involve an HDL receptor, such as the scavenger receptor type B class I (SR-BI), which is highly expressed in steroid producing tissues and the liver (for review see Ref. 18).

SR-BI is a well characterized HDL receptor that is highly expressed in the liver and steroidogenic tissues, including the adrenal, which is often affected during endotoxemia (19). Its human orthologue, CD36 and LIMPII analogous-1 (Cla-1), has also been shown as a human receptor for high density lipoprotein and apoprotein thymocytes (20). Despite the fact that Cla-1 has not been studied as extensively as rodent SR-BI, the physiological role of Cla-1 is generally assumed to be similar to that of rodent SR-BI. The primary function of SR-BI has been previously demonstrated to be a selective uptake of HDL-free cholesterol and cholesteryl ester without the concomitant uptake of HDL apolipoproteins, which serve as ligands for SR-BI (21, 22). The class A amphipathic α-helices of exchangeable apolipoproteins serve as the primary recognition motif for the interaction of HDL with SR-BI (23, 24). However, lipid composition (especially the presence of negatively charged phospholipids) impacts HDL binding with SR-BI. Moreover, phospholipid vesicles containing no apolipoproteins, only negatively charged aminophospholipids, such as phosphatidylserines and, phospholipids containing a negative charge such as phosphatidylethanolamines, as well as the phospholipid probe DiI are also effective ligands for SR-BI (22, 25). Lipid A, the most conserved portion of endotoxin, is a phosphorylated glucosamine-based phospholipid, which resembles the physicochemical properties of phospholipids containing a negative charge, and may function as an independent ligand for SR-BI in adrenal epithelial cells, macrophages, and hepatocytes, the cells that highly express SR-BI. Additionally SR-BI can be involved with the selective uptake and excretion of HDL-associated LPS in the liver, an important mechanism of LPS clearance (26). In this study, we examined the potential role of Cla-1 in LPS metabolism and demonstrate that Cla-1 mediates the binding, endocytosis, and the cellular accumulation of both monomerized, lipoprotein-free LPS as well as LPS associated with HDL.

**MATERIALS AND METHODS**

Lipopoly saccharides, *Escherichia coli* B4:0111, *Salmonella minnesota* Re 595, dipholosphoryl lipid A, and monophosphoryl lipid A were purchased from Sigma. Lipopoly saccharides from *E. coli* K12 strain LCD25 (unlabeled and 3H-metabolically labeled) were purchased from List Biological Laboratories. Rabbit anti-SR-BI antibody cross-reacting with the human homologue Cla-1 was from Novus Biological.

**Raw Cells**—Mouse monocyte-macrophages, RAW 264.7 (ATCC (American Type Culture Collection) TIB 71), were grown in 12-well plates in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 100 μg/ml G418. Cells were transfected with *Fu gene* (Roche Diagnostics), using the expression plasmid pTRE2 (Clontech), encoding a Cla-1 protein (pTRE2-CLA-1). Cells were co-transfected with pTRE2-CLA-1 and pTRE-Hyg (Clontech), using a 1:20 ratio, and selected with 400 μg/ml hygromycin. Hygromycin-resistant cells were screened for the expression of the CLA-1 protein by utilizing rabbit anti-SR-BI (Novus Biological, Inc.) by Western blotting.

**HDL, Apolipoprotein Isolation, and Labeling—**Human HDL (1.072 < d < 1.216) was isolated from the plasma of healthy donors by two repetitive centrifugations by the method of Ref. 27. The HDL was passed through an agarose-heparin column (HiTrap, Amersham Biosciences), and an apoE-free HDL fraction was collected. Apolipoproteins were purified from human plasma (28), and were over 99% pure, as determined by SDS-PAGE and amino-terminal sequence analysis. Labelling of HDL, apoA-I, and apoA-II with Na125I was performed by the *N*-bromosuccinimide method according to Sinn et al. (29). The specific radioactivities ranged from 1000 to 3000 cpm/μg of protein with more than 98% of the radioactivity being protein associated. Human HDL labeled with 125I-cholesterol oleoyl ether (CE), a nonhydrolyzable cholesteryl ester analogue, was prepared by a modification of the procedure of Miyazaki et al. (30). Fast protein liquid chromatography analysis demonstrated more than 95% of 125I-CE associated with the fraction corresponding to native HDL. The specific radioactivity for HDL-125I was 12–20 dpn/μg of HDL protein.

**Western Blot Analysis**—Western blot analysis was performed, as previously described (31). Cell proteins were extracted with 2% Triton X-100 in Tris-buffered saline, pH 7.4. The extracts were precipitated by adding methanol to a final concentration of 90%. Precipitated proteins were dissolved in 2× SDS-PAGE sample buffer and applied on a 7.5% SDSPAGE gels, after reducing gel conditions. Anti-SR-BI antibody at a dilution of 1:1000 was used as the first antibody, and a sheep anti-rabbit IgG antibody conjugated with alkaline phosphatase (Sigma) was used as the second antibody. For protein normalization, mouse anti-human β-actin antibody at a dilution of 1:2500 was used as the first antibody, and a sheep anti-mouse IgG antibody conjugated with alkaline phosphatase (Sigma) was used as the second antibody.

**HDL Binding and Cholesteryl Oleoyl Uptake Assays—**Saturation binding experiments were performed at 4°C using 125I-HDL concentrations between 1.25 and 40 μg/ml. The cells were incubated with ice-cold Hanks’ balanced salt solution (HBSS) containing 20 μg/ml BSA (HBSS/BSA) and labeled ligand in the presence or absence of a 20-fold excess cold ligand. After a 2-h incubation on ice, specific binding was determined as previously reported (32). HDL-125I uptake experiments were performed in serum-free DMEM containing 0.2% BSA. Cell monolayers were incubated with various concentrations of HDL-125I in the presence (nonspecific uptake) or absence (total uptake) of a 25-fold excess of the unlabeled HDL for 20 h. Specific uptake was determined as the difference between total and nonspecific uptake.

**LPS Binding Assay**—The lipopolysaccharide 0111:B4 (Sigma) was iodinated as reported earlier (32). Saturation binding experiments were performed at 4°C using 125I-LPS concentrations between 1.25 and 40 μg/ml. All incubations were performed in HBSS containing 20 μg/ml BSA (HBSS/BSA) and labeled ligand in the presence or absence of a 20-fold excess unlabeled LPS. After a 2-h incubation on ice, the cells were rinsed with ice-cold HBSS and utilized for radioactivity measurements as reported earlier (32). Specific binding was determined as the difference between total and nonspecific binding, and normalized by protein content.

**LPS Binding Experiments**—RAW cells were cultured for 24 h in serum-free DMEM before the experiment. After chilling on ice, cells were incubated in the presence of 5 μg/ml 125I-HDL, 1 μg/ml 125I-apoA-I, 1 μg/ml 125I-apoA-II, and increasing concentrations of cold ligands (HDL, apoA-I, apoA-II, and *E. coli* B4:0111:B4 LPS) for 1 h in HBSS/BSA. Cell radioactivity was measured as described under the *HDL Binding and Cholesteryl Oleoyl Uptake Assays.*

**LPS Uptake and Internalization Assays**—For measurement of LPS uptake and internalization, cells were incubated in a CO2 incubator for different time periods in DMEM (20 mg/ml BSA) containing 1 μg/ml 125I-LPS in the presence or absence of 200× excess of unlabeled ligand.
At specified time points, cells were chilled on ice and rinsed for three times with ice-cold PBS followed by a 20-min treatment with 0.05% trypsin, 5 mM EDTA, 150 mM NaCl solution on ice. Trypsin released radioactivity was determined as surface-bound ligand. Cell associated radioactivity counted after hydrolysis in 1 N NaOH was considered as internalized LPS. Specific binding and internalization were determined as the ratio between total and non-specific binding/internalization (the amount of radioactivity measured in the presence of ×200-fold excess of unlabelled ligand).

**Preparation of BODIPY-LPS and Alexa 568 HDL, Lipid-free Apo-A-I and Apo-A-II—HDL, apoA-I, and apoA-II were conjugated with Alexa 568/488, SE (Molecular Probes, protein labeling kit) following the kit instructions. The Alexa ligands were analyzed by 10–20% Tricine-SDS peptide gel electrophoresis. Gels were scanned using a Fluoroscan (model A, Hitachi). Alexa-labeled preparations of HDL and apolipoproteins were found in appropriate positions with molecular masses of 28 and 18 kDa for apoA-I and apoA-II, respectively (data not shown). Re-LPS was labeled using the BODIPY FL, SE labeling kit from Molecular Probes, Inc. following the manufacturer’s suggested procedure and modifications reported earlier (12).**

**Preparation of Bodipy-LPS/Alexa 488-Apolipoprotein-labeled HDL Complex—**Alexa 488-apolipoprotein-labeled HDL (5 mg) were mixed with Bodipy-LPS (5 μg) in a final volume of 1 ml followed by the addition of 2 ml of delipidated human plasma and incubated for 24 h at 37 °C. Bodipy-LPS/Alexa 488-apolipoprotein-labeled HDL complexes were re-isolated by a centrifugation in a NaBr gradient (1.072 g/ml) and incubated for 30 min followed by being washed with ice-cold PBS and fixed with 4% paraformaldehyde. Imaging was performed in PBS containing 0.4 mg/ml trypsin blue as the quenching agent. The effect of apoA-I on LPS uptake was studied by incubating HeLa cells with 0.5 μg/ml Bodipy-LPS in the presence of 100 μg/ml lipid-poor apoA-I for 1–2 h in a CO₂ incubator. For co-localization, Bodipy-LPS and Alexa 568-apoA-I were used at the same concentration of 0.5 μg/ml. A Nikon video-imaging system, consisting of a phase-contrast inverted microscope equipped with a set of objectives and filters for immunofluorescence and connected to a digital camera and image processor, was used for recording Alexa 568-HDL and Bodipy-LPS uptake. For co-localization experiments, fluorescence was viewed with a Zeiss 510 laser scanning confocal microscope, using a krypton-argon- Omnichrome laser with excitation wavelengths of 488 and 568 nm for Bodipy-LPS and Alexa 568, respectively.

**Uptake of BODIPY-LPS and Alexa 568-HDL, Apo-A-I/LPS Co-localization Experiments—**HeLa cells cultured on collagen-coated glass micro-slides were incubated with 5 μg/ml Alexa 568 HDL, 1–0.5 μg/ml Alexa 568 apoA-I, 1–0.5 μg/ml Alexa 568 apoA-II, or 0.5 μg/ml Bodipy-LPS for 1–2 h in a CO₂ incubator in DMEM containing 20 mg/ml BSA. For quenching experiments, BODIPY-LPS (1 μg/ml) was incubated with cells for 30 min followed by being washed with ice-cold PBS and fixed with 4% paraformaldehyde. Imaging was performed in PBS containing 0.4 mg/ml trypsin blue as the quenching agent. The effect of apoA-I on LPS uptake was studied by incubating HeLa cells with 0.5 μg/ml Bodipy-LPS in the presence of 100 μg/ml lipid-poor apoA-I for 1–2 h in a CO₂ incubator. For co-localization, Bodipy-LPS and Alexa 568-apoA-I were used at the same concentration of 0.5 μg/ml. A Nikon video-imaging system, consisting of a phase-contrast inverted microscope equipped with a set of objectives and filters for immunofluorescence and connected to a digital camera and image processor, was used for recording Alexa 568-HDL and Bodipy-LPS uptake. For co-localization experiments, fluorescence was viewed with a Zeiss 510 laser scanning confocal microscope, using a krypton-argon- Omnichrome laser with excitation wavelengths of 488 and 568 nm for Bodipy-LPS and Alexa 568, respectively.

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**Uptake of Bodipy-LPS/Alexa 488-Protein-labeled HDL Complex—**The surface binding of the LPS-HDL complex was studied by incubating 10 μg/ml doubly labeled HDL (Bodipy-LPS and Alexa 488-HDL) for 2 h with Clα-1 overexpressing or mock transfected HeLa cells at 4 °C and examined by confocal microscopy. Internalization of the complex was analyzed after three washings with ice-cold Ca²⁺/Mg²⁺-free PBS followed by incubation at 37 °C for a 4-h period in fresh serum-free culture medium. A separate sample of HDL (10 μg/ml) was incubated with HeLa cells at 37 °C for the 1- and 4-h periods.

**Preparation of ‘H-LPS/HDL and LPS’125I-HDL Complexes—**H-LPS was synthesized according to the instructions. The Alexa ligands were analyzed by 10–20% Tricine-SDS peptide gel electrophoresis. Gels were scanned using a Fluoroscan (model A, Hitachi). Alexa-labeled preparations of HDL and apolipoproteins were found in appropriate positions with molecular masses of 28 and 18 kDa for apoA-I and apoA-II, respectively (data not shown). Re-LPS was labeled using the BODIPY FL, SE labeling kit from Molecular Probes, Inc. following the manufacturer’s suggested procedure and modifications reported earlier (12). **Selecting LPS Uptake—**The selective LPS uptake was studied by incubating HeLa cells with 0.5 μg/ml Bodipy-LPS in the presence of 100 μg/ml lipid-poor apoA-I for 1–2 h in a CO₂ incubator. For co-localization, Bodipy-LPS and Alexa 568-apoA-I were used at the same concentration of 0.5 μg/ml. A Nikon video-imaging system, consisting of a phase-contrast inverted microscope equipped with a set of objectives and filters for immunofluorescence and connected to a digital camera and image processor, was used for recording Alexa 568-HDL and Bodipy-LPS uptake. For co-localization experiments, fluorescence was viewed with a Zeiss 510 laser scanning confocal microscope, using a krypton-argon- Omnichrome laser with excitation wavelengths of 488 and 568 nm for Bodipy-LPS and Alexa 568, respectively.

**Degradation of HDL—**Degradation of HDL was determined, using the following previously reported pulse-chase scheme (35). Briefly, cultured Clα-1 overexpressing and mock transfected HeLa cells were pulsed with 1 ng/ml radiolabeled 125I-HDL-LPS complexes. Cells were then cooled on ice and washed 3 times with binding buffer. Following the washes, cells were returned to 37 °C and chased for 2 h in binding buffer in the absence of radiolabeled lipoproteins. At the completion of the chase period, cell media was collected and trichloroacetic acid-precipitable counts were determined as a measurement of degradation (35). Cells were also lysed, and radioactivity and protein concentration was measured.

**Sites of LPS Delivery—**For studying the sites of LPS delivery, cells were incubated with 1 μg/ml Bodipy-LPS at 37 °C for 2 h, then washed and chased at 37 °C for 30 min in the presence of Bodipy-transferrin or Bodipy-ceramide BSA complex. In separate experiments, instead of BSA-monomerized Bodipy-LPS, the cells were incubated with 10 μg/ml HDL-bound Bodipy-LPS to determine the sites of LPS transport when associated with HDL.

**RESULTS**

**Competition of LPS with SR-BI Ligands—**The competition of LPS with HDL, which is known to bind to SR-BI, was analyzed in RAW cells, which have a high level of SR-BI expression (33). As seen at Fig. 1, LPS (E. coli B4:0111) competed with iodi

![Image](http://www.jbc.org/)
that when unlabeled apolipoproteins were used as competitors. Because the experiments were conducted on ice, which prevents the formation of a complex between LPS and lipoproteins (34), the HDL, apolipoproteins, and LPS interacted with HDL receptor as independent ligands.

Cla-1 Expression, HDL Binding, and Cholesterol Ester Uptake in Stably Transfected HeLa Cells—Because LPS was a potent competitor for SR-BI-related ligands, this suggests that SR-BI might function in LPS uptake through LPS binding and internalization. To evaluate this possibility, human HeLa cells were stably transfected with a vector containing the human SR-BI receptor, Cla-1. Western blot analyses of stably transfected HeLa cell extracts, using an anti-rodent SR-BI antibody that cross-reacts with Cla-1, revealed a single band with an estimated molecular weight of 83,000 (Fig. 3A). An ~10 times higher Cla-1 level was observed in Cla-1 overexpressing cells when compared with mock transfected HeLa cells. A 2-day incubation of Cla-1 overexpressing cells with 1 μg/ml tetracycline diminished the Cla-1 level close to that seen with mock transfected cells. As seen in Fig. 3, B and C, overexpressing cells have increased levels of 125I-HDL binding and HDL-CE uptake by more than 10-fold, observations demonstrating the HDL receptor activity of Cla-1. Scatchard analysis reveals a 10-fold increase of capacity for the HDL-binding site after transfection with a Kd = 1–2 μg/ml (data not shown).

Specific Binding of LPS to Cla-1—To examine the possible role of Cla-1 in LPS binding, we conducted ligand-binding analyses, using iodinated LPS (0111:B4). The Cla-1 overexpressing cells demonstrated a 4–5-fold increase of specific LPS binding (Fig. 4A). Scatchard analyses demonstrated a high affinity binding site with a Kd = 16 μg/ml and capacity of 150 ng/mg cell protein in the Cla-1 overexpressing cells. Despite the demonstration of saturable specific LPS binding in mock transfected cells, Scatchard analyses resulted in an indeterminable Kd because of low and variable amounts of specific binding.

The ability of O-antigen containing LPS (0111:B4) and O-antigen lacking LPS, Re 595, diphosphoryl lipid A, and monophosphoryl lipid A, to compete against 125I-LPS (0111:B4), was analyzed in Cla-1 overexpressing and mock transfected cells. As seen at Fig. 4, B and C, all unlabeled ligands competed against iodinated LPS in both cell lines. The increased LPS binding observed in Cla-1 overexpressing cells was effectively competed by the presence of ×100 excess of unlabeled ligand to a similar level observed in mock transfected cells. The exchangeable HDL apolipoproteins also effectively competed against LPS binding (Fig. 4B). Because essentially all lipid A variants competed for LPS binding this indicates that the acetylated digalactosamine interface is required for LPS binding rather than O-antigen.

Uptake and Internalization of Iodinated LPS—As seen in Fig. 5A, Cla-1 overexpressing HeLa cells demonstrate a substantial time-dependent increase in LPS uptake when analyzed at 37 °C. Specific binding and internalization of LPS were also increased when analyzed using trypsin treatment to discriminate between surface-bound (Fig. 5A) and internalized LPS (Fig. 5B). Similar results were observed upon FACS scan analyses of Bodipy-labeled LPS uptake (data not shown).

Uptake of Alexa-HDL and Bodipy-LPS in Cla-1 Overexpressing HeLa Cells—As seen in Fig. 6B, Cla-1 overexpressing HeLa cells demonstrate intensive membrane and intracellular staining upon the incubation with Alexa 568-HDL. Rare, very faint staining could be observed in some experiments when incubating with mock transfected cells (Fig. 6A). Cla-1 overexpression in HeLa cells increased Bodipy-LPS uptake (Fig. 6D) when compared with a mock transfected control (Fig. 6C). Cla-1 overexpression induced rapid Bodipy-LPS internalization and delivery into perinuclear cellular compartments, as determined in trypan blue quenching experiments comparing mock transfected (Fig. 6E) and overexpressing cells (Fig. 6F).

To determine whether both apoA-I and LPS are delivered to
in intracellular compartments via the same pathway, Cla-1 overexpressing HeLa cells were incubated with Bodipy-LPS in the presence of excess of unlabeled LPS. As seen at Fig. 7, the presence of high apoA-I excess (Fig. 7B) dramatically reduced Bodipy-LPS uptake and affected its distribution through intracellular compartments when compared with the absence of apoA-I (Fig. 7A). Smaller stained vesicles were eventually seen in the cytoplasm with significantly reduced staining in the perinuclear area. When incubating Cla-1 overexpressing HeLa cells with equal concentrations of 0.5 μg/ml Alexa 568-apoA-I (Fig. 7C) and Bodipy-LPS (Fig. 7D), a strong area of co-localization (yellow) was demonstrated on the cell surface as well as intracellularly (Fig. 7E). A similar co-localization of apoA-I and LPS was observed in the RAW cell model, indicating that both LPS and apoA-I perinuclear transportation is not an artifact of high Cla-1 expression or result of the use of a particular cell model (data not shown). Other Cla-1 ligands, such as HDL and apoA-II could be also extensively co-localized with LPS in Cla-1 overexpressing cells (data not shown).

Sites of Delivery of BSA-monomerized LPS—Co-localization experiments demonstrate that the majority of BSA-monomerized Bodipy-LPS enters the Golgi complex after rapid endocytosis (Fig. 8). Intensive co-localization of Bodipy-LPS with ceramide (Fig. 8C) indicates that the Golgi complex rather than endocytic recycling compartment is the primary site of LPS transport by Cla-1. However, a weaker yellow signal could be also detected when Bodipy-LPS-loaded cells were chased at 37 °C for 30 min in the presence of Bodipy-transferrin suggesting that some LPS was transported to the endocytic recycling compartment by Cla-1 (Fig. 8F).

Uptake of HDL-associated LPS in Cla-1 Overexpressing Cells—It has been demonstrated that LPS, an amphipathic molecule that forms micelles in aqueous buffers, is rapidly
monomerized by and forms complexes with plasma proteins in the plasma. In addition to serum albumin, another important plasma LPS-binding protein is HDL, the major ligand for Cla-1. Because HDL has also been demonstrated to neutralize LPS in both \textit{in vitro} and \textit{in vivo} experiments, Bodipy-LPS binding and internalization was studied while in a complex with Alexa 488 apolipoprotein-labeled HDL in Cla-1 overexpressing HeLa cells. This approach allows studying both holoparticle transport and intracellular sorting by directly observing the fluorescent signal from HDL Alexa 488-apolipoproteins and Bodipy-LPS. As seen at Fig. 9A, the LPS-HDL complex binds to the plasma membrane after a 2-h incubation at 4 °C as a holoparticle because Bodipy-LPS (red) and Alexa 488-HDL (green) merge at the cell surface as a bright yellow staining. No substantial HDL/LPS binding was detected when incubated with mock transfected cells (data not shown). After washing unbound ligand and incubating the cells at 37 °C for 4 h, very little co-localization was detected on the cell surface (Fig. 9B). The mostly green surface staining indicates the presence of HDL associated with the plasma membrane. Intracellularly, holoparticle internalization (yellow) was detected as co-trafficking of labeled components within HDL/LPS as well as a sorting of HDL and LPS to different intracellular compartments (red and green spotting). To determine whether the same sorting process occurs in the continuous presence of an HDL/LPS complex at 37 °C, cells were incubated for 1- and 4-h periods. As seen at Fig. 9C, an HDL-LPS complex resided initially on the plasma membrane and was rapidly internalized in Cla-1 overexpressing HeLa cells (yellow). Little sorting can be seen after a 1-h incubation. By 4 h of HDL/LPS binding, there is both surface and intracellular co-localization (yellow), as well as a sorting of HDL and LPS to different intracellular compartments (Fig. 9D). These data indicate that the metabolism of LPS associated with HDL closely resembles HDL endocytosis and selective apolipoprotein-cholesterol ester sorting by mouse SR-BI that has been recently reported (35), detailing mecha-
Followed by confocal microscopy. In panels C and D, respectively, at 37°C incubated with Bodipy-LPS/Alexa 488-apolipoprotein-labeled HDL for 1 h at 4°C (panel A), then washed and chased at 37°C for 4 h (panel B) followed by confocal microscopy. In panels C and D, the cells were incubated with Bodipy-LPS/Alexa 488-apolipoprotein-labeled HDL for 1 h and 4 h, respectively, at 37°C followed by confocal microscopy. Yellow represents a merging of Bodipy-LPS (green) and Alexa 488-apolipoprotein-labeled HDL (green) signals.

Fig. 9. HDL apolipoprotein and LPS colocalization in Cla-1 overexpressing HeLa cells. HeLa cells expressing Cla-1 were incubated with Bodipy-LPS/Alexa 488-apolipoprotein-labeled HDL for 1 h at 4°C (panel A), then washed and chased at 37°C for 4 h (panel B) followed by confocal microscopy. In panels C and D, respectively, at 37°C incubated with Bodipy-LPS/Alexa 488-apolipoprotein-labeled HDL for 1 h and 4 h, respectively, at 37°C followed by confocal microscopy. Yellow represents a merging of Bodipy-LPS (red) and Alexa 488-apolipoprotein-labeled HDL (green) signals.

In this paper we evaluated the role of Cla-1 in LPS binding, uptake, and intracellular transport when in lipoprotein-free form or in association with HDL (purified HDL-LPS complex). Lipoprotein-free LPS strongly competes with HDL and exchangeable lipid-poor HDL apolipoproteins for HDL-binding sites in RAW cells that highly express SR-BI (40). Whereas LPS binding was measured on ice and in the absence of plasma factors facilitating LPS exchange, conditions highly unfavorable for HDL-LPS complex formation, the possibility of an effect of a small amount of HDL-associated LPS on HDL binding cannot be excluded. However, the competition of LPS against iodinated apolipoproteins, which has a much lower ability than HDL to interact with LPS, also supports the idea that HDL and LPS compete as independent ligands and share the same type of specific binding sites(s).

To evaluate human SR-BI as a potential LPS-binding protein, a Cla-1 stably transfected HeLa cell line was created.
The individual localizations are seen for Bodipy-LPS-labeled HDL (red, panel A) and Bodipy-labeled transferrin (green, panel B). The merged image for LPS and transferrin is shown in panel C as yellow. The individual localizations are seen for Bodipy-LPS-labeled HDL (red, panel D) and Bodipy-ceramide-BSA complex (green, panel E). The merged image for LPS and ceramide is shown in panel F as yellow.

Analyses of HDL binding and selective cholesterol ester uptake were conducted to demonstrate functional Cla-1 activity in the cells. Both HDL binding and cholesterol ester uptake were elevated by 10-fold in Cla-1 overexpressing HeLa cells when compared with a mock transfected control. In addition, in agreement with previous data on rodent SR-BI, its human orthologue, Cla-1, induced a 4-fold increase of the initial cholesterol efflux to HDL (data not shown). Cla-1 overexpressing HeLa cells demonstrated a 3–4-fold increase of the specific LPS binding with a $K_d = 16 \mu g/ml$. It has been reported that LPS, an amphipathic molecule, exists in an aggregated form in aqueous buffers. Monomerization of LPS requires appropriate binding plasma proteins such as HDL or serum albumin (11) and a phospholipid transfer protein such as LBP. It has been reported that LPS association with lipoproteins at a low temperature or in the absence of serum factors such as LBP is inefficient (42). We have observed that the molecular mass of LPS when aggregated is ~1000 kDa as determined by fast protein liquid chromatography in the absence of BSA. Utilizing agarose gel electrophoresis we surprisingly found that a 10–30-min incubation of Bodipy-LPS or iodinated LPS in high BSA concentrations (1–20 mg/ml) monomerized LPS and resulted in forming BSA-LPS complexes even in the absence of serum phospholipid-transfer proteins (data not shown). Assuming that LPS may form a monomolecular complex with BSA ($M_r = 75,000–80,000$) or BSA dimers ($M_r = 150,000–160,000$), the estimated $K_d$ would be $1–2 \times 10^{-7}$ m. This value is in the range of the $K_d$ values previously determined for SR-BI utilizing HDL lipid-poor exchangeable apolipoproteins (20, 21). Because diverse LPS structures carry a highly conserved lipid A structure, the comparable potency among several forms of LPS, including diphosphoryl lipid A, indicates that Cla-1 provides a binding site for the lipid A moiety of LPS rather than the highly variable polysaccharide O-antigen portion.

Earlier reports indicated that the expression of HDL-binding proteins, such as SR-BI and ATP-cassette transporters are under strict negative control by LPS-related activation of NF-kB in monocyte cell lines and the rodent liver (34, 43, 44). Because the decreased endogenous level of Cla-1 or ABCA1 expression might be a confounding factor for the differences in LPS uptake in HeLa cells, we measured levels of Cla-1, IL-6, IL-8, and IL-1 in HeLa cells after LPS stimulation. In agreement with previous reports on the unresponsiveness of the HeLa cell to LPS (45), we have found no LPS effect when using a wide range of concentrations upon both IL-6 and IL-8 secretion as well as on Cla-1 expression by enzyme-linked immunosorbet assay and Western blotting, respectively (data not shown).

It was reported earlier that CD14-associated LPS after initially binding in caveolae is transported to the Golgi complex (45). It is significant that SR-BI is predominantly associated with caveolae (46), which have been demonstrated as the initial loci for membrane transfer of HDL cholesteryl esters (47), sphingomyelin, phosphatidylcholine, and phosphatidylethanolamine (25). It has been also been reported that SR-BI is directly involved with caveolae-like microvilliar channel formation in a heterologous SR-BI expressing cell system (48). In an agreement with data on specific $^{125}$I-LPS binding (Fig. 4), cellular $^{125}$I-LPS uptake followed by LPS internalization was dramatically increased in Cla-1 overexpressing HeLa...
cells when evaluated at 37 °C. In contrast to mock transfected HeLa cells, where the amount of internalized ligand decreased significantly by the third hour of incubation, cell-associated 125I-LPS remained steady after plateauing at 60 min. It was observed that Cla-1 overexpression in HeLa cells (LPS nonresponsive cells) did not affect NF-κB responsive genes as estimated by reverse transcriptase-PCR and enzyme-linked immunosorbent assay detection of IL-6, IL-8, and tumor necrosis factor α in culture media (data not shown). However, Cla-1 can also regulate caveolae structure as well as surface clustering of LPS/membrane CD14 and TLR4, which can affect LPS signaling. 

Previous studies suggested that Cla-1 might be involved with LPS efflux, the process of dissociation of LPS from the cell surface to HDL particles (50). This process is similar to passive cholesterol efflux, which is increased in mouse SR-BI overexpressing cells, and which might be pathophysio logically significant during endotoxemia (49). However, LPS release from the cell surface could also be the result of direct competition between HDL and LPS for HDL/HDL-binding sites. We have found that apoA-I, a Cla-1 ligand, at $\times 100$ excess, eliminated surface bound Bodipy-LPS in Cla-1 overexpressing cells. Moreover, when added at the same concentration, Alexa-apoA-I and Bodipy-LPS co-localized on the cell surface, suggesting a common binding site on the plasma membrane. Both ligands were predominantly transported into a perinuclear compartment, which was determined to be the Golgi network by a co-localization with the ceramide-BSA complex (Fig. 8). The finding of surface-bound LPS being intracellularly transported to the Golgi complex is consistent with previous reports that LPS is rapidly transported to a perinuclear compartment, which has been identified as the Golgi complex (46). Regarding the novel observation of the rapid endocytosis and intracellular transport of lipid-poor apoA-I we have found no reference for this observation in the literature. Importantly a human monocye cell line, THP-1, which expresses a high level of SR-BI, also demonstrated rapid Alexa-568 apoA-I endocytosis. This indicates that apoA-I perinuclear transportation is not an artifact of high Cla-1 expression or result of the use of a particular cell model. We speculate that Cla-1 related apoA-I binding and endocytosis can be one of the mechanisms involved with the accelerated apoA-I clearance reported for Tangier disease (50). The significant intracellular co-localization of LPS and apoA-I also suggests the same intracellular transporting mechanism(s) for both ligands. These observations also suggest that the effects of HDL and apoA-I infusion during endotoxemia, which have been shown to have a beneficial effect (13), could partially result from the direct competition between SR-BI ligands and LPS cellular binding sites. If there is binding competition, other Cla-1-related synthetic agonists, such as the double α-helical amphipathic peptides resembling the exchangeable apolipoproteins A-I and A-II, could be evaluated as potential treatments of septic shock. Supporting this possibility is an earlier study that demonstrated that phospholipid vesicles reconstituted with a 18A single α-helical amphipathic peptide as well as structurally related α-helical cationic antimicrobial peptides prevented LPS-related mortality in a murine model (13).

It appears that lipid transport and LPS neutralization utilize similar mechanisms. Recently it was shown that LBP together with cholesterol ester transfer protein and phospholipid transfer protein, the major proteins involved with HDL remodeling, belong to the same family of lipid transport proteins (51). A number of observations demonstrate that LPS association with HDL is an important step in LPS neutralization and clearance. Generally, LPS clearance from plasma is enhanced when LPS is associated with lipoproteins and results in increased biliary excretion (27).

Our data demonstrates that the LPS uptake from a HDL-LPS complex is significantly increased in Cla-1 overexpressing HeLa cells when compared with mock transfected HeLa cells. Similarly to selective CE and lipid uptake (22, 25), radiolabeled LPS were taken up at a higher rate than radiolabeled HDL apolipoproteins, indicating that selective LPS uptake takes place. Secreted HDL apolipoproteins were partially degraded in Cla-1-overexpressing cells, indicating that some portion of HDL may be delivered to lysosomes. The ratio of degraded versus secreted HDL apolipoprotein was observed to be increased when the uptake of HDL-LPS complex was examined (Fig. 11, C and D). It is possible that such an increase in HDL uptake and degradation may be one of the factors causing HDL levels to decrease during septic conditions. By utilizing confocal microscopy, Cla-1 overexpressing HeLa cells rapidly bind and internalize Bodipy-LPS/Alexa 488-protein-labeled HDL as a holoparticle into a perinuclear compartment. Upon longer incubations, LPS and apolipoproteins were sorted to distinct intracellular compartments that are clearly visible on confocal images (Fig. 9) by the segregation of green (HDL) and red (LPS) signals. SR-BI has been thought to mediate a selective uptake of HDL cholesterol ester without the endocytosis and degradation of the particle. In contrast to its accepted role as a surface functioning, non-endocytic receptor, rodent SR-BI has been recently shown to mediate HDL particle uptake, endocytosis, and lipid sorting in both transfected Chinese hamster ovary cells and hepatocytes (35). Internalized HDL particles entered the endocytic recycling compartment paralleling the movement of rodent SR-BI (35). In agreement with these data, Cla-1 overexpressing HeLa cells rapidly internalized both Alexa 568 apolipoprotein-labeled HDL and HDL-bound Bodipy-LPS. Lipoprotein bound label was transported to the endocytic recycling compartment as determined by colocalization experiments utilizing Bodipy-transferrin (Fig. 10C). In contrast to BSA-monomerized LPS, HDL-bound Bodipy-LPS did not significantly co-localize with the Bodipy-ceramide BSA complex (Fig. 10F). This indicates that an association of LPS with HDL results in re-compartmentalization of LPS taken up by the Cla-1 receptor from the Golgi complex to the endocytic recycling compartment. The significance of this observation is under investigation. 

In summary, we have demonstrated that Cla-1, a known HDL receptor involved with the trafficking of lipids and lipid-like molecules, is a potent LPS-binding protein, which mediates LPS binding and endocytosis. Lipoprotein-free LPS serves as an independent ligand like other SR-BI ligands including HDL, apoA-I, and apoA-II. Cla-1 expression dramatically increases the uptake, internalization, and intracellular accumulation of LPS associated with HDL in a process closely resembling HDL cholesterol ester uptake and intracellular sorting. These data strongly suggest that Cla-1 could be an important mechanism of liver LPS uptake and bile secretion. Following up our findings leads to multiple lines of future investigation, including the role of Cla-1 in LPS-induced cortical insufficiency and direct toxicity in adrenal glands, LPS-mediated signaling and LPS clearance by the liver. Knowledge regarding the functional relationship between of Cla-1 and LPS may also result in the development of new treatments for sepsis and septic shock.

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Binding and Internalization of Lipopolysaccharide by Cla-1, a Human Orthologue of Rodent Scavenger Receptor B1

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