Human scavenger receptor class B type I, CLA-1, mediates lipopolysaccharide (LPS) binding and internalization (Vishnyakova, T. G., Bocharov, A. V., Baranova, I. N., Chen, Z., Remaley, A. T., Csako, G., Eggerman, T. L., and Patterson, A. P. (2003) J. Biol. Chem. 278, 22771–22780). Because one of the recognition motifs in SR-B1 ligands is the anionic amphipathic α-helix, we analyzed the effects of model amphipathic α-helical-containing peptides on LPS uptake and LPS-stimulated cytokine production. The L-37pA model peptide, containing two class A amphipathic helices, bound with high affinity ($K_d = 0.94 \mu M$) to CLA-1-expressing HeLa cells with a 10-fold increased capacity when compared with mock transfected HeLa cells. Both LPS and L-37pA colocalized with anti-CLA-1 antibody and directly bound CLA-1 as determined by cross-linking. SR-BI/CLA-1 ligands such as HDL, apolipoprotein A-1, and L-37pA efficiently competed against iodinated L-37pA. Bacterial LPS, lipoteichoic acid, and hsp60 also competed against iodinated L-37pA. Model peptides blocked uptake of iodinated LPS in both mock transfected and CLA-1-overexpressing HeLa cells. Bound and internalized Alexa-L-37pA and BODIPY-LPS colocalized at the cell surface and perinuclear compartment. Both ligands were predominantly transported to the Golgi complex, colocalizing with the Golgi markers bovine serum albumin-ceramide, anti-Golgin97 antibody, and cholera toxin subunit B. A 100-fold excess of L-37pA nearly eliminated BODIPY-LPS binding and internalization. L-37pA and its D-amino analogue, D-37pA peptide were similarly effective in blocking LPS, Gram-positive bacterial wall component lipoteichoic acid and bacterial heat shock protein Gro-EL-stimulated cytokine secretion in THP-1 cells. In the same culture Ledia used for the cytokine stimulation study, neither L-37pA nor D-37pA affected the Limulus amebocyte lysate activity of LPS, indicating that LPS uptake and cytokine stimulation were blocked independently of LPS neutralization. These results demonstrate that amphipathic helices of exchangeable apolipoproteins may represent a general host defense mechanism against inflammation.

Sepsis is a systemic manifestation of infection and is classically associated with septic shock, disseminated intravascular coagulation, and multigorgan failure. Septic shock results from bacteria and their products entering the bloodstream and causing an overwhelming inflammatory response. Both bacterial proliferation and antibacterial therapy cause the release of endotoxins and other bacterial cell wall components, such as lipoteichoic acid (LTA) and peptidoglycan (1, 2). Endotoxins, or lipopolysaccharides (LPSs), the structural components of the outer membrane of Gram-negative bacteria, play a central role in the development and progression of septic 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 the production of inflammatory mediators, such as leukocyte adhesion molecules, soluble cytokines, and chemokines. LPS-activated phagocytes elevate plasma levels of TNF-α and IL-1β, contributing to microcirculatory damage, plasma leakage into tissue, hypotension, and later organ failure, which are major manifestations of septic shock (4, 5).

Binding of LPS to cell receptor(s) causes a pro-inflammatory cellular response and mediates endotoxin degradation and clearance. Recently, it has been demonstrated that the LPS-induced cytokine response primarily involves Toll-like Receptor 4 (TLR4) and plasma membrane CD14, which initiate downstream signaling to NF-κB followed by activation of LPS-responsive genes, including pro-inflammatory cytokines (6, 7). LPS uptake, clearance, and catabolism are mediated by a family of scavenger receptors (8–11). LPS binding to class A scavenger receptors, which is associated with classic clathrin-dependent internalization and delivery to lysosomal compartments, has been demonstrated to have only a minor role in LPS clearance in vivo (8).

In vitro cellular LPS uptake is mediated by several unrelated mechanisms, including formation of clathrin-coated vesicles (12), macropinocytosis (13), and via uncoated plasma mem-

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brane invaginations involving caveolae and microcystosis (14). The aggregation state of LPS determines the mechanism of LPS uptake. Aggregated LPS has been demonstrated to enter cells through clathrin-coated pits. Monomerized LPS is internalized by a mechanism involving uncoated plasma membrane invaginations and subsequent transportation to the Golgi complex (15).

The role of rafts and the Golgi complex in LPS-induced signaling is intensively being investigated, because the major LPS-signaling receptor, TLR4, resides within the Golgi complex. Furthermore, LPS-induced signaling requires LPS internalization in these cells (18, 19). Intestinal epithelial cells, hepatocytes, and antigen-presenting dendritic cells highly express class B scavenger receptors, such as CLA-1, which recently has been shown to bind, internalize, and transport LPS to the Golgi (20).

Rodent scavenger receptor, class B, Type I (SR-BI) and its human orthologue CD36 and LIMPII analogue-1 (CLA-1), are plasma membrane proteins that function as HDL receptors (21). SR-BI colocalizes with caveolin and is found in the detergent-resistant membrane fraction indicating its involvement with raft and caveola raft formation (21, 22). In transfected mammalian cells, overexpressed SR-BI induces a marked increase in HDL binding, HDL cholesterol ester uptake, and the translocation of the major structural component of the plasma membrane caveolin, caveolin-1, to the cell surface (23). Plasma membrane caveolae (rafts) have been recently demonstrated as the initial loci for the membrane transfer of HDL lipids (24) as well as for cell signaling (16).

Negatively charged phospholipids and anionic class A amphipathic α-helices of exchangeable apolipoproteins serve as two primary recognition motifs for HDL interaction with SR-BI/CLA-1 (25, 26). Both short-length model synthetic amphipathic helical peptides, which resemble class A amphipathic α-helices of exchangeable apolipoproteins, and LPS, which is an anionic glucosamine-based phospholipid, bind to CLA-1 with high affinity (20, 26).

The CLA-1 receptor associates with rafts and transports LPS to the Golgi, the two sites of TLR localization, we hypothesized that targeting SR-BI with synthetic amphipathic peptides might affect LPS-induced cytokine expression. In this study we compared the binding, internalization, and distribution pattern of a model synthetic helical peptide L-37pA to that of LPS and investigated the effect of the peptide on LPS binding, internalization, and LPS-induced cytokine production in HeLa cells and human monocyte THP-1 cells.

**MATERIALS AND METHODS**

Lipopolysaccharides *Escherichia coli* 0111:B4 and *Salmonella minnesota* Re595, LTA, and Gro-EL were purchased from Sigma. Rabbit anti-SR-BI antibody raised against the C-terminal CSIIARKTVQEAKL peptide and cross-reacting with the CLA-1, was from Novus Biological. Disuccinimidyl suberate was purchased from Pierce. All fluorescent probes and labels were from Molecular Probes.

**Synthesis of Amphipathic Helical Peptides**—The peptides were synthesized by a solid-phase procedure as previously reported (27, 28). t-Amino acids substituted for β-amino acids are underlined and the sequences of the peptides are shown in Table I.

| Peptide | Sequence |
|---------|----------|
| L-18pA  | DLKAFYDKVAEKLEAF |
| L-37pA  | DLKAFYDKVAEKLEAF-P-DWLKAFYDKVAEKLEAF |
| D-37pA  | DLKAFYDKVAEKLEAF-P-DWLKAFYDKVAEKLEAF |
| L1D-37pA | DLKAFYDKVAEKLEAF-P-DWLKAFYDKVAEKLEAF |
| L2D-37pA | DLKAFYDKVAEKLEAF-P-DWLKAFYDKVAEKLEAF |
| L3D-37pA | DLKAFYDKVAEKLEAF-P-DWLKAFYDKVAEKLEAF |

*α*-helical Peptides Block Pro-inflammatory Responses

Because CLA-1 associates with rafts and transports LPS to the Golgi, the two sites of TLR localization, we hypothesized that targeting SR-BI with synthetic amphipathic helical peptides might affect LPS-induced cytokine expression. In this study we compared the binding, internalization, and distribution pattern of a model synthetic helical peptide L-37pA to that of LPS and investigated the effect of the peptide on LPS binding, internalization, and LPS-induced cytokine production in HeLa cells and human monocyte THP-1 cells.

**Cross-linking Experiments—CLA-1-overexpressing and mock transfected HeLa cells grown to confluence in 6-well plates were incubated overnight in serum-free DMEM prior to the experiment. Fluorescently labeled ligands (Alexa 488-HDL, Alexa 568-L-37pA, and BODIPY-LPS) were added in 1 ml of DMEM containing 2 mg/ml lipid-free BSA, and the cells were incubated for 90 min at 37 °C in a CO2 incubator. Cells were washed three times with ice-cold PBS and incubated with 250 μM disuccinimidyl suberate (DSS) in PBS for 15 min at room temperature. Afterward, cells were washed additionally three times with PBS and lysed in 200 ml of 2% Triton X-100, 10 μg/ml aprotinin, 0.3 mM phenylmethylsulfonyl fluoride, and 5 mM EDTA for 10 min at room temperature, and centrifuged at 10,000 × g for 10 min at 4 °C. Supernatants were added with protein G-agarose (Sigma) and either 25 μl of rabbit anti-CLA-1 (raised against the C-terminal 15-amino acid peptide) or non-immune rabbit serum. After an overnight incubation at 4 °C, protein G-agarose was washed three times with PBS containing 0.5% NaCl and 0.5% BSA. Protein G-agarose was added with 40 μl of 2× SDS-PAGE sample buffer containing 5% β-mercaptoethanol and boiled for 3 min. After Tris/glycine SDS-PAGE, gels were immediately immersed in water and scanned for Alexa-488 and Alexa-568/BODIPY signals using a Typhoon 9200 imager. Cross-linked complexes were detected as fluorescent bands. To determine the molecular masses of the cross-linked bands, these were scanned in the visible spectrum and compared with positions of sea-blue pre-stained standards using an HP scanner 7400c scanner. Fluorescent and visible light images were superimposed to produce a combined image.

**Lipid Amebocyte Lysate Assay for LPS**—The LAL activity of LPS was determined using a CLyse assay as described previously (29). HeLa cells grown until 70% confluence in 6-well plates were incubated with iodinated L-37pA and increasing concentrations of cold ligands (LPS, L-37pA, and D-37pA) for 1 h. After washing with ice-cold PBS, the cells were hydrolyzed in 1N NaOH. Radioactivity was counted in an LKB-Wallac Ultragamma counter.

**Labeling of HDL, Lipid-free ApoA-I, L-37pA, L2D-37pA, L-18pA, and LPS—HDL, apoA-I, L-37pA, L2D-37pA, and L-18pA were conjugated with Alexa-568, 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 variable mode imager, Typhoon 9200 (Molecular Dynamics). Alexa-labeled preparations of HDL, apolipoproteins, and the peptides were found in appropriate positions with molecular masses of 28, 5, and 2.5 kDa for apoA-I, L-37pA and L-18pA, respectively (data not shown).

**Isolation of HDL and Apolipoprotein A-I—Human apolipoprotein E-free HDL (E-free HDL) and apolipoprotein A-I were isolated from the plasma of healthy donors as previously reported (20). L-37pA was labeled as previously reported for HDL and apoA-I (20).**

**125I-LPS Binding Assay—**LPS from *E. coli* 0111:B4 (Sigma) was iodinated as reported earlier (29). HeLa cells grown until 70% confluence in DMEM with 10% FCS were washed with PBS and cultured for 24 h in serum-free DMEM. After chilling on ice, cells were incubated in the presence of 1 μg/ml 125I-LPS and increasing concentrations of cold ligands (LPS, L-37pA, and D-37pA) for 1 h. After washing with ice-cold PBS, the cells were hydrolyzed in 1N NaOH. Radioactivity was counted in an LKB-Wallac Ultragamma counter.

**L-37pA Binding and Competition Experiments—**Transfected cells were plated in 12-well plates, grown to confluence, and incubated in serum-free DMEM prior to the binding experiments. Cells were incubated with iodinated L-37pA in the presence of 50-fold excess of cold ligand for 1 h on ice, and specific binding was measured as reported previously (20). For competition experiments, cells were incubated with 5 μg/ml iodinated L-37pA in the presence of increasing concentrations of tested ligands on ice. Kd and capacity were determined by Scatchard analysis.

**Labeling of HDL, Lipid-free ApoA-I, L-37pA, L2D-37pA, L-18pA, and LPS—**HDL, apoA-I, L-37pA, L2D-37pA, and L-18pA were conjugated with Alexa-568, 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 variable mode imager, Typhoon 9200 (Molecular Dynamics). Alexa-labeled preparations of HDL, apolipoproteins, and the peptides were found in appropriate positions with molecular masses of 28, 5, and 2.5 kDa for apoA-I, L-37pA and L-18pA, respectively (data not shown).

**S. minnesota Re-LPS was labeled using the BODIPY® FL, SE labeling kit from Molecular Probes, Inc. (Eugene, OR) following the manufacturer’s suggested procedure with previously reported modifications (30).**

**TABLE I**

| Peptide   | Sequence                      |
|-----------|-------------------------------|
| L-18pA    | DLKAFYDKVAEKLEAF              |
| L-37pA    | DLKAFYDKVAEKLEAF-P-DWLKAFYDKVAEKLEAF |
| D-37pA    | DLKAFYDKVAEKLEAF-P-DWLKAFYDKVAEKLEAF |
| L1D-37pA  | DLKAFYDKVAEKLEAF-P-DWLKAFYDKVAEKLEAF |
| L2D-37pA  | DLKAFYDKVAEKLEAF-P-DWLKAFYDKVAEKLEAF |
| L3D-37pA  | DLKAFYDKVAEKLEAF-P-DWLKAFYDKVAEKLEAF |

*α*-helical Peptides Block Pro-inflammatory Responses
preincubated with various peptides was quantitatively determined by a chromogenic LAL test (Kinetic-QCL, BioWhittaker, Walkersville, MD). The assay was carried out as recommended by the manufacturer and had an analytical sensitivity of 0.005 EU/ml (~0.5 pg of highly purified LPS/ml).

Uptake of BODIPY-LPS and Alexa 568 HDL and Colocalization of L-37pA/LPS and ApoA-I/HDLa—HeLa cells cultured on glass microslides 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 L-37pA, or 0.5 μg/ml BODIPY-LPS for 1–2 h in a CO2 incubator in DMEM containing 1 mg/ml BSA. The effect of L-37pA on uptake was studied by incubating HeLa cells with 0.5 μg/ml BODIPY-LPS in the presence of 100 μg/ml L-37pA for 1–2 h in a CO2 incubator. For colocalization, BODIPY-LPS and Alexa 568-apoA-I or BODIPY-LPS and Alexa 568-L-37pA were used at the same concentration of 0.5 μg/ml. 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 labels, respectively.

Sites of LPS, L-37pA, and ApoA-I Transport in CLA-1-overexpressing HeLa Cells—For studying the sites of LPS, L-37pA, and apoA-I delivery, cells were incubated with 1–2 μg/ml BODIPY-LPS or Alexa 568 ApoA-I, 1–0.5 μg/ml Alexa 568 L-37pA, or 0.5 μg/ml BODIPY-LPS for 1 h at 37°C followed by 4% paraformaldehyde fixing. Cells were permeabilized by incubating with 0.1% Triton X-100 in PBS for 10 min at room temperature, and further incubated with 10 mg/ml BSA, 1% goat serum in PBS to prevent nonspecific antibody absorption. CLA-1 and the Golgin-97 were detected utilizing rabbit anti-CLA-1 antiserum and mouse CD4 anti-human Golgin-97 IgG (Molecular Probes) as respective first antibodies. Alexa 488/568-labeled goat anti-rabbit IgG and goat anti-mouse IgG were used as secondary antibodies. Confocal images were acquired as described above.

CytoKines and Lactate Dehydrogenase Assays—Interleukins 6 and 8 (IL-6 and IL-8) and tumor necrosis factor-α (TNF-α) were measured in culture supernatants of THP-1 cells using commercial ELISA kits (BIO-SOURCE International). Lactate dehydrogenase activity was measured in the supernatants by a Hitachi 917 automated chemistry analyzer (Roche Applied Science).

Detection of Cytokine mRNA by RT-PCR—Expression of IL-8, IL-6, and TNF-α was determined by RT-PCR as reported earlier (31). Glyceraldehyde-3-phosphate dehydrogenase was used as a reference. Forward and reverse primers are shown in Table II.

Competition Experiments using BODIPY-LPS—The cells were incubated in 1 μg/ml BODIPY-LPS in the presence of increasing concentrations of various peptides. After a 2-h incubation, cells were washed with ice-cold PBS and lysed in 0.1% SDS. Fluorescence in the lysates was measured by a HTS7000 Bioassay reader (PerkinElmer Life Sciences) using 488 nm for excitation and 533 nm for emission monitoring.

Results

Table II: Forward and reverse primers used in RT-PCR

| Protein    | Forward (F) and reverse (R) primers |
|------------|-----------------------------------|
| TNF-α      | (F) 5'–TCC AGG ACC TTA CCA C3'    |
|            | (R) 5'–AGC TGA AGC AGC GC3'       |
| IL-6       | (F) 5'–AGC GCC CTC CCA GCC GTT GC3' |
|            | (R) 5'–TCC GAT TCG TGG GCA GC3'   |
| IL-8       | (F) 5'–AGG CTG GCC GTG CTC TGG C3' |
|            | (R) 5'–CAT AAT TTC GTC GTC GCC G3' |
| GAPDH      | (F) 5'–GTC TTC ACC ACC ATG GAG AAG-3' |
|            | (R) 5'–GCT TCA CCA CCT TGT TGT CAT C3' |

ACCEPTED GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

accumulation of BODIPY-LPS and L-37pA was found in the perinuclear compartment, which has been identified as a primary site for LPS accumulation in mononuclear cells (14, 15). Additionally, the uptake of the single amphipathic helix 18A and L2D-37pA containing 2-amino acid substitutions in each helix that disturb the helix structure, was not increased in CLA-1-transfected HeLa cells when compared with a mock transfected control (data not shown).

L-37pA Binding and Competition—It has been reported that synthetic amphipathic helical peptides such as L-37pA, which have no sequence homology to exchangeable HDL apolipoproteins, bind to the cells overexpressing mouse SR-BI with a high affinity of $K_d = 0.4 \mu g/ml$ and can be cross-linked to the receptor (26). This observation combined with the report demonstrating LPS binding to CLA-1 suggests that L-37pA might share the L-37pA binding site with LPS. Because L-37pA binding to CLA-1 has not been characterized, we analyzed the $125I$-L-37pA binding to CLA-1-overexpressing HeLa cells compared with a mock transfected controls. As seen in Fig. 2A, $125I$-L-37pA specifically and dose-dependently bound to CLA-1-overexpressing HeLa cells. More than a 10-fold increase of binding capacity was observed in CLA-1-overexpressing cells when compared with a mock transfected controls (220 versus 1% mg/mg of cell protein). Like its mouse ortholog, CLA-1 demonstrated high affinity L-37pA binding ($K_d = 0.94$ ng/ml) (Fig. 2B). Mock transfected HeLa cells demonstrated a similar ($K_d = 0.89$ ng/ml) and had readily detectable expression of CLA-1 by Western blotting (20).

To further characterize the L-37pA interaction with CLA-1, competition experiments were conducted utilizing CLA-1-overexpressing HeLa cells. As seen in Fig. 2C, along with L-37pA, several known CLA-1 ligands such as HDL and apoA-I competed against iodinated L-37pA in a dose-dependent manner indicating that an amphipathic $\alpha$-helical synthetic peptide shares the binding site with these ligands. D-amino acid substitutions, which disrupt helical structure, decreased the ability of L-37pA to compete for CLA-1.

It has been demonstrated that LPS, a negatively charged bacterial phospholipid, functions as a CLA-1 ligand (20). LTA, which represents another example of an amphipathic bacterial lipid and the bacterial hsp60, Gro-EL, which demonstrates a highly redundant amphipathic helical motif, are potential ligands for CLA-1. Both bacterial lipids and hsp60 competed with L-37pA for CLA-1 in the order of L-37pA > LTA > LPS > Hsp60 (Fig. 2D).

Cross-linking—To evaluate the potential direct interaction of CLA-1 with LPS and L-37pA, CLA-1-overexpressing and mock transfected HeLa cells were incubated with Alexa 568-labeled L-37pA, BODIPY-LPS, or Alexa 488-protein-labeled HDL as a positive control. Disuccinimidyl suberate (DSS) was used for cross-linking after 90-min ligand incubations in the presence or absence of a 20-fold excess of unlabeled ligand followed by immunoprecipitation using an anti-CLA-1 antibody directed against the C-terminal CLA-1 peptide. The utilization of fluorescently labeled ligands provided several advantages over the more commonly utilized iodinated ligands such as lower nonspecific background and direct scanning of the gel without drying. As seen in Fig. 3, cross-linked complexes of CLA-1 and HDL are present in at least two major bands. The bands in lane 1 with molecular masses of ~200 and ~280 kDa approximate the theoretical masses of HDL proteins (~100 kDa) cross-linked to CLA-1 monomer (~82 kDa) or dimer forms (~164 kDa). Incubations in the presence of 20-fold excess HDL drastically reduced the signal intensity in these bands, indicating a direct competition for CLA-1 binding (lane 2). The signals for both BODIPY-LPS and Alexa 488-L-37pA with similar molecular masses of ~5 kDa were predominantly detected in two
bands with molecular masses of ~90 and ~180 kDa. This indicates cross-linking of the ligands to both monomeric and dimeric CLA-1 forms (lanes 3 and 5). The intensity of the signals was significantly reduced in both bands when the fluorescent ligands were incubated in a 20-fold excess of unlabeled ligand (lanes 4 and 6). These observations implicate CLA-1 as a binding protein for HDL, L-37pA, and LPS. Mock transfected cells demonstrated detectable complexes only when the cell extracts were prepared from 10-fold more cells (data not shown).

CLA-1 Colocalization with BODIPY-LPS and Alexa 488-L-37pA—To further characterize the interaction of CLA-1 with L-37pA and bacterial LPS, CLA-1-expressing and mock transfected HeLa cells were incubated with fluorescently labeled LPS or L-37pA. After a 1-h incubation, cells were fixed with 4% paraformaldehyde, permeabilized, and stained using anti-CLA-1 antibody. As seen at Fig. 4, a significant portion of bacterial LPS (panel A) and L-37pA (panel D) was internalized into the perinuclear compartment. CLA-1 was located on the cell surface as well as the intracellular perinuclear compartment (panels B and E). Merge images demonstrate that a majority of the ligands (yellow) are strongly colocalized with CLA-1 at the cell surface and intracellularly, indicating a direct role of CLA-1 in LPS/L-37pA surface binding as well as ligand internalization (panels C and F).

L-37pA, ApoA-I, and Monomeric LPS Colocalize with Golgi Complex Markers—In contrast to LPS aggregates, monomeric LPS is known to be transported to the Golgi complex (15). Confocal microscopy demonstrated similar staining patterns for BODIPY-LPS, Alexa568-ApoA-I, and Alexa568-L-37pA (Fig. 5). Extensive overlap was observed for fluorescent ceramide and fluorescent SR-BI ligands in the perinuclear compartment, thus confirming a predominant accumulation of LPS, apoA-I, and L-37pA in the Golgi apparatus. These results were confirmed using anti-COP1 antibody, another trans-Golgi marker (data not shown). A much less extensive overlap was observed between fluorescent transferrin and L-37pA or LPS indicating that the endocytic recycling compartment was a secondary transporting site for CLA-1 ligands.

Colocalization of LPS and L-37pA with Golgi Apparatus by Anti-Golgino-97 Antibody—Because ceramide is rapidly converted to sphingomyelin and glucosylceramide, and further to glycosphingolipids, (15) the fluorescent signal of ceramide can be redistributed to locations such as the endosomal and lysosomal compartments, complicating the interpretation of colo-
calization studies with ceramide. To further support the results of Golgi localization studies, internalized BODIPY-LPS and Alexa 488-L-37pA distributions were also assessed with an anti-Golgin-97 antibody. Golgin-97 is a member of granin family of proteins located on the cytoplasmic face of the Golgi apparatus (32). Golgin-97- and ceramide-positive compartments (the Golgi) are known to be a site of HDL cholesterol accumulation and play a central role in lipid compartmentalization in mouse adipocytes. As seen in Fig. 6, both LPS (panel C) and L-37pA (panel F) signals extensively overlapped with anti-Golgin-97 staining confirming that both ligands were distributed to the Golgi complex.

Colocalization of LPS and L-37pA with Cholera Toxin B Subunit—It has been demonstrated that a B subunit of cholera toxin (CTB) binds with high affinity to ganglioside GM1 located in plasma membrane rafts or detergent-resistant plasma membrane domains. CTB is internalized by a vesicular clathrin-independent membrane-to-Golgi transport (33). Bound and internalized CTB are predominantly recovered in a detergent-resistant membrane fraction indicating that CTB may function as a raft-dependent plasma membrane-to-Golgi internalization. To further analyze the internalization of LPS and L-37pA, fluorescently labeled ligands were incubated with CLA-1-expressing HeLa cells on ice. After washing to remove free ligands, bound LPS and L-37pA were allowed to internalize in the presence of Alexa 488-CTB/BSA complex. With little surface-bound LPS detected (Fig. 7A), the majority of internalized LPS was colocalized with CTB (Fig. 7C) in the perinuclear compartment. This was similar to that observed for other Golgi markers such as ceramide and Golgin-97 (Figs. 5 and 6). A strong merge signal was also found when L-37pA was colocalized with CTB (Fig. 7F). No substantial internalization was found for either LPS or L-37pA in mock transfected HeLa cells.

Colocalization of LPS, L-37pA, and ApoA-I in CLA-1-overexpressing HeLa Cells—To determine whether LPS is internalized and transported to the same compartment(s) as classic SR-BI ligands such as apolipoprotein A-I and class A helical amphipathic peptides, uptake and colocalization of BODIPY-LPS with labeled SR-BI ligands were analyzed. As seen in Fig. 8, coincubation of BODIPY-LPS and Alexa 568-apoA-I for 1 h at 37 °C...
L-37pA and D-37pA Prevent LTA- and Gro-EL-stimulated Production of IL-8—It has been reported that various bacterial components, including LTA, a cell wall component of Gram-positive bacteria, and cytoplasmic bacterial heat shock protein, chaperonin 60 or Gro-EL, elicit their effect by inducing downstream signaling by activating receptors, which belong to the TLR family. To study whether their effects can be blocked by L-37pA treatments, THP-1 cells were incubated with 1 μg/ml LTA or 5 μg/ml Gro-EL in the presence of the L-37pA, L-18pA, or L-37pA peptides with single (L1D-37pA), double (L2D-37pA), or triple (L3D-37pA) d-amino acid substitutions, which progressively abolish helical peptide structure. As seen in Fig. 14, a 10- to 200-fold excess of L-37pA blocked production of IL-8 and IL-6 elicited by LTA and Gro-EL. Peptides synthesized with single, double, or triple d- to l-amino acid substitutions were not effective blockers of either LPS- or LTA-induced IL-8 secretion, indicating that an amphipathic helical structure plays a critical role in the blocking efficiency of the peptides. The low blocking efficiency of 18A indicates a requirement of at least a double helix for blocking.
Fig. 8. Colocalization of BODIPY-LPS with Alexa 568-L-37pA and Alexa-apoA-I in CLA-1-overexpressing HeLa cells. CLA-1-overexpressing HeLa cells were incubated with 1 μg/ml of each, BODIPY-LPS and Alexa 568-L-37pA, or BODIPY-LPS and Alexa 568-apoA-I for 1 h followed by confocal microscopy.

Fig. 9. Amphipathic double helix containing peptides compete for LPS-binding sites in CLA-1-overexpressing and mock transfected HeLa cells. LPS-binding was measured in CLA-1-overexpressing and mock transfected HeLa cells after incubation with 1 μg/ml ¹²⁵I-LPS in the presence of various concentrations of unlabeled LPS (A), L-37pA (B), or D-37pA (C).

Fig. 10. Amphiphatic peptides containing a double helix block BODIPY LPS uptake in HeLa cells. Fluorescent microscopy images of 1 μg/ml BODIPY-LPS uptake in CLA-1-overexpressing HeLa cells are shown in the absence (A) or presence (B) of 100-fold excess L-37pA. In C, BODIPY-LPS uptake measured by a fluorescent spectrophotometer is shown in the presence of various concentrations of L-37pA, D-37pA, L2D-37pA, and L-18pA in CLA-1-overexpressing HeLa cells.
DISCUSSION

Bacterial interaction with pattern-recognizing cellular molecules, including scavenger receptors, and initiation of an inflammatory reaction are important parts of the innate immune system, which protects an organism during the initial contact with an infectious entity. Gene knock-out experiments indicate that mice deficient in the expression of various scavenger receptors, TLR, or receptors recognizing bacterial cell wall components such as CD14 exhibit increased sensitivity to bacterial infections (7, 39).

Scavenger receptors are a family of cell surface glycoproteins, including Class A, B, and D (SR-A, SR-B, and SR-D), which are able to bind modified lipoproteins and HDL. This receptor family is characterized by wide ligand specificity and predominantly resides in phagocytes, hepatocytes, and steroid hormone-producing cells. Multiple studies have established an important role of class A scavenger receptors in bacterial binding and internalization (40) and antigen presentation and cell adhesion (41), processes involved with host defense during infections. In contrast, a role for the class B scavenger receptor, especially human orthologue CLA-1, has not been extensively studied in infection and/or inflammation.

The physiological importance of the interaction of SR-BI/CLA-1 with its ligands, such as HDL (apoA-I), has been established by a variety of in vivo studies, primarily using rodent models. SR-BI affects the structure and composition of plasma HDL, including the cholesterol and cholesterol ester content of HDL. SR-BI/CLA-1 also regulates cholesterol levels in the adrenal gland, ovary, and bile by mediating selective cholesterol ester uptake in these SR-BI/CLA-1 abundantly expressing organs. Recent observations also indicate that SR-BI/CLA-1 expression is regulated by LPS in monocyte cell lines (31) and that SR-BI/CLA-1 binds and internalizes LPS (20). Because overexpression of SR-BI/CLA-1 causes LPS to be transported to the trans-Golgi network, SR-BI/CLA-1 appears to function as an endocytic LPS receptor. In this study, CLA-1-overexpressing cells demonstrated an increased uptake and internalization into the Golgi complex of both BODIPY-LPS and L-37pA, a synthetic model peptide mimicking α-helices of exchangeable proteins, including Class A, B, and D (SR-A, SR-B, and SR-D), which are able to bind modified lipoproteins and HDL. This receptor family is characterized by wide ligand specificity and predominantly resides in phagocytes, hepatocytes, and steroid hormone-producing cells. Multiple studies have established an important role of class A scavenger receptors in bacterial binding and internalization (40) and antigen presentation and cell adhesion (41), processes involved with host defense during infections. In contrast, a role for the class B scavenger receptor, especially human orthologue CLA-1, has not been extensively studied in infection and/or inflammation.

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apolipoproteins. L-37pA specifically bound to CLA-1-expressing HeLa cells with high affinity ($K_d = 0.94 \mu g/ml$) and a 10-fold increased capacity when compared with vector-transfected control cells. Due to its amphipathic nature, L-37pA is oligomerized in micelles in physiological saline solution. This fected control cells. Due to its amphipathic nature, L-37pA is 10-fold increased capacity when compared with vector-transfected control cells. Due to its amphipathic nature, L-37pA is oligomerized in micelles in physiological saline solution. This effectively increases its estimated molecular mass to 40–100 kDa when self-aggregated. Using these estimated molecular masses, L-37pA has an approximate $K_d$ of $2.5 \times 10^{-8}$ to $1 \times 10^{-8} M$, which is slightly more avid than the previously reported $K_d$ of $1.6 \times 10^{-7}$ to $8 \times 10^{-8} M$ for BSA-monomerized LPS-CLA-1 high affinity binding (20). These results are consistent with cross-competition experiments demonstrating that 4-fold higher concentrations of LPS were required to compete with $^{125}$I L-37pA (Fig. 2D), whereas lower concentrations of L-37pA were found to effectively compete against $^{125}$I-LPS (Fig. 9). Similar high affinity L-37pA binding has been previously reported for mouse SR-BI with a $K_d$ of 0.4 $\mu g/ml$ (26).

A direct interaction between CLA-1, L-37pA, and LPS was further supported by the results of the cross-linking experiments. Both LPS and L-37pA cross-linked to monomer and dimer forms of CLA-1 when evaluated with reducing 10% SDS-
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apoproteins neutralize LPS pro-inflammatory activity and prevent LDL oxidation (47). In addition, HDL and exchangeable apoproteins also inhibit the production of IL-1β and TNF-α by blocking contact-mediated activation of monocytes by T lymphocytes (48) and block cytokine-induced expression of E-selectin, ICAM-1, and VCAM-1 on endothelial cells at the transcriptional level (46, 49).

Class A amphipathic helical peptides such as the model peptide L-37pA, have also been demonstrated to mimic the anti-inflammatory properties of HDL in vivo and in vitro (35). When injected into an animal as a phospholipid vesicle, one of the analogues prevented endotoxic shock induced by LPS (50). A class A amphipathic helical peptide has also been reported to protect mice from diet-induced atherosclerosis (35), as well as dramatically reducing atherosclerosis in LDL receptor-deficient mice independently of the plasma cholesterol level (51). It has been recently demonstrated that another amphipathic peptide mimicking apoA-I, D-4F, prevented a marked increase in activated macrophage traffic into the aortic arch induced by an influenza viral infection (52). The mechanisms responsible for such anti-inflammatory properties of peptides are unknown. However, our recent demonstration of human SR-BI/CLA-1 (CLA-1) being an endocytic LPS receptor suggests that apoA-I-mimicking peptides, which are also SR-BI/CLA-1 ligands (26), can elicit their anti-inflammatory effect by targeting SR-BI/CLA-1 (20). A direct competition of L-37pA and D-37pA with LPS in CLA-1-overexpressing HeLa cells suggests that peptides can elicit their anti-inflammatory effect by competing with pro-inflammatory compounds, such as LPS. When the effect of the peptides was studied in THP-1 cells, a marked decrease of LPS-stimulated cytokine expression was observed. Importantly, 18A, a less potent SR-BI/CLA-1 ligand as well as L2D-37pA, which do not bind to SR-BI/CLA-1, were without effect.

A number of amphipathic helical peptides based on anti-bacterial proteins have been reported to protect animals against endotoxic shock by forming a complex and neutralizing LPS (35, 37). To exclude the possibility that neutralization of LPS by L-37pA is a major factor, we studied the effect of LPS/L-37pA incubation on LPS activity using the LAL test. The outcome of this experiment indicates that L-37pA does not neutralize LPS when incubated in the same calcium containing media used for cytokine detection experiments in THP-1 cells (RPMI 1640 containing 1% FCS). In the absence of bivalent cations (PBS containing 1% serum versus DPBS that contains bivalent cations) L-37pA bound LPS and decreased LPS activity in the LAL test. These data are in agreement with the general observation that bivalent cations dramatically reduce LPS neutralization by plasma components (38, 39). Interestingly, L2D-37pA, a peptide lacking helical structure, also decreased LPS activity in the absence of bivalent cations and had no effect in the presence of bivalent cations. Because L2D-37pA appeared to block neither LPS uptake nor LPS/LTA/Gro-EL-induced interleukin (IL-6 and IL-8) secretion while being a relatively effective LPS-neutralizer, it appears that the neutralization effect seen in the absence of calcium is likely to result from a nonspecific lipid-binding activity of the peptides (53).

To determine if L-37pA is a toxic peptide, the effect of the peptide on the TNFα-induced IL-6 as well as IL-8 secretion was measured in THP-1 cells. Neither TNFα-induced secretion of IL-6 or IL-8, lactate dehydrogenase release (data not shown), nor β-actin expression were affected by incubation with 20 μg/ml L-37pA, indicating intact inflammatory pathways and no apparent toxic effect in THP-1 cells. These results contrast with anti-bacterial helical cathelicin-derived peptides, which being highly positively charged and bactericidal (54), are toxic to mammalian cells at high concentrations (55).

This study also demonstrates that L-37pA blocks the pro-inflammatory response induced by LTA, an amphipathic membrane component of Gram-positive bacteria. In contrast to L-37pA, no effect was observed for the non-helical peptides L1D-37pA, L2D-37pA, or L3D-37pA. Importantly, the peptides made with a mixture of L- and D-amino acids had lower lipid affinity, as assessed by monitoring their ability to act as detergents in the solubilization of dimyristoylphosphatidylcholine vesicles in the order L-37pA > L1D-37pA > L2D37pA > L3D-37pA (56). However, a similar ability to stimulate cholesterol efflux was demonstrated in HeLa cells with these peptides (56). Because the L- to D-substituted peptides are neither CLA-1 ligands nor effective anti-inflammatory blockers, it appears that non-selective cholesterol depletion was not a factor in L-37pA-related blockade of LPS/LTA/Gro-EL-stimulated cytokine production.

The amphiphilic properties of LTA as well as recent data demonstrating LTA localization to SR-BI expressing in the liver (56) suggest that SR-BI/CLA-1 can act as an endocytic LTA receptor. LTA as well as Gro-EL strongly compete for CLA-1 against L-37pA further supporting this hypothesis (Fig. 2D). Amphipathic helical ligands also blocked pro-inflammatory responses induced by Gro-EL, cytoplasmic bacterial chaperon 60. It is likely that other pro-inflammatory bacterial and animal compounds may also use this receptor, because IL-6 and IL-8 secretion induced by human HSP60, a highly helical pro-inflammatory molecule (57), was also blocked by L-37pA (data not shown). We speculate that, in addition to its well established role in HDL metabolism (21) and HDL-related signaling (58), SR-BI/CLA-1 may play an important role in the intracellular trafficking of various bacterial and mammalian pro-inflammatory components and could also participate in their signaling.

In summary, we demonstrated that SR-BI/CLA-1 targeting by model synthetic amphipathic helical peptides blocks LPS as well as LTA and Gro-EL-induced pro-inflammatory responses in THP-1 cells. The effect on LPS appears to result from a competition of the L-37pA with LPS for the LPS-endocytic receptor, CLA-1. The data indicate that SR-BI/CLA-1 targeting by L-37pA eliminates LPS binding to the plasma membrane and transport to the Golgi complex, two major sites of TLR receptor localization. The observed model amphipathic peptide interactions with CLA-1, mechanisms of peptide internalization, and raft-dependent peptide trafficking provide important insights into the mechanisms of the anti-inflammatory and anti-infection properties seen with plasma high density lipoproteins and exchangeable apolipoproteins. Because the effects of different bacterial compounds were blocked by CLA-1 ligands, the amphipathic helical motif of exchangeable apolipoproteins may represent a general host defense mechanism against inflammatory reactions. Additionally, agents targeting CLA-1 may represent a new class of therapeutics for infections and inflammation.

REFERENCES

1. Cohen, J., and McConnell, J. S. (1985) Lanecet 2, 1069–1070
2. Perrit, P., and Mauze, T. (1999) Int. J. Antimicrob. Agents 12, 97–105
3. Morrison, D. C., and Ryan, J. L. (1987) Annu. Rev. Med. 38, 417–432
4. Ulevitch, R. J. (2000) Immunol. Res. 21, 49–54
5. Adem, A., and Ulevitch, R. J. (2000) Nature 406, 782–787
6. Lien, E., Means, T. K., Heine, H., Yoshimura, A., Kusumoto, S., Fukase, K., Fenton, M. J., Okawa, M., Qureshi, N., Monks, B., Finberg, R. W., Ingalls, R. R., and Golenbock, D. T. (2000) J. Clin. Investig. 105, 497–504
7. Chow, J. C., Young, D. W., Golenbock, D. T., Christ, W. J., and Gusovsky, F. (1999) J. Biol. Chem. 274, 10699–10692
8. van Oosten, M., van Amersfoort, E. S., van Berkel, T. J., and Kuiper, J. (2001) J. Endotoxin. Res. 7, 381–384
9. Shnya, A., and Lindberg, A. A. (1995) Infect. Immun. 63, 865–873
10. Seternes, T., Dalmo, R. A., Hoffman, J., Bogwald, J., Zykova, S., and
