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Class B Scavenger Receptor Types I and II and CD36 Mediate Bacterial Recognition and Proinflammatory Signaling Induced by Escherichia coli, Lipopolysaccharide, and Cytosolic Chaperonin 60

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Class B scavenger receptors (SR-B) are lipoprotein receptors that also mediate pathogen recognition, phagocytosis, and clearance as well as pathogen-induced signaling. In this study we report that three members of the SR-B family, namely, CLA-1, CLA-2, and CD36, mediate recognition of bacteria not only through interaction with cell wall LPS but also with cytosolic chaperonin 60. HeLa cells stably transfected with any of these SR-Bs demonstrated markedly (3- to 5-fold) increased binding and endocytosis of Escherichia coli, LPS, and chaperonin 60 (GroEL) as revealed by both FACS analysis and confocal microscopy imaging. Increased pathogen (E. coli, LPS, and GroEL) binding to SR-Bs was also associated with the dose-dependent stimulation of cytokine secretion in the order of CD36 > CLA-2 > CLA-1 in HEK293 cells. Pathogen-induced IL-6-secretion was reduced in macrophages from CD36- and SR-BI/II-null mice by 40–50 and 30–40%, respectively. Intravenous GroEL administration increased plasma IL-6 and CXCL1 levels in mice. The cytokine responses were 40–60% lower in CD36/−/− relative to wild-type mice, whereas increased cytokine responses were found in SR-BI/II/−/− mice. While investigating the discrepancy of in vitro versus in vivo data in SR-BI/II deficiency, SR-BI/II/−/− mice were found to respond to GroEL administration without increases in either plasma corticosterone or aldosterone as normally seen in wild-type mice. SR-BI/II/−/− mice with mineralocorticoid replacement demonstrated an ~40–50% reduction in CXCL1 and IL-6 responses. These results demonstrate that, by recognizing and mediating inflammatory signaling of both bacterial cell wall LPS and cytosolic GroEL, all three SR-B family members play important roles in innate immunity and host defense. The Journal of Immunology, 2012, 188: 000–000.

The class B scavenger receptor (SR-B) family is a group of integral membrane proteins that includes SR-BI, its splicing variant SR-BII (respectively, CLA-1 and CLA-2 in humans), thrombospondin receptor CD36, and lysosomal integral membrane protein II. Apart from lysosomal integral membrane protein II, which is not found at the cell surface, these receptors have been demonstrated to interact with multiple ligands, including those involved with atherosclerosis (1–5), inflammation (6, 7), host defense (8–10), angiogenesis (11), viral Ag presentation (12), and removal of apoptotic cells (13, 14). SR-BI/BI and CD36 have many common features, including high structural homology and localization in plasma membrane caveolae-like domains, which facilitate lipid exchange and cell signaling (15). These receptors also share ligands, including native and modified lipoproteins, negatively charged phospholipids, various bacteria, and viruses (12–14, 16, 17). SR-BI/II and CD36 play a critical role in lipid metabolism where SR-BI/II mediate both selective high-density lipoprotein (HDL) cholesterol ester uptake and HDL-mediated cholesterol efflux (18, 19). The SR-BII isoform also mediates holopartical uptake and internalization of HDL (20). CD36 is a well-known lipoprotein receptor and a long chain fatty acid transporter (21). CD36 also binds oxidized low-density lipoprotein, facilitating foam cell formation and development of atherosclerosis (1, 2, 22, 23). However, the role of these receptors in pathogen recognition remains to be elucidated.

The organ distribution of various SR-Bs is different, with SR-BI and SR-BII being predominantly expressed in the liver and sterioxidogenic tissues, whereas CD36 expression is primarily in adipose tissue and heart (21, 24). Importantly, both SR-BI/II and CD36 are abundantly expressed in phagocytic cells such as mature monocytes, macrophages, dendritic cells, and neutrophils, which represent cells significantly involved with inflammation as well as innate and adaptive immune responses to infection.

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Abbreviations used in this article: cpn60, chaperonin 60; HDL, high-density lipoprotein; hsp, heat shock protein; LTA, lipoteichoic acid; PM, plasma membrane; SR-B, class B scavenger receptor; WT, wild-type.
SR-Bs mediate binding (adhesion), internalization, and phagocytosis of various bacteria (8, 9, 25, 26), in part due to their ability to interact with bacterial cell wall lipids such as LPS and lipoteichoic acid (LTA) (9, 26, 27). Although the involvement of CD36 in signal transduction induced by various bacteria and LPS has been demonstrated by several groups (9, 25–27), the role of LPS binding to SR-BI/II in signal transduction has been only hypothesized (10, 28) and requires further assessment. In addition to bacterial surface LTA and LPS, bacterial and eukaryotic cytosolic proteins such as chaperonin 60 (cpn60/heat shock protein [hsp] 60) have been reported as potent activators of the macrophage cytokine response (29–32). Cpn60 proteins are highly conserved cytosolic oligomers, facilitating protein folding in prokaryotic and eukaryotic cells. Under steady-state, cpn60 is highly expressed, with its levels being greatly increased in response to physical or chemical stress (33, 34). Importantly, cpn60 could be released due to loss of bacterial cell wall integrity upon antibiotic treatment or inflammatory response.

Cpn60 from Escherichia coli (29, 30), Mycobacterium tuberculosis (31), and Chlamydia trachomatis (32) has been shown to induce proinflammatory cytokine production, alter the expression of cellular adhesion molecules, modulate signal transduction pathways involving ERK and p38 kinases, and induce transcription factors such as NF-κB (32, 35, 36). Moreover, the GroEL homolog cpn60.1 of M. tuberculosis has been recently reported to be required for bacterium-induced proinflammatory responses in M. tuberculosis-infected animals (37). Cpn60/hsp60 serves as a chemoattractant for neutrophils as well as a phagocytosis enhancer of opsonized E. coli particles (38), which could also help to activate innate and adaptive immune responses. Despite the important role of cell wall LPS and LTA as well as cytosolic cpn60 in innate immune responses, the role of scavenger receptors, especially SR-BI and II and CD36, in cpn60-induced inflammation has not yet been investigated. Recent studies have been primarily focused on the role of TLR2, TLR4, and CD14, which were initially characterized as receptors for microbial LTA (39–41) and LPS (42–44). These data suggest that the proinflammatory effects of cpn60/hsp60 could be the result of activation of TLRs (35, 45–48). It has been also demonstrated that TLRs are not involved with hsp60 binding to mouse macrophages (49–52) and receptor-mediated signaling via TLR2 and TLR4 (53–55). Because SR-Bs are widely expressed in reticuloendothelial cells such as macrophages, dendritic cells, and endothelial cells (56), they potentially play important roles in bacterial recognition by mediating binding, internalization, and intracellular signaling of surface LPS, LTA, and cytosolic cpn60.

We hypothesized that bacteria, LPS, and GroEL could be the potent ligands for SR-Bs contributing to the overall response toward bacterial pathogens, where SR-Bs serve as pattern recognizing receptors complementary to the TLR system. The aim of our study was to assess the role of SR-Bs in pathogen recognition and proinflammatory signaling induced by bacteria such as E. coli as well as by its major outer membrane component LPS and cytosolic protein, GroEL. To our knowledge, our findings are the first to determine that bacterial cpn60, GroEL, and LPS could potentially contribute to bacteria-induced inflammation in vitro and vivo, acting via SR-Bs.

Materials and Methods

Reagents

All media, sera, reactive fluorescent dyes, and antibiotics were obtained from Invitrogen (Carlsbad, CA). Recombinant mouse M-CSF, fluoroacetone acetate, LPS from Salmonella enterica (serotype Minnesota), and recombinant cpn60 (GroEL) obtained from an overproducing E. coli strain were purchased from Sigma-Aldrich (St. Louis, MO). Synthetic amphipathic peptides were synthesized by a solid-phase procedure (57, 58). Peptide sequences of these peptides were reported previously (28).

Animals

The National Institutes of Health criteria for laboratory animal care were used in this study. C57BL/6 mice (24–30 wk old) were purchased from the National Cancer Institute/Division of Cancer Treatment and Diagnosis (Frederick, MD). Two pairs of CD36 knockout mice were provided by Dr. K.J. Moore’s laboratory (Lipid Metabolism Unit, Harvard Medical School, Boston, MA) and grown into a colony at the National Institutes of Health animal facility. SR-BI–/––deficient mice (strain B6. 129S2-Scarb1tm1Kri/J) were obtained from The Jackson Laboratory (Bar Harbor, ME) and a colony was established at the National Institutes of Health.

Cell culture

HeLa (Tet-off) cells (Clontech, Palo Alto, CA) stably expressing CLA-1, CLA-2, and CD36 were generated, selected, and cultured as previously reported (52, 53). Human embryonic (epithelial) kidney cells (HEK293) were obtained from the American Type Culture Collection and were stably transfected to express CLA-1 and CLA-2 (CLA-1-HEK293 and CLA-2-HEK293). The HEK293 cell line was also stably transfected with CD36 pIREs-hrGFP-2a plasmid (Strategene, La Jolla, CA) followed by cell selection according to the highest GFP expression. HeLa and HEK293 SR-B–expressing clones with similar expression levels of each SR-B (assessed by RT-PCR; see Supplemental Fig. 1A) were selected and used in this study. Murine SR-BI/II–/–, CD36–/–, and wild-type (WT) macrophages were isolated from mouse bone marrow cells obtained from SR-BI/II–/– mice, CD36–/– mice, and control WT strain, respectively. The macrophages were differentiated by culturing in RPMI 1640 supplemented with 10% FCS, in the presence of 10 ng/ml mouse M-CSF and 10 ng/ml mouse IL-4 for 7–10 d.

Subcellular localization of SR-Bs in CLA-1–, CLA-2–, and CD36-overexpressing HeLa cells visualized by confocal microscopy

The subcellular localization of SR-Bs was analyzed by indirect immunofluorescence staining using specific SR-B Abs and fluorescein isothiocyanate conjugated secondary Abs. The specific markers used for cellular compartment staining were Alexa Fluor 594 wheat germ agglutinin for plasma membrane (PM) and LysoTracker Red DND-99 for lysosomes. Briefly, the cells were grown on glass slides (Nalge Nunc International, Rochester, NY) until 20–50% confluence, subjected to a 30-min staining with fluorescently labeled organelle markers (PM or lysosomal), washed, and fixed in 4% paraformaldehyde in PBS. Fixed cells were permeabilized with Triton X-100 (0.05% in PBS) for 15 min at room temperature and incubated in blocking buffer (5% BSA/PBS/0.1% Tween 20/5% goat serum). The localization of SR-Bs was visualized after incubations with CLA-1– or CLA-2–specific custom Abs or anti-CD36 mAb (JC63.1; Abcam) and secondary Alexa Fluor 488-labeled Abs (Invitrogen).

Confocal microscopy of Alexa Fluor 488 GroEL binding and internalization in SR-B–overexpressing HeLa cells

The cells were grown on glass coverslips until 20–50% confluence and incubated with Alexa Fluor 488 GroEL (10 μg/ml for 30 min) in DMEM, containing 2 mg/ml BSA and 20 mM HEPES. Cells were washed with PBS and then either stained with a PM marker (red fluorescent Alexa Fluor 594 wheat germ agglutinin) or chased for 1 h in DMEM/BSA/HEPES medium and then stained with LysoTracker Red DND-99 (50 nM for 20 min; Molecular Probes, Eugene, OR); nuclei were stained with Hoechst 33342 (1 μg/ml). Cells were washed with PBS and imaged using a confocal microscope. To assess subcellular localization, images were obtained with a Zeiss 510 confocal system (Zeiss, Jena, Germany). Images were acquired sequentially by using a 488-nm laser line and emission between 505 and 580 nm for Alexa Fluor 488, a 561-nm laser line and emission between 575 and 615 nm for Alexa 568, and blue fluorescent Hoechst 33342 dye (excitation/emission maxima when bound to DNA of ~350/461 nm). High-resolution (100 nm/pixel) images were obtained throughout the cells with a ×63, 1.4 numerical aperture Plan-Apochromat oil immersion objective under conditions avoiding bleed-through.

Fluor-labeled ligand uptake

Lipoproteins, BSA, cpn60, and E. coli were conjugated with Alexa Fluor 488 using a protein labeling kit (Invitrogen) following the manufacturer’s instructions. Salmonella minnesota Re 595 LPS was labeled with a BODIPY FL, SE labeling kit from Molecular Probes following the...
manufacturer’s suggested procedure with previously reported modifications (19). HeLa cells were incubated with fluorescently labeled ligands in serum-free DMEM containing 2 mg/ml BSA and 10 mM HEPEs at 37°C for 1 h. The cells were then washed extensively with PBS, detached with Cellstripper dissociation solution (Mediatech, Herndon, VA), fixed with 4% paraformaldehyde, and analyzed by FACS (model A; Hitachi).

Dose-dependent Alexa Fluor 488 GroEL uptake and competition studies

WT, CLA-1–, CLA-2–, and CD36-overexpressing HeLa cells grown in 24-well plates were incubated with increasing concentrations (0–50 μg/ml) of Alexa Fluor 488 GroEL at 37°C for 2 h in the presence (nonspecific uptake) or absence (total uptake) of 200 μg/ml GroEL. All incubations were performed in DMEM containing 2 mg/ml BSA and 20 mM HEPEs. Specific uptake was determined as the difference between total and nonspecific uptake and was normalized for protein content. For competition studies CLA-1–, CLA-2–, and CD36-overexpressing HeLa cells grown in 24-well plates were incubated with 10 μg/ml Alexa Fluor 488 GroEL without or with increasing concentrations of unlabeled ligands at 37°C for 2 h. Cells were then washed three times with PBS and detached from the plate surface by incubation in 200 μl Cellstripper solution for 30 min at room temperature with continuous rocking. Aliquots of cell suspensions were transferred into 96-well black microplates and were read using a fluorescence plate reader (Wallac VICTOR 1420 multilabel counter; PerkinElmer, Boston, MA).

RNA isolation and RT-PCR

The total RNA was isolated from WT and SR-B–overexpressing clones of HeLa and HEK293 cells or from differentiated mouse bone marrow cells using the TRZol reagent (Invitrogen) according to the manufacturer’s recommended protocol. RNA samples were reverse-transcribed by Moloney murine leukemia virus reverse transcriptase (SuperScript reverse transcriptase; Invitrogen) and oligo(dT)12 primers (Promega, Madison, WI). Fluorescein–labeled primers were obtained from Operon Biotechnologies (Huntsville, AL). cDNA was amplified using System 2400 DNA thermal cycler (PerkinElmer Life Sciences) with 25, 30, or 35 cycles for GAPDH, SR-B, and the TLR primers, respectively, and annealing temperatures 50°C (for GAPDH, SR-Bs) and 55°C (for TLRs). Sequences for sense/antisense primer pairs were as follows: GAPDH, 5′-GCTTCCACCACATGAGAGA-AG-3′, 5′-GCTTTACACCCTGAGTGCATC-3′; CLA-1, CLA-2, 5′-GAAATCTGTCGCAGGCA TTGGAC-3′, 5′-AG-3′; TLR7, 5′-GGGGTTCAACAGCCATGTGCT-3′, 5′-CGAGG-CAATTTCCACTTAGG-3′; CD36, 5′-GCCACGATTATGGTCAGATGC-3′, 5′-GATGGCCTGACCAATAGTGTC-3′; TLR2, 5′-GGCGACGTTGGTAAAGTGAACAAA-3′, 5′-AGCCCGAGAAGAACAAGAAGA-3′; TLR4, 5′-GATGGTGCTAGGAAGACAGAC-3′, 5′-GACAAGAAGATGATCGACAGAC-3′; TLR8, 5′-GGGTCACAAGCCATGTGCT-3′, 5′-CGAGGCAATTTCCACTTAGG-3′. The total RNA was isolated from WT and SR-B–overexpressing clones of HeLa cells. After a 20-h incubation in serum-free medium with or without BSA (2 mg/ml) Alexa Fluor 488 GroEL at 37˚C for 2 h. Cells were then washed three times with PBS and detached from the plate surface by incubation in 200 μl Cellstripper solution for 30 min at room temperature with continuous rocking. Aliquots of cell suspensions were transferred into 96-well black microplates and were read using a fluorescence plate reader (Wallac VICTOR 1420 multilabel counter; PerkinElmer, Boston, MA).

Analysis of cytokine and corticosterone production

The IL-8 secretion by HEK293 cells was analyzed in culture supernatants after a 20-h incubation in serum-free medium with or without BSA (2 mg/ml) utilizing a commercial ELISA kit for human IL-8 (Invitrogen). Cytokine levels in culture supernatants of the murine macrophages and sera were measured using ELISA kits for mouse IL-6 (Invitrogen) and mouse CXCL1/KC (R&D Systems) following the manufacturer’s instructions. Serum levels of corticosterone, cortisol, and aldosterone were quantified with corresponding ELISA kits from Stressgen (Ann Arbor, MI). All samples and standards were measured in duplicate.

Limulus amebocyte lysate assay

The endotoxin activity of recombinant GroEL was determined by an automated Limulus amebocyte lysate assay (Kinetic-QCL; Lonza, Walkersville, MD) according to the manufacturer’s recommendations.

Statistical analyses

All data are expressed as mean values ± SD. Differences between groups were analyzed by the nonparametric Mann–Whitney U test with a p value of <0.05 considered as significant.

Results

Colocalization of SR-Bs with a PM marker in HeLa cells overexpressing CLA-1, CLA-2, and CD36

To assess subcellular localization of SR-Bs in HeLa cells overexpressing each of these receptors, we used fluorescent confocal imaging after colabeling cells with an Alexa Fluor 594-conjugated PM marker and corresponding anti–SR-B Abs. The results show considerable overlap of CLA-1 and to a lesser extent of CD36 with a PM marker at the cell surface. CLA-2 was predominantly visible intracellularly with minor localization on the cell surface (Supplemental Fig. 2). The CLA-1 and CLA-2 subcellular localizations are in a good agreement with the previously published data of Eckhardt et al. (20), demonstrating that the SR-B1 isofrom was primarily expressed at the cell surface, whereas 80–90% of the SR-BII protein was expressed intracellularly. With CD36, however, PM staining was observed along with the staining of various intracellular structures throughout the cells. Our observations that CD36 resides on the cell surface and to some extent within the cells are also consistent with the previously reported findings of Eyre et al. (60) indicating that CD36 could be found associated with both the PM and intracellularly located lipid rafts.

Uptake of Alexa Fluor 488-labeled bacterial ligands is increased in HeLa cells stably overexpressing SR-Bs

Previously we and others demonstrated that SR-BI/BII and CD36 function as endocytic receptors mediating intracellular bacterial and LPS uptake (8, 9, 25). We first evaluated the ability of SR-Bs to bind bacteria and LPS. Using FACS analyses, we demonstrated a several-fold increased uptake of fluorescently labeled E. coli (K12) bacteria and LPS by stably transfected HeLa cells expressing all three SB-Rs (Fig. 1). Based on the presence of multiple amphipathic helical domains within cnp60 and the known recognition of these motifs by SR-Bs, we predicted that GroEL might be another candidate ligand for SR-Bs. Similar to E. coli and LPS, GroEL uptake was increased in SR-B–overexpressing HeLa cells. No appreciable differences were observed in Alexa Fluor 488 BSA uptake (used as negative control) between SR-B–overexpressing and control cells. In addition to the FACS data, further evidence for the affinity of SR-Bs toward GroEL was obtained by comparing mock-transfected cells and the utilization of an alternative technique for quantifying fluorescence GroEL uptake (via use of a VICTOR multilabel counter; see Materials and Methods). As shown in Fig. 2A, all three types of SR-B–overexpressing cells demonstrated markedly (3- to 5-fold) increased dose-dependent specific uptake of the fluorescently labeled GroEL versus mock-transfected control cells.

SR-B agonists efficiently compete with Alexa Fluor 488 GroEL for uptake in CLA-1–, CLA-2–, and CD36-expressing HeLa cells

Because we and others previously demonstrated the role of SR-Bs in LPS and bacteria uptake (8, 9, 25), in this study we focused on the analysis of a novel SR-B ligand, GroEL. As seen in Fig. 2B, unlabeled GroEL inhibited Alexa Fluor 488 GroEL uptake in a dose-dependent manner. HDL, oxidized 1-palmitoyl-2-arachidonoyl-glycerol-3-phosphorylcholine, and L-37pA peptide also potently blocked the uptake of labeled GroEL by as much as 60–80% (Fig. 2C–E). In contrast, the L3D-37pA peptide, previously shown by the FACS assay as a poor ligand for SR-B (25, 61), did not compete with Alexa Fluor 488 GroEL uptake (Fig. 2F). Our experiments did not reveal a statistically significant inhibition of GroEL uptake by LPS in SR-B–overexpressing cells, indicating separate LPS and GroEL binding sites within the extracellular loop of SR-Bs (data not shown).
Intracellular trafficking of Alexa Fluor 488 GroEL in mock-transfected and SR-B–overexpressing HeLa cells

The role of each of the three SR-Bs in GroEL binding and internalization was additionally assessed via confocal microscopy by analyzing Alexa Fluor 488 GroEL colocalization with Alexa Fluor 594-conjugated organelle markers. As seen in Fig. 3B–D following a 30-min incubation, Alexa Fluor 488 GroEL could be clearly observed on the cell surface of all three SR-B–overexpressing cell types, colocalizing with the PM marker. Next we analyzed Alexa Fluor 488 GroEL distribution after a 1-h chase period, allowing for internalization and intracellular transport of bound Alexa Fluor 488 GroEL within the cells. As shown in Fig. 3F in CLA-1–overexpressing cells, only a minor colocalization of Alexa Fluor 488 GroEL with the lysosomal intracellular tracker could be detected, with the major portion of the bound ligand still remaining on the cell surface. These results correlate with our data (28) and those of others (20) demonstrating CLA-1 as a receptor with a predominantly cell surface localization. In contrast, in CLA-2–overexpressing cells, all of the Alexa Fluor 488 GroEL appeared to be internalized, colocalizing with LysoTracker (Fig. 3G), additionally indicating the important role of CLA-2 as a GroEL endocytic receptor. Analysis of the Alexa Fluor 488 GroEL distribution in CD36-overexpressing cells revealed partial colocalization of internalized ligand (Fig. 3H) and LysoTracker, possibly suggesting more than one CD36-dependent intracellular trafficking pathway for GroEL. No visible GroEL-associated fluorescence was observed in mock-transfected cells (Fig. 3A, 3E).

HEK293 cells overexpressing SR-Bs demonstrate increased IL-8 secretion upon treatment with bacteria, LPS, and GroEL

Several earlier studies provided experimental evidence for an important role of the CD36 receptor in proinflammatory cytokine signaling induced by various bacteria and bacterial wall-derived compounds, such as LTA and LPS (9, 25–27). The role of SR-BI and SR-BII as potential mediators of bacteria/LPS-initiated cytokine release, however, remains contradictory. In this study we observed a moderate 2- to 3-fold increase of IL-8 secretion in both CLA-1– and CLA-2–overexpressing versus WT HEK293 cells following a 20-h cell treatment with LPS (Fig. 4A). A much greater increase (6- to 8-fold) of IL-8 secretion was observed when cells were treated with live E. coli K12 bacteria (Fig. 4B). At the same time, CD36-overexpressing cells demonstrated markedly (6- to 8-fold) higher levels of IL-8 release induced by either LPS or bacteria when compared with WT cells.

Bacterial cpn60 has been previously reported as a potent proinflammatory cytokine-inducing protein in both phagocytic and endothelial cells (29–32). In this study, we also compared GroEL effects upon IL-8 secretion in WT HEK293 cells versus those stably transfected with CLA-1, CLA-2, or CD36. The results presented in Fig. 4C demonstrate a marked increase in IL-8 secretion induced by bacterial cpn60 in CLA-II– (∼5- to 6-fold) and CD36– (∼7- to 8-fold) expressing cells and a less pronounced (2- to 3-fold) elevation of cytokine levels in CLA-1–overexpressing cells over those detected in WT cells. The data for the most part indicate that proinflammatory activity of LPS and GroEL can be described as CD36 > CLA-2 > CLA-1.

Importantly, the amphipathic helical peptide, L-37pA, a known SR-B ligand (25, 28, 62, 63), efficiently and in a dose-dependent manner blocked IL-8 release induced by E. coli (Fig. 5A), LPS (Fig. 5B), and GroEL (Fig. 5C) in CLA-1–, CLA-2–, and CD36– expressing HEK293 cells. The peptide L3D-37pA, containing three D-amino acid substitutions that are known to disturb the amphipathic α-helical motif, did not affect pathogen-induced IL-8 secretion and was used as a negative control.

Heat treatment significantly reduces the cytokine-inducing SR-B–dependent activity of GroEL

We have checked the potential contamination of recombinant GroEL with LPS that might contribute to the observed GroEL-induced cytokine release using the Limulus amebocyte lysate assay. The en-
dotted toxin content of GroEL was in the range 2–6 endotoxin units/mg protein (0.02–0.06%). Considering that various cells were exposed to GroEL in the range of concentrations of 50–500 ng/ml, the maximal concentration of endotoxin (∼0.3 ng/ml) potentially present in our GroEL preparations appears to be too low to induce measurable levels of cytokine release in either cell types used in this study.

To further dissociate the proinflammatory activity of bacterial cell wall LPS and cytosolic protein GroEL, the effects of heat treatment on the cytokine-inducing activity of GroEL as well as of LPS were analyzed using HEK293 cells expressing three types of SR-Bs. Heat-induced denaturation for the indicated time intervals (from 1 to 45 min) at 100˚C resulted in a rapid (starting after 2 min heating) and significant reduction (by ∼70–80%) of the IL-8–inducing activity of GroEL (Fig. 4D). In contrast, a much weaker and delayed effect of boiling upon the cytokine-inducing activity of the LPS was observed. Thus, we think that GroEL-induced cytokine secretion observed in our study is associated mostly with the protein activity rather than with LPS contamination.

Proinflammatory cytokine release induced by bacterial stimuli is markedly reduced in macrophages from both SR-BI/II−/− and CD36−/− mice

The role of SR-B in mediating proinflammatory bacteria-, LPS-, and GroEL-induced signaling was further tested using cultured

![FIGURE 2. Dose-dependent uptake of Alexa Fluor 488 GroEL in mock-transfected and SR-B–expressing HeLa cells (A). Cells were incubated with the indicated concentrations of Alexa Fluor 488 GroEL for 2 h at 37˚C without (total uptake) or in the presence of 200 µg/ml unlabeled GroEL (nonspecific uptake). B–F, Competition of SR-B ligands with Alexa Fluor 488 GroEL uptake in CLA-1–, CLA-2–, and CD36-overexpressing cells. Cells were incubated with 7.5 µg/ml Alexa Fluor 488 GroEL or with or without the indicated concentrations of unlabeled competitors for 2 h at 37˚C. Unlabeled GroEL was used as a control. Cell-associated fluorescence was estimated using a fluorescence plate reader (see Materials and Methods). The inhibition curves are expressed as percentage of maximal Alexa Fluor 488 GroEL uptake in cells incubated in the absence of unlabeled competitor. The data (mean ± SD) represent one of three separate experiments that yielded similar results and were performed in triplicates. oxPAPC, oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine.](http://www.jimmunol.org/)

![FIGURE 3. GroEL trafficking in mock-transfected and CLA-1–, CLA-2–, and CD36-overexpressing HeLa cells by confocal fluorescent microscopy. Following a 30-min pulse with Alexa Fluor 488 GroEL (10 µg/ml), cells were stained either immediately with the PM marker Alexa Fluor 594 wheat germ agglutinin (A–D) or following a 1-h chase in Alexa Fluor 488 GroEL-free medium (E–H) were stained with the lysosomal marker LysoTracker Red DND-99. The cell type is specified at the top. Merged images are shown, where arrows indicate colocalization (yellow) of Alexa Fluor 488 GroEL (green) with the corresponding (PM or lysosomal) marker (red). Nuclei (blue) were stained with Hoechst 33342. Scale bars, 5 µm.](http://www.jimmunol.org/)
bone marrow–derived macrophages from WT, SR-BI/II−/−, and CD36−/− mice. Because macrophages possess various pathogen-recognizing receptors such as TLRs, LOX, and class A and class B scavenger receptors, the relative impact of SR-B was analyzed in bone marrow cells treated with murine M-CSF for 5 d. Following a 20-h cell treatment with increasing concentrations of GroEL, LPS, and bacteria, the levels of IL-6 secretion in normal, SR-BI/II−/−, and CD36−/− macrophages were determined. Genetically modified SR-BI/II−/− and CD36−/− macrophages demonstrated ∼45 and ∼50% reductions, respectively, of IL-6 secretion upon GroEL stimulation when compared with macrophages from WT mice (Fig. 6A). A similar extent of cytokine production impairment was observed in the absence of functional SR-BI/II or CD36 receptors upon cell stimulation with LPS and E. coli K12, indicating that these two receptors could account for up to 50% of total IL-6 secretion in phagocytes (Fig. 6B, 6C).

To rule out possible alteration of expression of TLRs in macrophage cells derived from genetically modified mice, specifically SR-BI/II−/− and CD36−/−, versus WT cells, we performed a comparative expression assessment of four TLRs known to be primarily involved in recognition of various pathogen-derived ligands, including LPS and GroEL (43–45, 48), using RT-PCR analyses. As shown in Supplemental Fig. 1B, macrophages originating from WT, SR-BI/II−/−, and CD36−/− mice demonstrated similar levels of mRNA expression for all four TLRs being analyzed in our assay.

Corticosteroid hormone responses induced by GroEL in the absence of SR-BI/II or CD36 scavenger receptors in vivo

Corticosteroids are known to play an important role in inflammatory responses and sodium reabsorption, regulating blood pressure and reducing inflammation-induced hypotension. SR-BI–dependent uptake of cholesterol into adrenals is critical for depositing cholesterol for immediate adrenal steroid hormone synthesis associated with acute inflammation or stress (63). It has been demonstrated that SR-BI/II knockout mice do not respond to acute inflammation by producing increasing amounts of corticosterone (7), the principal glucocorticoid of mice and rats (64), which has some mineralocorticoid activity. This relative adrenal insufficiency has been associated with an exaggerated inflammatory response induced by LPS administration or by sepsis (7, 65). However, corticosteroid replacement was insufficient to rescue mice from the inflammatory hyperreactivity in both cases. Because the absence of SR-BI/II could lead to aldosterone deficiency associated with poor sodium reabsorption and reduced blood pressure, we evaluated the levels of corticosteroids including cortisol, aldosterone, and cortisone in SR-BI/II–deficient mice after GroEL administration. As demonstrated in Fig. 7A, plasma levels of cortisol (a common precursor for both cortisol and aldosterone biosynthesis) in control and CD36-deficient mice showed similar (5- to 10-fold) increases in response to a GroEL injection. In SR-BI/II–deficient mice, however, the serum corticosterone levels remained practically unaltered with GroEL administration. Likewise, the mineralocorticoid hormone aldosterone levels were increased (∼3-fold) in GroEL-injected normal and CD36-deficient mice but were unchanged in SR-BI/II–deficient mice (Fig. 7B). In contrast, plasma levels of the glucocorticoid hormone cortisol remained practically unchanged in both normal and CD36-deficient GroEL-challenged mice, but they appeared to be even slightly elevated over the basal level in SR-BI/II–deficient mice following GroEL injection (data not shown). These results clearly indicate the impairment of the adrenal steroid production in SR-BI/II–deficient mice during GroEL-induced acute inflammation.

Proinflammatory cytokine responses induced by GroEL in the absence of SR-BI/II or CD36 scavenger receptors in vivo

To determine the influence of SR-BI/II and CD36 scavenger receptors on GroEL-induced inflammation in vivo, WT, SR-BI/II−/−, and CD36−/− mice were injected i.v. with GroEL (20 μg/mouse) and serum levels of IL-6 and the chemokine CXCL1 (KC) were determined by ELISA at various times following injection. As shown in Fig. 8, GroEL injection resulted in a strong induction of both IL-6 and KC secretion that peaked after 1 h in CD36-deficient mice and in 2 h in normal and SR-BI/II–deficient mice. CD36−/− mice demonstrated ∼50 and ∼55% reduction of IL-6 and KC serum levels, respectively, when compared with normal mice (Fig. 8C, 8F). The IL-6 concentrations were slightly (1.3-fold) higher, whereas the KC levels were slightly lower, although not statistically significant, in SR-BI/II–deficient versus WT mice (Fig. 8A, 8D). When fluocortisone acetate (a corticosteroid with moderate glucocorticoid and much greater mineralocorticoid activity) was provided through drinking water for 24 h prior to GroEL
injection, the levels of both cytokines, IL-6 and KC, were markedly reduced in SR-BI/II–deficient mice compared with untreated SR-BI/II–deficient mice. The peak plasma levels of IL-6 measured in SR-BI/II–deficient mice treated with fludrocortisone acetate were similar to those in normal mice (Fig. 8B), whereas a 30–55% reduction was observed in KC concentrations of SR-BI/II–/– mice versus WT mice (Fig. 8E). A reduced cytokine response in CD36–deficient mice compared with control mice was evident at each time point tested, whereas no cytokine response was observed in mice injected with PBS alone (data not shown). These results are in agreement with the findings of several other studies demonstrating an important role for CD36 as a mediator of the proinflammatory signaling induced by various pathogenic agents, including LPS, LTA, and bacteria (9, 25–27). In contrast, SR-BI/II–deficient mice were shown to be more sensitive to LPS-induced septic shock (7, 64) and CLP-induced sepsis (65). Previous studies demonstrated that corticosterone replacement alone was insufficient to completely correct an abnormal inflammatory state due to adrenal insufficiency and to protect SR-BI/II knockout mice against septic death (65). In our experiments mineralocorticoid supplementation alone rescued SR-BI/II–deficient animals from the GroEL-induced hyperinflammatory response.

**Discussion**

Increasingly there has been a greater interest in SR-Bs, primarily known as key mediators in lipoprotein metabolism, due to their newly discovered role as pattern recognition receptors involved with innate immune responses to bacteria, viruses, and other pathogens (9, 12, 25, 66, 67). SR-Bs CLA-1 (a human ortholog of rodent SR-BI), its splicing homolog CLA-2/SR-BII, and CD36 have been recently demonstrated to mediate uptake and endocytosis of a broad range of pathogens including Gram-positive and Gram-negative bacteria (8, 9, 26, 66). We and others have previously demonstrated that uptake of Gram-negative bacteria through CLA-1/2 is mediated in part through an interaction of bacterial cell wall LPS with these receptors (66). LPS is a well-known inducer of endotoxic shock and inflammation and is a potent ligand for CLA-1 expressed in HeLa cells (10). Bacterial recognition and innate immune responses could also be induced by intracellular proteins that are released during bacterial death due to antibiotic treatment and/or complement-dependent bacterial targeting. Several studies have demonstrated bacterial cpn60 as a potent stimulator of inflammatory cytokine synthesis in both human and murine myeloid cells (29–32). By analogy with the LPS, bacterial cpn60 has been reported to activate TLRs such as TLR4 and/or TLR2 (35, 44, 45).
Because other unknown receptors potentially bind cpn60 (49, 52), we envisioned that GroEL, known to contain amphipathic helices, a structural motif recognized by class B scavenger receptors, could be a ligand for CLA-1/2 and CD36. Moreover, our earlier data showed that GroEL- as well as LPS- and LTA-induced inflammatory effects in phagocytes could be blocked by SR-B antagonists such as the peptide L-37pA (61). However, to our knowledge, until this report, only CD36 has been identified as the single class B receptor mediating proinflammatory responses to LTA and LPS, functioning either as a TLR coreceptor or as an independent signaling molecule (6, 9, 25, 27, 68). No direct data have been reported regarding the role of CLA-1 and CLA-2 in bacterial recognition and inflammatory signaling.

To our knowledge, our uptake studies are the first to demonstrate a markedly increased uptake of E. coli, LPS, and GroEL in HeLa cells due to overexpression of CLA-1, CLA-2, and CD36 receptors. Further analyses of GroEL uptake demonstrated that SR-B ligands, including various lipoproteins and amphipathic helical peptides, efficiently compete for Alexa Fluor 488 GroEL uptake in all three types of SR-B-overexpressing cells. Similar to other studies, the role of SR-B proteins in bacteria-, LPS-, and GroEL-induced proinflammatory signaling was analyzed in HEK293 cells, characterized by low inflammatory IL secretion due to low or absent expression of TLRs (25, 59, 69). These cells demonstrated greatly elevated IL-8 secretion in response to a challenge with E. coli, LPS, and GroEL upon transfection with SR-B-expressing plasmids. Although responses to E. coli were comparable between HEK293 cells stably expressing CLA-1, CLA-2, and CD36, IL-8 secretion was increased in the order CD36 > CLA-2 > CLA-1 when cells were treated with LPS or GroEL. Importantly, our results demonstrate that SR-B–dependent inflammation induced by the bacterial pathogens could be inhibited by SR-B antagonists such as the amphipathic helical peptide L-37pA, and even more efficiently (by 90–95%) when used in association with phosphatidylcholine.

Because bacterial recognition could be also associated with the binding of bacterial cytosolic proteins to SR-B, GroEL–induced proinflammatory responses were further analyzed using murine bone marrow-derived macrophages. Because cultured phagocytes express various pattern recognizing receptors, including the Fc receptors, TLRs, COX, SR-A, and SR-B, it was important to establish the contribution of SR-B deficiency on the overall phagocyte response toward bacteria and their products. Our findings indicate that the proinflammatory effects of bacteria, LPS, or GroEL were reduced by 30–40 and 40–60% in SR-BI/II– and CD36-deficient cells, respectively. These data suggest that in cultured macrophages bacteria-induced inflammation is partially CD36- and SR-BI/II–dependent, with other receptors such as TLR2 and TLR4 also contributing to cytokine secretion induced by bacterial cpn60 or LPS. The macrophage response in vitro is known to be critically dependent on cultivation conditions. Likely reflecting cultivation conditions, our results, in contrast to those of Guo et al. (65),

**FIGURE 7.** GroEL-induced corticosteroid hormone responses in WT, SR-BI/II–null, and CD36-null mice. WT, SR-BI/II+/−, and CD36−/− mice (n = 6/group) were injected with E. coli cpn60 (20 µg/mouse, i.v.) or PBS as a negative control (not shown). Blood was drawn at 1, 2, and 3 h after injection, and serum samples were prepared and analyzed for corticosterone (A) and aldosterone (B) levels by ELISA. The results are shown as means ± SD.

**FIGURE 8.** GroEL-induced proinflammatory responses in normal, SR-BI/II–, and CD36-deficient mice. A, C, D, and F, Levels of IL-6 (A, C) and KC (D, F) in the same serum samples obtained as described in the legend for Fig. 7 were analyzed by ELISA. B and E, Experiments were conducted as described above, but after WT (n = 7) and SR-BI/II–deficient mice (n = 9) were given drinking water supplemented with flu-drocortisone acetate (2 × 10−5 M) for 24 h prior to GroEL injection. The results are shown as the mean ± SD. *p < 0.05, **p < 0.01 versus WT.
demonstrate that SR-BI/II receptors potently contribute to bacteria-mediated inflammatory responses in cultured bone marrow cell-derived phagocytes (Fig. 5). To distinguish between culture conditions and pathophysiologically important responses, we further investigated pathogen-induced inflammation in vivo. Because the effects of both LPS and bacteria have been previously investigated, we studied the effect of bacterial GroEL on SR-B–mediated inflammation. The results of in vivo experiments revealed that the proinflammatory cytokine/chemokine responses induced by bacterial cpn60 were 30–50% lower in CD36-deficient mice than in control animals, fully consistent with the in vitro observations indicating a proinflammatory role of CD36. At the same time, our in vivo data were quite similar to Cai et al. (7), demonstrating no difference or mildly increased plasma cytokine levels in SR-BI–null mice (Fig. 8A). However, there was an inconsistency between our in vivo and in vitro results observed in SR-BI–deficient macrophages. In our opinion, this discrepancy could be easily explained considering earlier reported results revealing that adrenal glucocorticoid deficiency occurring in SR-BI–deficient mice could greatly affect immune cell response to LPS or other proinflammatory stimuli in vivo. As demonstrated previously by Cai et al. (7), SR-BI–deficient mice show an abnormally high inflammatory cytokine response to LPS. However, without appropriately compensating for adrenal corticosteroid deficiency, the true role of SR-BI during LPS-induced inflammation might be misinterpreted.

In view of the well-recognized relative adrenal insufficiency and abnormal stress-induced glucocorticoid/cortisol response in SR-BI/II–null mice, as well as the known clinical inefficiency of glucocorticoid therapy (70), we investigated the GroEL-induced mineralocorticoid response in SR-BI/VII–/– versus WT mice (Fig. 7). In contrast to responsive WT mice, a GroEL challenge was associated with no aldosterone release in SR-BI/II–null mice (Fig. 7B). Consequently, the effect of mineralocorticoid replacement given prior to cpn60 injection was evaluated. The synthetic fluorocortisone reduced IL-6 plasma levels in both normal and SR-BI/VII–/– mice treated with GroEL, whereas CXCL1/KC levels were reduced (to 40–50% of normal mice levels) only in SR-BI/II–deficient mice. Previous studies demonstrated that administration of the exogenous corticosterone, although improving the survival rate of SR-BI/VII–/– mice during LPS-induced endotox shock (7), failed to completely reverse mortality of the CLP-treated SR-BI–deficient mice (65), indicating that glucocorticoid therapy alone is insufficient to correct the aberrant inflammatory response. Additionally, clinical trials have demonstrated that combined hydrocortisone/fludrocortisone treatment markedly reduced the risk of death in patients with septic shock and relative adrenal insufficiency (70). Our findings demonstrate that supplementation with the mineralocorticoid fluorocortisone treatment markedly reduced the risk of death in patients with septic shock and relative adrenal insufficiency. These results provide experimental evidence that mineralocorticoid replacement therapy, known to regulate blood pressure, may improve the outcomes of clinical sepsis in patients with complete or partial adrenal insufficiency.

In conclusion, our study indicates that SR-B receptors play an important role in bacterial recognition and mediate bacteria-associated inflammation and signaling. Additionally, SR-B receptors function as TLR-independent mediators of bacteria-induced signaling triggered by the bacterial wall compound LPS, as well as by cytosolic proteins such as bacterial cpn60, GroEL.

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Disclosures The authors have no financial conflicts of interest.

References

10

CLASS B SCAVENGER RECEPTORS AND BACTERIAL RECEPTOR ACTIVATION


Supplemental Figures Legends

**Suppl. Fig. 1.** Reverse transcription-PCR analysis of the SR-Bs and TLRs expression. **A.** mRNA expression levels of CLA-1, CLA-2 and CD36 in SR-B-expressing clones of HeLa and HEK 293 cells. Lanes 1, 6 (wild type cells), 2, 7 (CLA-1-expressing clones) and 3, 8 (CLA-2-expressing clones) indicate PCR products being produced when using CLA-1/CLA-2 pairs of primers. Lanes 4, 9 (wild type cells) and 5, 10 (CD36-expressing clones) indicate PCR products generated when CD36 primers were used. **B.** Major TLRs’ mRNA expression in macrophages derived from BMC of wild type, SR-BI/II-/- and CD36-/- mice. Each sample was analyzed for glyceraldehyde-3-phosphate dehydrogenase (GADPH) mRNA as a housekeeping gene.

**Suppl. Fig. 2.** Confocal images of SR-Bs co-localizing with a PM marker in HeLa SR-B-overexpressing cells. Live cells pre-stained with the PM marker Alexa Fluor® 594 WGA (red) for 30 min prior to fixation were subjected to indirect immunofluorescence staining using a specific anti-SR-B first antibody and appropriate secondary Alexa Fluor 488-conjugated antibody (green). Nuclei (blue) were stained with Hoechst 33342. The cell type is specified at the top. The scale bars correspond to 5μm.