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Association of SIGNR1 with TLR4–MD-2 enhances signal transduction by recognition of LPS in gram-negative bacteria

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Abstract

SIGNR1, a member of a new family of mouse C-type lectins, is expressed at high levels in macrophages (MΦ) within the splenic marginal zone, lymph node medulla, and in some strains, in peritoneal cavity. We previously reported that SIGNR1 captures gram-negative bacteria, such as Escherichia coli and Salmonella typhimurium, as well as Candida albicans. We have now investigated the precise ligands and innate responses that involve SIGNR1. The interaction of SIGNR1 with FITC–dextran and E. coli was completely inhibited by LPS from E. coli and Salmonella minnesota. Using LPS from various types of rough mutants of Salmonella, we found that SIGNR1 primarily recognizes oligosaccharides in the non-reductive end of the LPS core region. In transfectants, expression of SIGNR1 enhanced the oligomerization of Toll-like receptor (TLR) 4 molecules as well as the degradation of IκB-α after stimulation with E. coli under low-serum conditions. The enhanced TLR4 oligomerization was inhibited by pre-treatment of the cells with anti-SIGNR1 mAb or with mannan. A physical association between SIGNR1 and the TLR4–MD-2 complex was also observed by immunoprecipitation. Finally, we found that transfection of SIGNR1 into the macrophage-like RAW264.7 cells resulted in significant augmentation of cytokine production. These results suggest that SIGNR1 associates with TLR4 to capture gram-negative bacteria and facilitate signal transduction to activate innate MΦ responses.

Introduction

Most cell types in the body express C-type lectins, but macrophages (MΦ) and dendritic cells (DCs) are of special interest because of their major roles in innate immunity (1). C-type lectins, which are pattern recognition molecules, can internalize various glycosylated substances and microbes, leading in vitro to clearance and presentation of antigens. MΦ and DCs express other pattern recognition molecules, particularly Toll-like receptors (TLRs), which mediate innate responses to various components of pathogens, e.g. LPS, peptidoglycan, non-methylated CpG DNA and single- and double-stranded viral RNA (2, 3).

Interest in MΦ and DC lectins was enhanced by the identification of human (h) DC-SIGN (CD209), a type II transmembrane lectin with a single C-terminus carbohydrate recognition domain. This lectin interacts with several different pathogens including several viruses [HIV-1 (4), HCV (5), dengue virus (6, 7), CMV (8), Sindbis virus (9) and other microbes [mycobacteria (11, 12), Leishmania]
SIGNR1 enhances TLR4 response

Pneumoniae in vivo pathogenic Candida albicans ovary cells (CHO) and macrophage-like RAW264.7 cells were conducted according to institutional guidelines. Female BALB/c, C3H/HeN and C3H/HeJ mice were purified...condition under specific pathogen-free conditions. Female BALB/c, C3H/HeN and C3H/HeJ mice were purified...peritoneal cavity with 10 ml of ice-cold PBS containing 5 mM EDTA. For the preparation of exudate Mφ, mice were inoculated with either 2 ml of 10% proteose peptone (PP) for 3 days, or 2 ml of 4% of thioglycollate (TGC) (both from Difco, Detroit, MI, USA) for 4 days.

Flow cytometry analyses of peritoneal cells

Peritoneal cells were pre-incubated with anti-CD16/32 (2.4G2) mAb for 30 min to block FcγR and then stained with PE–CD11b (clone M1/70; BD PharMingen, San Diego, CA, USA) followed by biotin–ER-TR9 (BMA Biomedicals, Augst, Switzerland) (29) with streptavidin–Cy-Chrome (BD PharMingen) to detect SIGNR1 expression. Biotin–DX5 (CD49b; BD PharMingen) was used as an isotype-matched control of mouse IgM. To analyze FITC–dextran binding, peritoneal cells stained with PE–CD11b at (2 × 105 cells per well) were incubated with 80 μg ml−1 of FITC–dextran (molecular weight, 2000 kDa; Sigma–Aldrich, Irvine, CA, USA) in HBSS containing 1% BSA and 0.1% Na3 (HBBS-BSA) for 1 h at 4°C. Flow cytometry was performed on a FACSCalibur system (Becton Dickinson, Mountain View, CA, USA) and analyzed by FlowJo software (Tree Star Inc., San Carlos, CA, USA). To inhibit SIGNR1, FC-blocked resident peritoneal cells (1 × 105 cells per well) were pre-incubated with ER-TR9 (25 μg ml−1; Bachem Bioscience, King of Prussia, PA, USA), control rat IgM (25 μg ml−1; clone R4-22; BD Pharmingen), mannan (Sigma–Aldrich; from Saccharomyces cerevisiae, 1 mg ml−1), EDTA (20 mM), E. coli LPS (80 μg; smooth strain, serotype 0111: B4; Sigma–Aldrich) or Salmonella minnesota LPS (80 μg; smooth strain, Sigma–Aldrich) in HBBS-BSA. After a 1-h incubation on ice, cells were further incubated with 80 μg ml−1 of FITC–dextran for 1 h followed by washing with HBBS-BSA. FITC–dextran binding was analyzed by flow cytometry.

Inhibition of microbe binding with various types of LPS to Ba/F3 transfectants

The cDNA-encoding SIGNR1 (15) was cloned into the retrovirus packaging cell line Plat-E, a kind gift from Kitamura, University of Tokyo) (30). For virus production, the packaging cell line Plat-E (31) was transfected using FuGene (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s protocol. Ba/F3 cells were infected with the virus supernatant including 10 μg ml−1 of polybrene (Sigma–Aldrich) under centrifugation (300 × g) for 2 h at 32°C. Cells expressing SIGNR1 were obtained by limiting dilution. The Ba/F3 (2 × 105 cells) transfectants were then pre-treated with mannan (1 mg ml−1), E. coli LPS, S. minnesota LPS, S. typhimurium TV119 LPS (rough strain, Ra mutant), S. typhimurium SL684 LPS (Rc mutant), S. typhimurium SL1181 LPS (Re mutant), 2-keto-3-deoxy octonate (KDO) and lipid A from S. minnesota Re-595 (100 μg ml−1; all from Sigma–Aldrich) as well as capsular pneumococcal polysaccharide (CPS) type 3 and type 14 (100 μg ml−1; both from American Type Culture Collection, Manassas, VA, USA) in HBBS-BSA for 30 min on ice. Then the

Methods

Mice

Female BALB/c, C3H/HeN and C3H/HeJ mice were purchased from Japan SLC (Hamamatsu, Shizuoka, Japan). The mice were maintained under specific pathogen-free condition and used at 8–12 weeks of age. All experiments were conducted according to institutional guidelines.

Cells and cultures

Human embryonic kidney (HEK) 293T cells, Chinese hamster ovary cells (CHO) and macrophage-like RAW264.7 cells were maintained in DMEM containing 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U ml−1 penicillin and 100 μg ml−1 streptomycin. RAW264.7 transfectants were maintained in 10 μg ml−1 of blasticidin (Invitrogen, Carlsbad, CA, USA). The mouse pro-B cell line Ba/F3 and its transfectants were maintained in RPMI containing 10% FCS, 2 mM L-glutamine, 100 U ml−1 penicillin, 100 μg ml−1 streptomycin as well as 1/2000 volume of conditioned medium from the X63-mL3 line (28). The retrovirus packaging cell line Plat-E, a kind gift from Kitamura, University of Tokyo, was maintained in DMEM containing 10% FCS, 2 mM L-glutamine, 1 μg ml−1 puromycin and 10 μg ml−1 blasticidin. Resident peritoneal cells were obtained by lavage of peritoneal cavity with 10 ml of ice-cold PBS containing 5 mM EDTA. For the preparation of exudate Mφ, mice were inoculated with either 2 ml of 10% proteose peptone (PP) for 3 days, or 2 ml of 4% of thioglycollate (TGC) (both from Difco, Detroit, MI, USA) for 4 days.
cells were further incubated with Alexa488-labeled E. coli (Molecular Probes, Eugene, OR, USA; 2 x 10^5 particles) for 4 h at 4°C. After washing with PBS containing 1% FCS, cells were analyzed by flow cytometry. To examine blocking efficiency, SIGNR1 transfectants were pre-incubated with the graded doses of LPS and CPS. Percent inhibition was calculated according to the formula: [1 – (percentage of SIGNR1 transfectant-binding microbe in the presence of inhibitor – percentage of non-transfectant-binding microbe)/(percentage of SIGNR1 transfectant-binding microbe – percentage of non-transfectant-binding microbe)] x 100. Inhibitor itself did not reduce E. coli binding of non-transfectant.

**Detection of oligomerization of TLR4**

Oligomerization of TLR4 after stimulation with E. coli was performed as described (32), using Ba/F3 transfectants (3 x 10^7 cells) expressing TLR4-Flag, TLR4-GFP, MD-2Flag and CD14 with or without SIGNR1. The cells were incubated with 2% formalin-fixed E. coli (XL-1 Blue K-12 strain lacking O-antigen; Stratagene, La Jolla, CA, USA) at various doses in the presence or absence of serum. After incubation for 30 min at 37°C, cells were lysed in 1 ml of lysis buffer 1 [50 mM Tris–HCl (pH 7.6), 150 mM NaCl, 25 mM CaCl_2, 0.5% Triton X-100, 1 mM phenylmethylsulphonylfluoride and 20 μg ml⁻¹ aprotinin] for 30 min on ice. The lysate was incubated with 1 μg of anti-GFP (Molecular Probes) antibody and 30 μl of Protein A/G agarose (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 12 h at 4°C. Then the agarose beads were washed twice with 1 ml of the lysis buffer 1 and once with binding buffer (lysis buffer 1 without Triton X-100) including 0.05% Triton X-100. Proteins bound to the agarose beads were separated by 7.5% SDS-PAGE under denaturing conditions, transferred to Immobilon-P membrane, we conducted experiments to block FITC–dextran binding to SIGNR1 with LPS, using peritoneal cells, of which possibility that SIGNR1 recognizes LPS on the bacterial outer terminus as described (16). After 48 h, cells were harvested and lysed in 1 ml of lysis buffer 1 for 30 min on ice. After removing cell debris by centrifugation at 12,000 × g for 20 min, the lysate was diluted 10-fold with binding buffer, and one-tenth was incubated with 1 μg of rabbit polyclonal anti-SIGNR1 (PAB-C13) (17), mouse anti-Xpress, rat anti-mTLR4–MD-2 complex (clone MTSS10; eBioscience, San Diego, CA, USA) or anti-Flag (clone M2; Sigma–Aldrich) along with 30 μl of Protein A/G agarose. After a 12 h incubation at 4°C, the agarose beads were washed five times by 1 ml of the binding buffer supplemented with 0.05% Triton X-100. Bound proteins were subjected to western blot analysis with HRP-labeled anti-Flag or anti-Xpress. In the case of Ba/F3 transfectants, 2 x 10⁷ cells are subjected to immunoprecipitation with the rabbit polyclonal PAB-C13 followed by detection using anti-Flag antibody with HRP-labeled bovine anti-mIgG₁ (clone AM6; Cosmo Bio, Tokyo, Japan).

**Cytokine production by RAW transfectants and resident peritoneal Mφ after E. coli stimulation**

RAW264.7 cells were transfected with pcDNA4/HisMax or pcDNA4/HisMax-SIGNR1 (16) by Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer’s protocol. RAW264.7 transfectants expressing SIGNR1 were obtained by limiting dilution. The transfectants (5 x 10⁷ cells per well) were cultured overnight in serum-free medium, X-VIVO15 (BioWhittaker, Walkersville, MD, USA) and stimulated with S. typhimurium KUB5001 (3 microbes per cell; clinically isolated by Mitsuyama, Kyoto university) for 1 h at 37°C. After washing, cells were further incubated for 7 h at 37°C. Resident peritoneal Mφ were negatively enriched by depletion of CD3-, B220-, CD19-, Gr-1- and CD49b-expressing cells using LumiGLO chemiluminescent substrate (Cell Signaling Technology, Beverly, MA, USA) according to the manufacture’s protocols. Precipitation of TLR4-GFP was confirmed using HRP–anti-GFP antibody (Santa Cruz Biotechnology). For inhibition assays, cells were incubated with hamster anti-SIGNR1 mAb (22D1) (20) or hamster IgG at 25 μg ml⁻¹ or mannan at 100 μg ml⁻¹ for 30 min at 37°C before the addition of E. coli (XL-1 Blue).

**IkB-α degradation assay**

Ba/F3 transfectants (1 x 10⁶ cells) expressing SIGNR1 alone, or TLR4-Flag, TLR4-GFP, MD-2Flag and CD14 with or without SIGNR1, were stimulated with formalin-fixed E. coli (XL-1 Blue) at 37°C in 1 ml of RPMI1640 containing 0.01% serum. Lysates equivalent to 5 x 10⁵ cells were separated by 10% SDS-PAGE under denaturing conditions and subjected to western blot analysis using anti-IκB-α (clone C-2; Santa Cruz Biotechnology) followed by HRP-labeled goat anti-rabbit IgG.

**Detection of an association of SIGNR1 with TLR4–MD-2**

HEK 293T cells in 35-mm dishes were transiently transfected with pEFBOS/mTLR4flaghis, pEFBOS/mMD-2flaghis and pcDNA4/HisMax-SIGNR1 tagged with Xpress at the N-terminus as described (16). After 48 h, cells were harvested and lysed in 1 ml of lysis buffer 1 for 30 min on ice. After removing cell debris by centrifugation at 12,000 × g for 20 min, the lysate was diluted 10-fold with binding buffer, and one-tenth was incubated with 1 μg of rabbit polyclonal anti-SIGNR1 (PAB-C13) (17), mouse anti-Xpress, rat anti-mTLR4–MD-2 complex (clone MTSS10; eBioscience, San Diego, CA, USA) or anti-Flag (clone M2; Sigma–Aldrich) along with 30 μl of Protein A/G agarose. After a 12 h incubation at 4°C, the agarose beads were washed five times by 1 ml of the binding buffer supplemented with 0.05% Triton X-100. Bound proteins were subjected to western blot analysis with HRP-labeled anti-Flag or anti-Xpress. In the case of Ba/F3 transfectants, 2 x 10⁷ cells are subjected to Immunoprecipitation with the rabbit polyclonal PAB-C13 followed by detection using anti-Flag antibody with HRP-labeled bovine anti-mlgG₁ (clone AM6; Cosmo Bio, Tokyo, Japan).

**Results**

**LPS inhibits FITC–dextran binding via SIGNR1**

In a previous study, we demonstrated that SIGNR1 transfectants captured gram-negative bacteria, such as E. coli and S. typhimurium, as well as FITC–dextran (16). To examine the possibility that SIGNR1 recognizes LPS on the bacterial outer membrane, we conducted experiments to block FITC–dextran binding to SIGNR1 with LPS, using peritoneal cells, of which a fraction express SIGNR1 (16, 33). We first confirmed SIGNR1 expression on resident peritoneal cells compared with elicited Mφ by PP and TGC (Fig. 1A). The results revealed that SIGNR1 was expressed only on resident, but not elicited, CD11b⁺ Mφ and that neither of these elicited Mφ-bound FITC–dextran. Binding of FITC–dextran to resident Mφ was shown to depend on SIGNR1, since it was completely abolished by
SIGNR1 expression patterns of the CD11bhigh cells were stained by PE–anti-CD11b and anti-SIGNR1 antibody, ER-TR9, and FITC–dextran. Resident, PP- or thioglycollate-elicited peritoneal CD11bhigh cells is also depicted (right panels). After PE–anti-CD11b staining, the peritoneal cells were incubated with FITC–dextran (80 µg ml⁻¹) at 4°C for 1 h. (B) Resident peritoneal cells were pre-incubated with ER-TR9, control IgM (25 µg ml⁻¹ each), mannann (1 mg ml⁻¹), EDTA (20 mM), Escherichia coli LPS (80 µg ml⁻¹) or Salmonella minnesota LPS (80 µg ml⁻¹) LPS for 30 min at 4°C before adding FITC–dextran. Histograms indicate FITC–dextran binding of the CD11bhigh cells.

Fig. 1. FITC–dextran binding by SIGNR1 on resident peritoneal Mφ is blocked by LPS. (A) Resident Mφ specifically express SIGNR1 and bind FITC–dextran. Resident, PP- or thioglycollate-elicited peritoneal cells were stained by PE–anti-CD11b and anti-SIGNR1 antibody, ER-TR9. SIGNR1 expression patterns of the CD11bhigh cells were indicated as histograms (middle panels). Open histograms showed staining patterns with control antibody. Binding of FITC–dextran by the CD11bhigh cells is also depicted (right panels). After PE–anti-CD11b staining, the peritoneal cells were incubated with FITC–dextran (80 µg ml⁻¹) at 4°C for 1 h. (B) Resident peritoneal cells were pre-incubated with ER-TR9, control IgM (25 µg ml⁻¹ each), mannann (1 mg ml⁻¹), EDTA (20 mM), Escherichia coli LPS (80 µg ml⁻¹) or Salmonella minnesota LPS (80 µg ml⁻¹) LPS for 30 min at 4°C before adding FITC–dextran. Histograms indicate FITC–dextran binding of the CD11bhigh cells.

The polysaccharide portion of LPS is crucial for the recognition by SIGNR1

LPS binding was also tested using Ba/F3 cells expressing SIGNR1 at 4°C in HBSS supplemented with BSA to avoid possible involvement of serum component, such as LPS-binding protein (LBP). The Ba/F3 transfectants captured fluoresceinated E. coli, and the binding was completely blocked by pre-incubation with mannan (Fig. 2A), suggesting that the polysaccharide on E. coli surface was recognized by SIGNR1. As expected, microbial binding to SIGNR1 transfectants was abolished by excess amounts of LPS (Fig. 2B). In addition, the binding of E. coli was also inhibited by type 14, but not type 3 CPS, being consistent with the prior observation that type 14 CPS has much higher blocking activity for the uptake of FITC–dextran via SIGNR1 (20). When blocking efficiency for E. coli binding was compared, S. minnesota LPS was equally potent to type 14 CPS and far more potent than E. coli LPS (Fig. 2C).

To define the region of LPS recognized by SIGNR1, we performed experiments to inhibit E. coli binding using various types of LPS from rough mutants of S. typhimurium, since the structure of the core region of LPS is shared by each other (34) (Fig. 3A). As shown in Fig. 3(B), both LPS from the Ra mutant that lacks O-antigen and mannan suppressed the binding of E. coli, whereas LPS from the Rc and Re mutants as well as the LPS components, KDO and lipid A, did not inhibit the binding. Similarly, binding of zymosan to CHO cells transfected with SIGNR1 was effectively suppressed by LPS from the smooth strains and Ra mutant, but not by the others (data not shown).

SIGNR1 enhances TLR4 oligomerization upon stimulation with E. coli

The TLR4–MD-2 complex is a crucial receptor for transducing signals from LPS by recognizing lipid A in LPS in concert with CD14 (35). The results in Fig. 3 had indicated that SIGNR1 recognizes the core polysaccharide portion, but not lipid A of LPS, during binding of E. coli. To elucidate a potential role of SIGNR1 for TLR4-mediated signaling, we used a newly developed system to detect oligomerization of TLR4 molecules after LPS stimulation (32). We transduced SIGNR1 cDNA into Ba/F3 cells expressing TLR4-Flag, TLR4-GFP, MD-2Flag and CD14. The expression levels of the TLR4–MD–2 complex and CD14 were not affected by SIGNR1 expression (Fig. 4A). In the presence of serum (0.5%), there were no differences in TLR4 oligomerization regardless of the presence of SIGNR1 after stimulation with graded numbers of E. coli (Fig. 4B, left panels). Similar results were obtained at higher concentrations of serum (10%) and by stimulation with LPS instead of E. coli (data not shown). However, under lower conditions (0.01%), TLR4 oligomerization was hardly detected in the transfectants lacking SIGNR1 (Fig. 3B, right upper panel), whereas SIGNR1 transfectants were able to oligomerize TLR4 in response to E. coli (Fig. 3B, right lower panel). In contrast, only trace TLR4 oligomerization was evident when 3 µg ml⁻¹ of LPS was used instead of E. coli microbes (data not shown). SIGNR1-mediated TLR4 oligomerization by E. coli was also confirmed by blocking the recognition of LPS with mannan and anti-SIGNR1 mAb (Fig. 4C). These results strongly suggest that SIGNR1 is involved in TLR4–MD–2-mediated signaling particularly by microbes.

SIGNR1 enhances downstream signaling of TLR4 after stimulation with E. coli

Next we examined TLR4–MD–2-mediated intracellular signaling by analyzing iκB-α degradation in response to E. coli...
under low-serum conditions. Following stimulation of transfec-
tants expressing TLR4–MD-2–CD14 and SIGNR1 with
E. coli (10 particles per cell), the level of I\(\kappa\)B-\(\alpha\) expression
was decreased to 27% at 30 min and recovered to 84% at
120 min, relative to unstimulated controls. However, there was
no decrease in the amount of I\(\kappa\)B-\(\alpha\) at any time point in
parental Ba/F3 cells with or without SIGNR1 (Fig. 5A). Upon
stimulation with a larger number of E. coli (20 particles per
cell), cells expressing TLR4–MD-2–CD14 and SIGNR1 ex-
hibited rapid and strong I\(\kappa\)B-\(\alpha\) degradation (40% at 10 min and
13% at 30 min) and prompt recovery (91% at 120 min) (Fig.
5B). In parallel, cells expressing TLR4–MD-2–CD14 without

SIGNR1 showed significant degradation (44% at 30 min and
25% at 60 min) but slow recovery (29% at 120 min). These
results support the notion that SIGNR1 facilitates signal
transduction through TLR4–MD-2.

Association of SIGNR1 with TLR4–MD-2 complex
It has been shown that lung SP-A binds directly to TLR2 and
influences TNF-\(\alpha\) production in response to the lectin ligands,
peptidoglycan (36) and zymosan (23). Thus, we analyzed the
interaction of SIGNR1 with the TLR4–MD-2 complex without
stimulation. Cell lysates of HEK 293T transfected with TLR4-
Flag-, MD-2Flag- and Xpress-tagged SIGNR1 were subject to
immunoprecipitation. The rabbit anti-SIGNR1 (PAb-C13) and
anti-Xpress antibodies co-precipitated TLR4 and MD-2, show-
ing an association of SIGNR1 with TLR4 and MD-2 (Fig. 6A).
Conversely, anti-Flag and MTS510 also co-precipitated
SIGNR1 (Fig. 6B). Using two independent stable transfectants
of Ba/F3, we confirmed the co-precipitation of SIGNR1 with
TLR4 and MD-2 (Fig. 6C). Since the MTS510 antibody is
specific for the TLR4–MD-2 complex, and TLR4 is only

SIGNR1 enhances TLR4 response

Fig. 2. Binding of E. coli via SIGNR1 and its inhibition by LPS and
CPS. (A) Ba/F3 cells expressing SIGNR1 were incubated with
Alexa488-labeled E. coli at 4°C for 4 h. Binding was analyzed by
flow cytometry. For inhibition assays, the transfec-
tants were pre-
incubated with mannan (1 mg ml\(^{-1}\)) at 4°C for 30 min. (B) Ba/F3 cells
expressing SIGNR1 and parent cells were pre-incubated with LPS
from E. coli or S. minnesota, or type 3 or type 14 CPS at 100 \(\mu\)g ml\(^{-1}\) as
in (A). After washing, binding of Alexa488-labeled E. coli was analyzed
4 h later as described. (C) SIGNR1 transfectants and parental Ba/F3
cells were pre-incubated with the graded doses of LPS or CPS, and
then binding of Alexa488-labeled E. coli was analyzed as in (B).
Percent inhibition was calculated as described in Methods.

Fig. 3. SIGNR1 recognizes the core polysaccharide portion LPS
from gram-negative bacteria. (A) Structures of outer and inner core
region of LPS from S. typhimurium. Sugar components deleted from
LPS in various S. typhimurium rough mutants are shown. GlcNAc,
N-acetyl-glucosamine; Glc, glucose; Gal, galactose; Hep, L-glycero-
D-manno-heptose; KDO. (B) As in Fig. 3(A), transfectants were
pre-incubated with 100 \(\mu\)g ml\(^{-1}\) of various types of LPS from rough
mutants, KDO, lipid A or 1 mg ml\(^{-1}\) of mannan at 4°C for 30 min.
Binding of Alexa488-labeled E. coli was analyzed.
expressed on the cell surface by forming heterodimers with MD-2 (37), SIGNR1 complexes with cell surface TLR4–MD-2. To further analyze the role of SIGNR1 in cell activation, we established macrophage-like RAW264.7 cells expressing SIGNR1 by gene transfection and examined the cytokine production following stimulation with \( S. \) \textit{typhimurium} (3 microbes per cell) in serum-free medium. Unlike previous studies of other C-type lectins, such as hDC-SIGN, MR, and BDCA-2 with TLR9, SIGNR1 markedly augmented the production of IL-6, TNF-\( \alpha \) and MCP-1 in response to microbial stimulation (Fig. 7). The increased production of cytokines in SIGNR1-transfected cells was inhibited by anti-SIGNR1 but not by control IgG. In this system, IL-10 and IL-12p70 were below the detection levels. It should be noted that mannan and type 14 CPS in liquid phase have no inhibitory effect on augmented production of cytokines mediated by SIGNR1 (data not shown). Anti-SIGNR1 also remarkably blocked cytokine productions, including IL-10, of C3H/HeN resident peritoneal M\( \phi \) (Fig. 8). However, peritoneal M\( \phi \) from C3H/HeJ mice, which are defective in TLR4-mediated signaling, showed no significant cytokine production by microbes, although they express comparable levels of SIGNR1 to those from C3H/HeN, indicating that any TLRs other than TLR4 is not involved in cytokine production induced by \( E. \) \textit{coli} in association with SIGNR1. These findings indicate that SIGNR1 can enhance the TLR4-dependent innate cytokine response to microbes in M\( \phi \).

**Discussion**

Accumulating evidence indicates that C-type lectins, such as hDC-SIGN, MR and BDCA-2, can deliver negative signals that interfere with TLR-mediated cellular responses against pathogens. In contrast, in the present study, we demonstrate that SIGNR1, a mouse homologue of hDC-SIGN, recognizes the core saccharide portion of LPS in gram-negative bacteria and facilitates signal transduction and subsequent pro-inflammatory cytokine production, possibly through an
observed physical association between SIGNR1 and TLR4–MD-2 complex on the plasma membrane.

In a previous paper, we reported that only SIGNR1, but not several other homologues of hDC-SIGN, bound gram-negative bacteria (16). This binding activity of SIGNR1 is now found to be directed to the LPS from a rough strain (K-12 E. coli) and a smooth strain (clinically isolated S. typhimurium). The outermost component of LPS is called O-antigen, which is a polymer of O-subunits. Each O-subunit is typically composed of four to six sugars depending on the particular O-antigen. Variation in the O-antigen results from a variation in the sugar components of the O-subunit, including a variation in the nature of the covalent bond between the sugars of the subunit, and a variation in the nature of the linkage between the O-subunits that form the O-antigen polymer (38, 39). In addition, the E. coli K-12 strain used in this study lacks O-antigen. Therefore, it is unlikely that the O-antigen is recognized by SIGNR1. Bacteria in the Enterobacteriaceae family include E. coli and S. typhimurium and have similar but not identical core polysaccharides (34). We took advantage of this and used commercially available LPS from rough mutant strains of S. typhimurium and S. minnesota and E. coli K-12 to define the sugars responsible for interactions with SIGNR1. Our results revealed that SIGNR1 recognizes the non-reductive outer end of the core polysaccharide of LPS. Oligosaccharide structures of this portion in S. typhimurium and S. minnesota are [GlcNAc]GlcGal (40, 41), whereas the corresponding oligosaccharide structure of E. coli K-12 is [GlcNAcHep]GlcGlc (34). These differences in the oligosaccharide structure between E. coli and S. typhimurium and S. minnesota possibly account for their different efficiencies for inhibiting microbial binding, as seen in Fig. 2(B and C). In addition, the LPS of the Rc mutant
lacking [GlcNAc]GlcGal was not inhibitory at all (Fig. 3B). We have also observed in the previous study that GlcNAc was inhibitory for FITC–dextran uptake by SIGNR1 (16). Therefore, SIGNR1 most likely recognizes GlcNAc or [GlcNAc]Glc in the oligosaccharide. During the preparation of the manuscript, it was reported that hDC-SIGN recognizes the non-reductive end of \textit{E. coli} LPS using HeLa cells expressing hDC-SIGN and various strains of \textit{E. coli} (42). Their results demonstrate that hDC-SIGN recognized \textit{E. coli} K-12, but not \textit{waaR} mutant bacteria lacking oligosaccharide [GlcNAcHep]Glc at the non-reductive end of the core polysaccharide. Together, there may be a common mechanism for the recognition of gram-negative bacteria by SIGNR1 and hDC-SIGN.

SIGNR1 expression clearly augments TLR4 oligomerization and \textit{IxB-\alpha} degradation in response to microbes, but only when serum concentration was limiting (Figs 4 and 5). In the absence of SIGNR1, TLR4 oligomerization was observed in the presence of serum (Fig. 4B), whereas little or no TLR4 oligomerization was detected in the absence of serum, unless SIGNR1 was also co-expressed. We do not yet understand the basis for this serum effect, but in the absence of serum, the cooperation with SIGNR1 possibly occurs through the observed constitutive association between SIGNR1 and TLR4–MD-2 on the plasma membrane (Fig. 6). However, SIGNR1 showed no augmentative effect on TLR4 oligomerization of Ba/F3 cells expressing TLR4–MD-2 but lacking CD14, a receptor of LBP (data not shown), although SIGNR1 associated with the TLR4–MD-2 complex in the absence of CD14. As in the case of TLR4–MD-2 (32), SIGNR1 was not detected to physically associate with CD14 in the presence or absence of microbial or LPS stimulation (data not shown). In addition, microbial stimulation did not significantly enhance association of SIGNR1 and TLR4–MD-2 (data not shown). CD14 and LBP are well known to be crucial for effective transfer of LPS to TLR4–MD-2 complex (35). Therefore, it is not conceivable that the lipid A portion in LPS bound to SIGNR1 is directly transferred to the TLR4–MD-2 complex without the aid of CD14. Molecular mechanisms underlying LPS transfer from CD14 with LBP in concert with SIGNR1 remain to be elucidated. However, these results suggest a possible interaction of SIGNR1 with CD14 on the plasma membrane. Our observation that SIGNR1 expression substantially augmented pro-inflammatory cytokine production during culture in serum-free medium (Fig. 7) may support this possibility.

Notably, anti-SIGNR1 mAb blocked the augmentation in cytokine production by bacteria (Figs 7 and 8). However, pretreatment with type 14 CPS and mannan in liquid phase had no inhibitory effect on cytokine production mediated by SIGNR1 (data not shown), even though these agents effectively blocked \textit{E. coli} binding (Fig. 2). The reasons for this apparent discrepancy in the blocking of cytokine production and microbial binding are not clear. One possibility is the temperature for the assay: the binding assay takes place at 4°C, whereas cytokine production occurs at 37°C after pre-incubation with blocking agents. Temperature affects membrane fluidity, leading to the association/dissociation of molecules. Cross-linking with anti-SIGNR1 mAb causes rapid internalization of SIGNR1 molecules from the cell surface, resulting in depletion of functional SIGNR1 molecules. Another possibility is the difference in affinity of SIGNR1 for blocking agents relative to the microbe of \textit{S. typhimurium}. Specific mAb once bound to an epitope is not dissociated under physical conditions, but the situation is different in the case of competitive inhibitors.

Accumulating evidence is revealing that TLR-mediated signaling and cell activation are regulated by various C-type...
lectins (12, 21–25). Prior to this study, SIGNR1 has been thought to primarily act as an endocytic receptor but not to transduce signals in cell activation, since SIGNR1 has no putative signaling motif in its cytoplasmic tail except for a dileucine motif to form clathrin-coated pit for endocytosis (15, 16). Neither ligation by anti-SIGNR1 mAb (22D1) nor stimulation with mannan and type 14 CPS induced cytokine production by RAW264.7 expressing SIGNR1 (data not shown), so it appears that a ligand, e.g., the microbe, must have components that can be recognized by both SIGNR1 and TLR4. Precise molecular mechanisms that augment TLR-mediated signals are currently being investigated.

The virulence of gram-negative bacteria is known to depend on the O-antigen that confers the resistance to complement-mediated killing and phagocytosis (43–45). Interestingly, Klena et al. proposed that O-antigen acts as an anti-phagocytic factor by blocking the hDC-SIGN-mediated contact of the ligand with the host (42). However, our previous study demonstrated that smooth strains of Salmonella with O-antigen as well as zymosan could be phagocytosed by COS-7 cells expressing SIGNR1 (16). Therefore, a possibility still remains that SIGNR1, unlike hDC-SIGN, is able to recognize part of O-antigen. Taken altogether, under physiological conditions, SIGNR1 may rapidly capture gram-negative bacteria to internalize and sequester the microbe from inducing an excessive response through the LBP and TLR4-MD-2 complex, but also supports signal transduction for innate defense.

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Abbreviations

CPS capsular pneumococcal polysaccharide
DC dendritic cell
DC-SIGN dendritic cell-specific intercellular adhesion molecule
HEK human embryonic kidney
hDC-SIGN human DC-SIGN
KDO 2-keto-3-deoxyoctonate
LBP LPS-binding protein
Mφ macrophages
MR mannose receptor
PP protease peptone
SP-A surfactant protein-A
TGC thiglycylolate
TLR toll-like receptor
TNF tumor necrosis factor

References

SIGNR1 enhances TLR4 response

[References]