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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), Ebola virus (9), Sindbis virus (10)] and other microbes [mycobacteria (11, 12), Leishmania

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(13) and candida species (14)]. Recently, we identified five mouse homologues of hDC-SIGN (15) and demonstrated their reactivities with microbial polysaccharides, including dextran and mannan (16, 17). Among these new lectins, SIGNR1 is abundant on particular subsets of Mφ in the marginal zone of spleen, the medulla of lymph nodes and in BALB/c mice, the resident peritoneal cavity (17, 18), suggesting that SIGNR1^{+} Mφ play a role as sentinels against pathogenic microbes. In fact, SIGNR1 can capture encapsulated *Streptococcus pneumoniae in vivo* (19, 20), and in culture recognizes pathogenic *Candida albicans*, *Escherichia coli* and *Salmonella typhimurium* (16).

Lipoarabinomannan from mycobacteria is a ligand for TLR2 that inhibits LPS-induced IL-12 production and enhances IL-10 production by human DCs. Lipoarabinomannan also targets DC-SIGN (12) and the mannose receptor (MR) (21). Ligation of blood dendritic cells antigen-2 (BDCA-2), a novel type II C-type lectin that is primarily expressed on human plasmacyotid DCs, suppressed type I IFN production induced by the TLR9 ligand, CpG-oligodeoxynucleotides (22). Surfactant protein-A (SP-A), which belongs to the collectin subgroup of C-type lectins, down-regulates TLR2-mediated signaling and *tumor necrosis factor* (TNF)-α secretion stimulated by zymosan, by attenuating the binding of zymosan to TLR2 (23). In the case of the lectin, dectin-1, a receptor for β-glucan, cooperation with TLR2 has been shown to generate pro-inflammatory responses to fungal pathogens (24, 25). All of these results indicate that two types of pattern recognition receptors, lectins and TLRs, can interact at the molecular level to positively and negatively regulate innate cellular responses (26, 27).

In this report we will show that recognition of the non-reductive portion of core polysaccharides of LPS on gram-negative bacteria by SIGNR1 enhances TLR4-mediated responses, such as TLR4 oligomerization, IκB-α degradation and pro-inflammatory cytokine production. Pre-treatment of SIGNR1-expressing cells with mannan or anti-SIGNR1 mAb abrogates these responses, possibly through an observed physical association between SIGNR1 and TLR4–MD-2 on the plasma membrane.

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-mIL3 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} blasticidine. 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.

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 × 10^5 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% NaN$_3$ (HBSS-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 × 10^5 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 HBSS-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 HBSS-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 vector pMX-puromycin (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 × 10^5 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 µl^{-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 HBSS-BSA for 30 min on ice. Then the

cells were further incubated with Alexa488-labeled *E. coli* (Molecular Probes, Eugene, OR, USA; 2×10^5 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)] × 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 ($3 \sim 5 \times 10^7$ 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 (Millipore, Bedford, MA, USA), probed by HRP-labeled anti-Flag (Sigma–Aldrich) or anti-Xpress antibody (Invitrogen) with HRP–anti-mouse IgG (Amersham Bioscience, Uppsala, Sweden) and visualized by 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).

IκB-α degradation assay

Ba/F3 transfectants (1×10^6 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 5×10^4 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 $12\,000 \times g$ 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 MTS510; 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, 2×10^7 cells are subjected to immunoprecipitation with the rabbit polyclonal PAb-C13 followed by detection using anti-Flag antibody with HRP-labeled bovine anti-mIgG$_1$ (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 (5×10^5 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 biotinylated mAbs with avidine–IMAg (BD PharMingen) and stimulated at 1 *E. coli* to 10 Mφ for 6 h at 37°C in X-VIVO15 medium. Then culture supernatants were collected and assessed for cytokine production using Cytometric Bead Array for mouse inflammation kit (BD Biosciences, Franklin Lakes, NJ, USA). For the inhibition assay, transfectants were incubated with 25 µg ml^{-1} of anti-SIGNR1 mAb (22D1) or control hamster IgG for 30 min at 37°C before the addition of *S. typhimurium*.

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
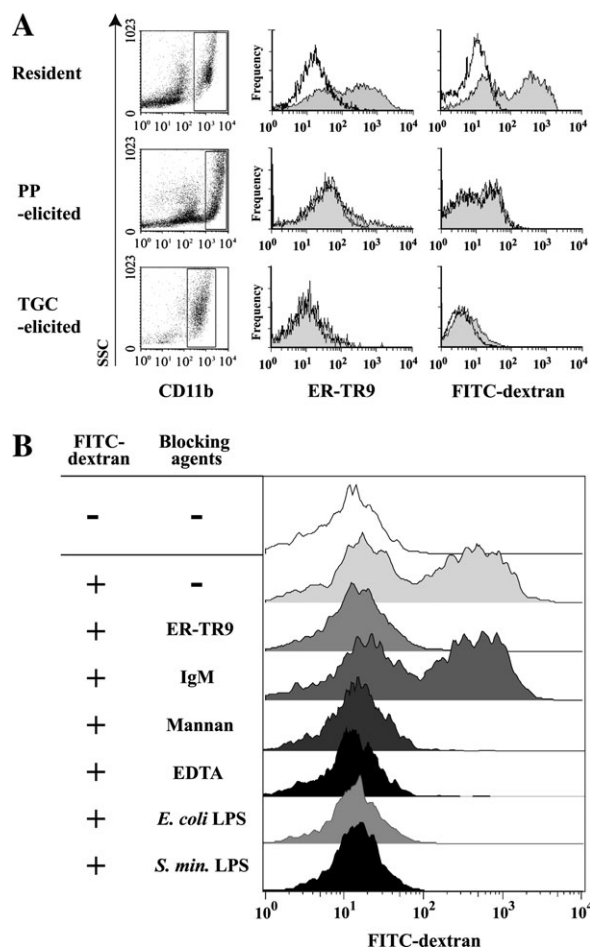
Fig. 1. FITC–dextran binding by SIGNR1 on resident peritoneal Mφ is blocked by LPS. (A) Resident Mφ specifically express SIGNR1 and bind FITC–dextran. Resident, PP- or thioglycollate-elicited peritoneal cells were stained by PE–anti-CD11b and anti-SIGNR1 antibody, ER-TR9. SIGNR1 expression patterns of the CD11b^{high} cells were indicated as histograms (middle panels). Open histograms showed staining patterns with control antibody. Binding of FITC–dextran by the CD11b^{high} 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), mannan (1 mg ml^{-1}), EDTA (20 mM), *Escherichia coli* LPS (80 μg ml^{-1}) or *Salmonella minnesota* (80 μg ml^{-1}) LPS for 30 min at 4°C before adding FITC–dextran. Histograms indicate FITC–dextran binding of the CD11b^{high} cells.

pre-incubation of Mφ with ER-TR9 anti-SIGNR1 mAb, mannan and EDTA (Fig. 1B). Furthermore, LPS from smooth strains of *E. coli* and *S. minnesota* entirely abrogated FITC–dextran binding. These results indicate that SIGNR1 can recognize LPS of gram-negative bacteria.

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 trans-fectants 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 mole-cules 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 pres-ence 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 low-serum 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 oligo-merization 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 signal-ing by analyzing IκB-α degradation in response to *E. coli*
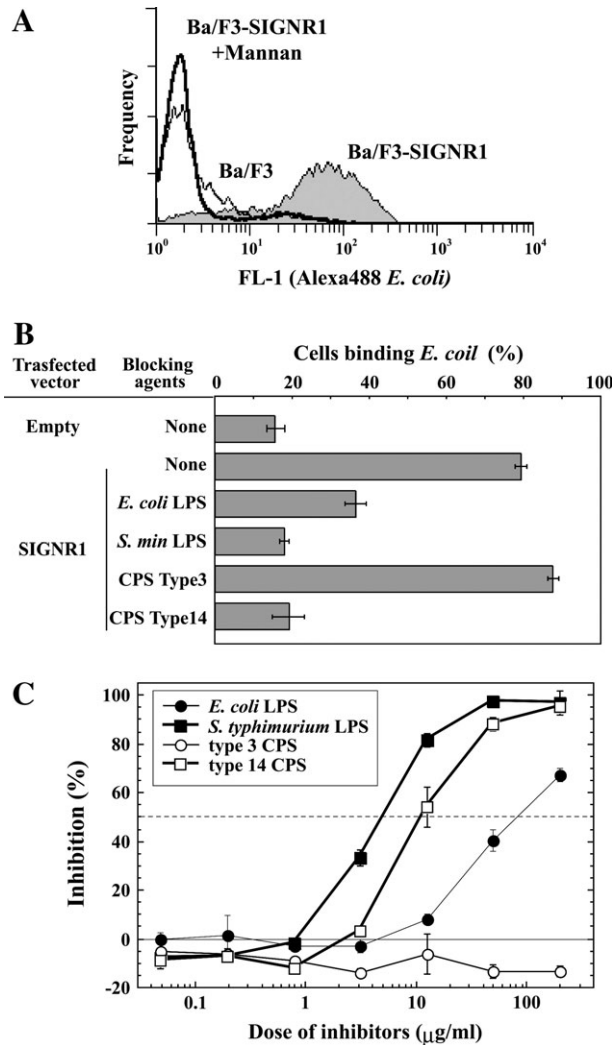
Fig. 2. Binding of *E. coli* via SIGNR1 and its inhibition by LPS and CPS. (A) Ba/F3 cells expressing SIGNR1 were incubated with Alexa488-labeled *E. coli* at 4°C for 4 h. Binding was analyzed by flow cytometry. For inhibition assays, the transfectants were pre-incubated with mannan (1 mg ml^{-1}) at 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 μg 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.
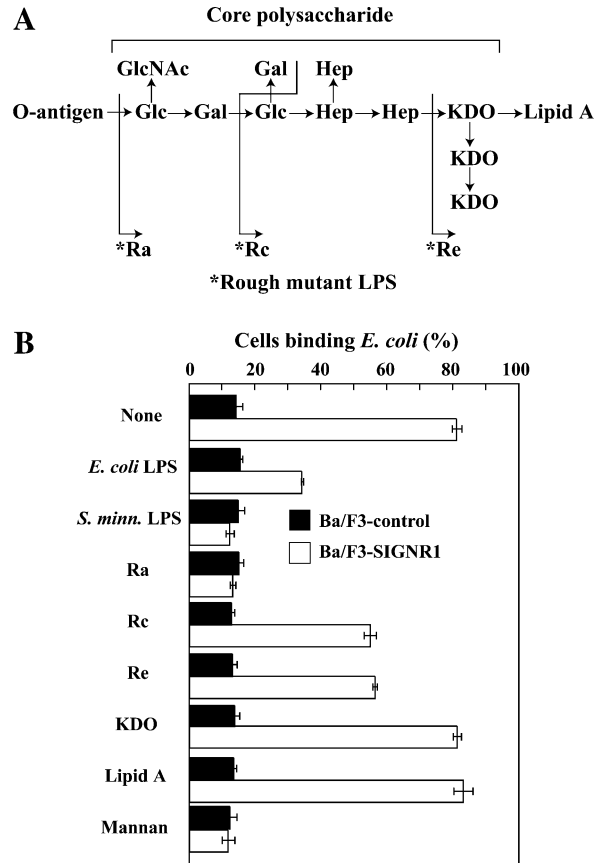

Fig. 3. SIGNR1 recognizes the core polysaccharide portion LPS from gram-negative bacteria. (A) Structures of outer and inner core region of LPS from *S. typhimurium*. Sugar components deleted from LPS in various *S. typhimurium* rough mutants are shown. GlcNAc, *N*-acetyl-glucosamine; Glc, glucose; Gal, galactose; Hep, L-glycero-D-manno-heptose; KDO. (B) As in Fig. 3(A), transfectants were pre-incubated with 100 μg 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.

under low-serum conditions. Following stimulation of transfectants expressing TLR4–MD-2–CD14 and SIGNR1 with *E. coli* (10 particles per cell), the level of IκB-α 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 IκB-α 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 exhibited rapid and strong IκB-α 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-α 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, showing 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
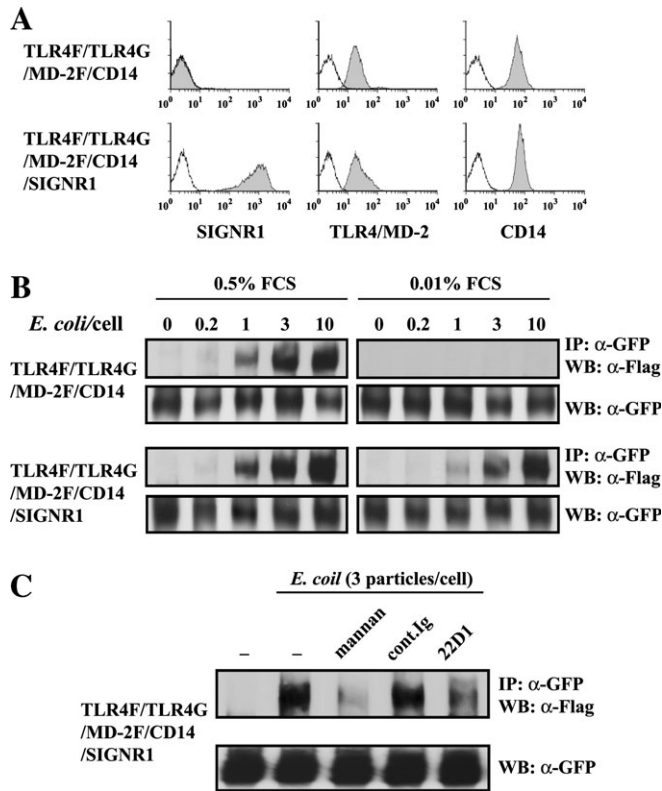
Fig. 4. SIGNR1 enhances TLR4 oligomerization upon stimulation with *E. coli*. (A) Ba/F3 transfectant expressing TLR4-Flag, TLR4-GFP, MD-2Flag and CD14 was transduced with SIGNR1 gene and stained with anti-SIGNR1 (ER-TR9), anti-TLR4–MD-2 (MTS510) or anti-CD14 (Sa2-8). Open histograms represent staining profiles with control antibodies. (B) Transfectants were stimulated with *E. coli* at various numbers of microbes for 30 min at serum concentrations indicated. The cell lysates were subjected to immunoprecipitation with anti-GFP for TLR4. Co-precipitation of TLR4-Flag was detected by western blotting with anti-Flag. Precipitation of TLR-GFP was confirmed by anti-GFP antibody. (C) The transfectants prepared in (A) were pre-treated with mannan, hamster IgG or anti-SIGNR1 (22D1), followed by stimulation with *E. coli* in the presence of 0.01% serum.

Fig. 5. SIGNR1 enhances IκB-α degradation after stimulation with *E. coli*. *Escherichia coli* bacteria were added to cell cultures at 10 and 20 microbes per cell in (A) and (B), respectively. After incubation for various time periods in the presence of 0.01% serum, IκB-α and β-actin in whole-cell lysates were analyzed by western blotting.

expressed on the cell surface by forming heterodimers with MD-2 (37), SIGNR1 complexes with cell surface TLR4–MD-2.

SIGNR1 augments cytokine production of RAW264.7 and resident peritoneal Mφ

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. 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-α 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
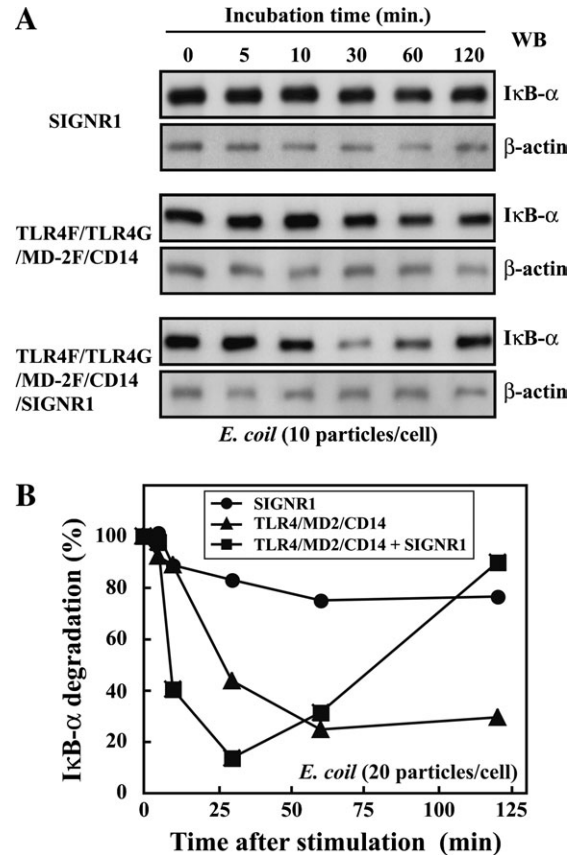
production of cytokines mediated by SIGNR1 (data not shown). Anti-SIGNR1 also remarkably blocked cytokine productions, including IL-10, of C3H/HeN resident peritoneal Mφ (Fig. 8). However, peritoneal Mφ from C3H/HeJ mice, which are defective in TLR4-mediated signaling, showed no significant cytokine 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. coli* in association with SIGNR1. These findings indicate that SIGNR1 can enhance the TLR4-dependent innate cytokine response to microbes in Mφ.

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 though an
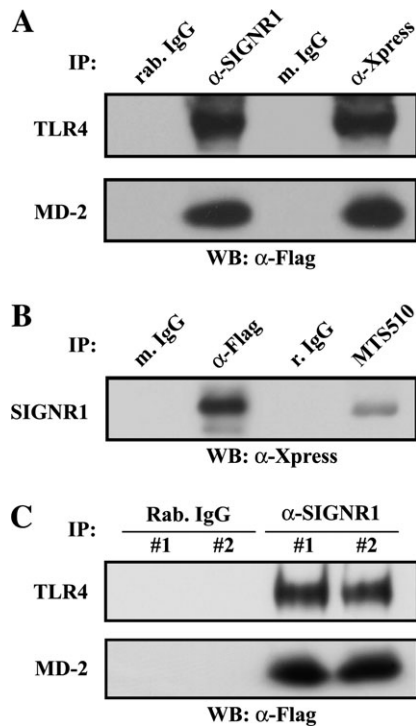
Fig. 6. Association of SIGNR1 with the TLR4–MD-2 complex. (A) Cell lysates of 293T transiently transfected with pEFBOS/mTLR4flaghis, pEFBOS/mMD-2flaghis and pcDNASIGNR1 (Xpress tagged) were subjected to immunoprecipitation with antibodies indicated, followed by western blot analyses by anti-Flag antibody against TLR4-Flag and MD-2Flag. (B) As in (A), cell lysates were precipitated with anti-Flag or anti-TLR4–MD-2 complex (MTS510), followed by blotting SIGNR1-Xpress using anti-Xpress antibody. (C) Cell lysates of stable Ba/F3 transfectants expressing TLR4-Flag, MD-2Flag and SIGNR1 was precipitated with anti-SIGNR1 (PAb-C13), followed by western blot analysis using anti-Flag antibody. Numbers indicate two independent clones.

Fig. 7. Augmented cytokine production by the macrophage-like cell line, RAW264.7, via SIGNR1 in response to *S. typhimurium*. RAW264.7 transfectants (5×10^5 cells per well) were cultured overnight in serum-free medium, X-VIVO15, and stimulated with *S. typhimurium* (3 microbes per cell) for 1 h at 37°C. After washing, cells were further incubated for 7 h at 37°C. For the inhibition assay, cells were pre-treated with 25 µg ml^{-1} of anti-SIGNR1 (clone 22D1) or control hamster IgG for 30 min at 37°C before the addition of microbes. Then culture supernatants were collected and assessed for inflammatory cytokine production using Cytometric Bead Array.

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
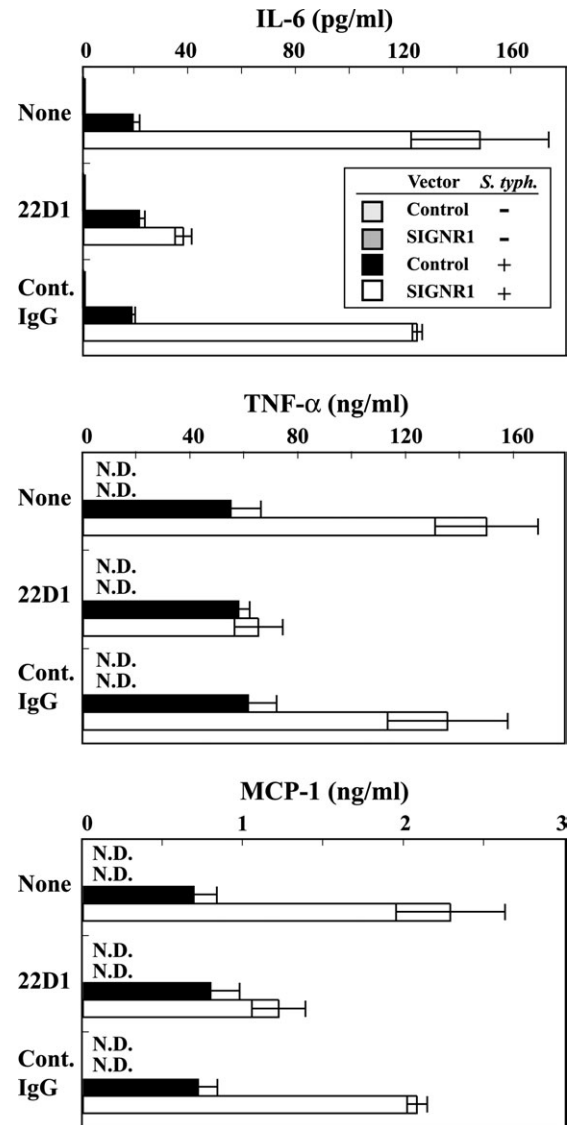
S. typhimurium to define the sugars responsible for interactions with SIGNR1. Our results revealed that SIGNR1 recognizes the non-reductive outer end of the core polysaccharide of LPS. Oligosaccharide structures of this portion in *S. typhimurium* and *S. minnesota* are [GlcNAc]GlcGal (40, 41), whereas 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
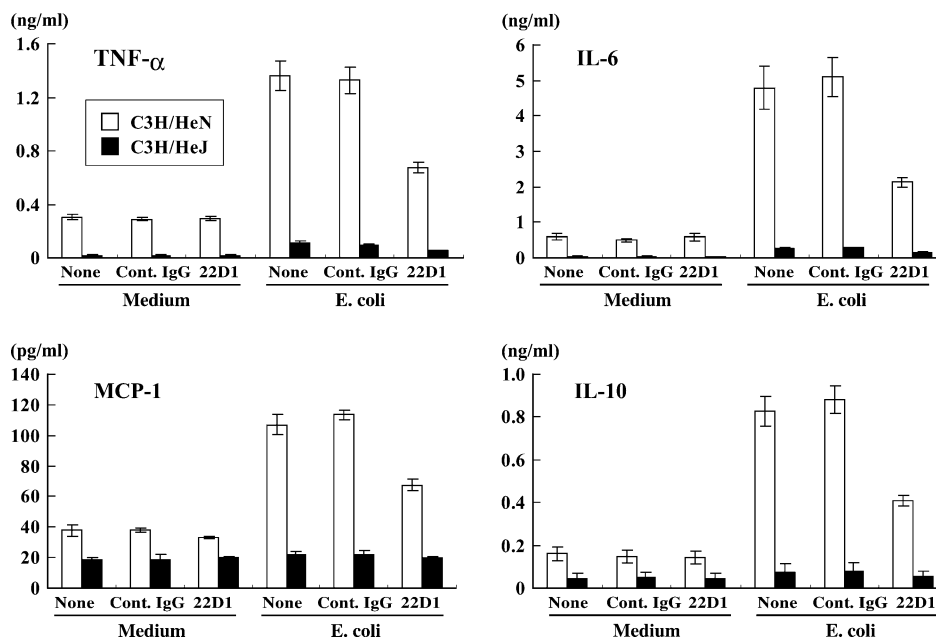
Fig. 8. SIGNR1-dependent cytokine production induced by *E. coli*. C3H/HeN- and C3H/HeJ-derived resident peritoneal Mφ enriched negatively by magnetic beads were stimulated with *E. coli* at 1 microbe per 10 cells in X-VIVO15 medium for 6 h, and cytokine activities in culture supernatants were assessed by Cytometric Bead Array. Inhibition assay was carried out as in Fig. 7.

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 *E. coli* LPS using HeLa cells expressing hDC-SIGN and various strains of *E. coli* (42). Their results demonstrate that hDC-SIGN recognized *E. coli* K-12, but not *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 IκB-α 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, pre-treatment 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 *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 *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 endocytotic 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 serves as an anti-phagocytic factoring 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.

Acknowledgements

Abbreviations

CPS	capsular *pneumococcal* polysaccharide
DC	dendritic cell
DC-SIGN	dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin binding receptor
HEK	human embryonic kidney
hDC-SIGN	human DC-SIGN
KDO	2-keto-3-deoxy octonate
LBP	LPS-binding protein
Mφ	macrophages
MR	mannose receptor
PP	proteose peptone
SP-A	surfactant protein-A
TGC	thioglycollate
TLR	Toll-like receptor
TNF	tumor necrosis factor

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