Biochemical and Biophysical Research Communications 386 (2009) 202–206

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Cross-linking of SIGNR1 activates JNK and induces TNF-α production in RAW264.7 cells that express SIGNR1

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ARTICLE INFO

Article history:
Received 16 May 2009
Available online 9 June 2009

Keywords:
JNK
Lectin-like receptors
Lipid raft
Macrophages
SIGNR1
Src family kinases
TNF-α

ABSTRACT

In this study, we evaluated the signaling ability of SIGNR1 in murine macrophage-like RAW264.7 cells that stably expressed FLAG-tagged SIGNR1 (SIGNR1–FLAG). Cross-linking of SIGNR1–FLAG expressed on the cells by an anti-FLAG antibody induced JNK phosphorylation without induction of phosphorylation of ERK1/2 and p38 MAP kinase, and led to phosphorylations of Src family kinases (SFKs) and Akt. The SIGNR1–FLAG molecules in the cells were found in lipid raft-enriched membrane fractions, and the tyrosine kinases Lyn, Hck, and Fgr co-precipitated with SIGNR1–FLAG in the lipid raft fractions. The antibody-induced JNK phosphorylation was inhibited by inhibitors of SFKs and tyrosine kinases. Furthermore, cross-linking of SIGNR1 led to production of TNF-α, and the JNK inhibitor inhibited the antibody-induced TNF-α production. These results show that cross-linking of SIGNR1 triggers phosphorylation of SFKs, which leads to activation of the JNK pathway and induction of TNF-α production in macrophage-like RAW264.7 cells.

Antigen-presenting cells (APCs) such as macrophages and dendritic cells express an array of pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) including lipids, carbohydrates, and proteins. One of the best-studied families of PRRs is the Toll-like receptor (TLR) family. PAMPs such as lipopolysaccharides signal through TLRs, leading to induction of inflammatory cytokines and maturation of APCs, which guide innate and adaptive immune responses. The family of TLRs has emerged as a critical axis in the innate and adaptive immune systems, but other families of PRRs can also bind pathogens and regulate the immune system. C-type lectin-like receptors (CLRs) expressed on myeloid cells act as PRRs in pathogen recognition through binding to pathogen-associated carbohydrate structures, function as signaling molecules, and play an important role in the immune system [1]. Specific ICAM-3 grabbing nonintegrin related 1 (SIGNR1), a murine homolog of human DC-SIGN [2], is expressed on marginal zone macrophages and resident peritoneal macrophages (PEMs), and is essential for clearance of *Streptococcus pneumoniae*-derived capsular polysaccharides by marginal zone macrophages [3,4]. SIGNR1 acts as a major mannose receptor for *C. albicans* and yeast-derived zymosan particles [5], and mice deficient in SIGNR1 exhibit increased susceptibility to *S. pneumoniae* [6], indicating that SIGNR1 plays a role in host defense. However, little is known regarding the biological function of SIGNR1.

Recently, we have shown that PEMs undergo maturation with enhanced expression of MHC class II and co-stimulatory molecules, and induce IL-12 production in response to specific uptake of liposomes coated with oligomannose residues (OMLs) [7,8]. To understand the maturation mechanisms of PEMs induced by OMLs, we established murine macrophage-like RAW264.7 cells that stably expressed SIGNR1 and showed that SIGNR1 mediates specific uptake of OMLs in both SIGNR1-transfected cells and PEMs [9]. These results led us to the hypothesis that maturation of PEMs in response to OML uptake might be triggered in part by SIGNR1-initiated intracellular signals. However, these signals remain obscure in macrophages [1], and therefore we examined SIGNR1-initiated intracellular signaling in the established SIGNR1-expressing macrophage-like RAW264.7 cells. Here, we present evidence that SIGNR1 is associated with Src-related tyrosine kinases in lipid rafts and that cross-linking of SIGNR1 molecules by an antibody triggers activation of c-Jun N-terminal kinase (JNK) without activation of extracellular signal-related kinase 1/2 (ERK1/2) and p38 MAP kinase, and induction of TNF-α production.

Materials and methods

Generation of macrophage-like RAW cells with stable expression of SIGNR1–FLAG. Mouse macrophage-like RAW264.7 cells were maintained in DMEM containing 10% heat-inactivated fetal calf serum,

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doi:10.1016/j.bbrc.2009.06.011

2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. A cDNA construct encoding SIGNR1 tagged with a FLAG epitope at the C-terminal was generated as described previously [9]. The construct was inserted into a pCMV expression vector (Clontech, Palo Alto, CA) to generate the pCMV–SIGNR1–FLAG plasmid. SIGNR1–FLAG-expressing RAW cells (RAW-SIGNR1 cells) were generated by electroporation of the plasmid, selection in DMEM with 1000 μg/mL G418 (Gibco, Grand Island, NY), and cell sorting with an anti-FLAG rabbit antibody. RAW-mock cells stably transfected with an empty pCMV vector were generated using a similar procedure. The transfectants were maintained in DMEM containing 10% heat-inactivated fetal calf serum, 2 mM glutamine, and 1000 μg/ml G418.

SIGNR1-cross-linking. RAW-SIGNR1 cells were washed extensively and cultured in serum-free DMEM for 18 h to reduce the basal level of activation. The resulting cells (1×10^6) were suspended in ice-cold serum-free DMEM and treated with 1 μg/ml of an anti-FLAG antibody (Rabbit IgG; Rockland, Gilbertsville, PA) on ice for 5 min. Anti-rabbit Fc antibody (1 μg/ml) was then added and the mixture was transferred to a 37 °C water bath and incubated for the indicated time. After stimulation, cells were immediately lysed with 200 μl of a solution containing 20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 200 mM Na_3VO_4, 0.5% Brij 58, and protein inhibitor cocktail (Sigma–Aldrich, St Louis, MO). The cell lysates were centrifuged at 3000 rpm for 10 min and the supernatants were collected and stored at −30 °C until assayed. An aliquot of each supernatant was used to determine the protein concentration using a modified Lowry protein assay reagent (Pierce, Rockford, IL). In some experiments, dimethylsulfoxide (DMSO) solutions of specific signal transduction inhibitors of JNK (SP600125; 10 μM), p38-MAPK (SB202190; 25 μM), ERK1/2 (PD98059; 10 μM), PI3 K (Ly294002; 25 μM), Syk (piceatannol; 25 μM), protein tyrosine kinases (genistein; 10 μM), and Src family tyrosine kinases (PP2; 10 μM) were added 30 min prior to stimulation with the antibody [10]. All inhibitors were purchased from Calbiochem. For assessment of cytokine production, the cells were treated with antibodies and incubated overnight at 37 °C, and TNF-α, IL-6, and IL-10 secreted in the culture supernatants were quantified by ELISA using a commercial ELISA kit (Pierce, Rockford, IL).

Western blot. Cell lysates (10 μg) were subjected to SDS–PAGE on 4–20% gradient gels under reducing conditions and transferred onto a PVDF membrane. After blocking with 3% BSA in 50 mM Tris–HCl (pH 7.6), 150 mM NaCl, and 0.1% Tween-20, the membranes were incubated with primary antibodies and then with horseradish peroxidase-conjugated secondary antibodies. Detection of phosphotyrosine, phospho-MAPKs, phospho-Akt, and phospho-Src family kinases was carried out using a mouse monoclonal anti-phosphotyrosine antibody (4G10, Upstate, Lake Placid, NY), rabbit polyclonal anti-phospho-ERK1/2 antibody (T202/Y204, Cell Signaling, Beverly, MA), rabbit polyclonal anti-phospho-p38-MAPK antibody (T180/Y182, Cell Signaling), rabbit polyclonal anti-phospho-JNK antibody (T183/Y185, Cell Signaling), rabbit polyclonal anti-phospho-Akt antibody (S473, Santa Cruz Biotechnology, Santa Cruz, CA), and rabbit polyclonal anti-phospho-Src family antibody (T416, Cell Signaling). As a control for protein loading, membranes were probed with rabbit polyclonal antibodies against ERK, p38-MAPK, JNK (all from Cell Signaling), and α-tubulin (Santa Cruz Biotechnology). SIGNR1 was detected using a goat polyclonal anti-mouse CD209b antibody (goat IgG, R&D Systems).

Isolation of detergent-insoluble cell membrane fractions. Detergent-insoluble membrane (DIM) domains were separated from cell lysates as described previously [11]. RAW-SIGNR1 cells (5×10^7) were homogenized on ice with 4 volumes of 0.5% Brij 58-containing lysis buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 200 μM Na_3VO_4, 0.5% Brij 58, and protease inhibitor cocktail). The cell lysates were centrifuged at 3000 rpm for 5 min. The supernatants were adjusted to 42.5% sucrose by adding an equal volume of 85% sucrose. Samples (4 ml) were placed in an ultracentrifuge tube and overlaid with a gradient of 4 ml of 30% sucrose followed by 4 ml of 5% sucrose. The gradients were centrifuged at 40,000 rpm for 18 h in an SW41 rotor (Beckman Coulter). Twelve fractions (each 1 ml) were harvested from the top of the gradient and numbered 1–12 from top to bottom. With this procedure, DIMs were located mainly in fractions 4 and 5. Aliquots (10 μl) were analyzed by Western blotting after SDS–PAGE and transferred to PVDF membranes for detection of the membrane distribution of SIGNR1–FLAG, and SFKs using antibodies against SIGNR1, Lyn, Hck, and Fgr (Santa Cruz Biotechnology), respectively.

Immunoprecipitation. Immunoprecipitation of protein assemblies was performed by incubation of the DIM fraction with an anti-FLAG rabbit antibody (1 μg) (Santa Cruz Biotechnology) followed by capture of the immune complexes on Protein G Sepharose (20 μl; GE Healthcare Bio-Science) at 4 °C, as described elsewhere [11]. As a control, a non-immunized rabbit IgG (1 μg) was used instead of the anti-FLAG rabbit antibody. Alternatively, the DIM fraction and the concentrated detergent-soluble fraction (fraction 12) were first adsorbed with a non-immunized rabbit IgG (1 μg) and Protein G Sepharose, and the resulting supernatant was immunoprecipitated with the anti-FLAG rabbit antibody. The resins were recovered by centrifugation and washed with buffer, and the immune complexes were subjected to SDS–PAGE and analyzed by Western blotting.

Results and discussion

To evaluate signaling ability of SIGNR1, we first established RAW264.7 cells that stably expressed SIGNR1 tagged with a FLAG epitope at the C-terminal (RAW-SIGNR1 cells) to allow cross-linking of SIGNR1 molecules using an anti-FLAG antibody. The RAW-SIGNR1 cells exhibited significant cell surface expression of both SIGNR1 and FLAG epitopes (Fig. 1A). Activation of mitogen-activated protein kinases (MAPKs) is implicated in the inflammatory response [12] and cross-linking of DC-SIGN with a specific antibody induces activation of ERK in human monocyte-derived dendritic cells (DCs) [13]. Therefore, we first investigated activa-

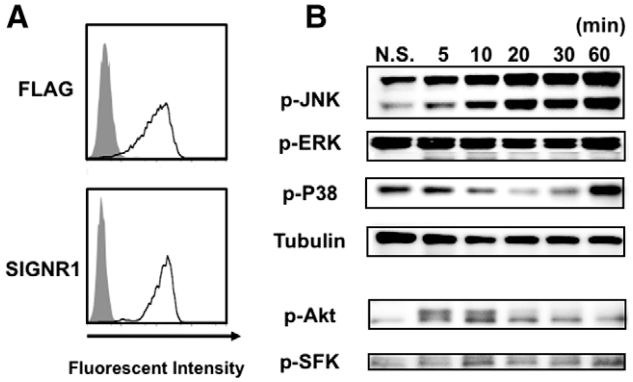

Fig. 1. SIGNR1-cross-linking leads to JNK phosphorylation. (A) Expression of SIGNR1 and FLAG epitopes on the surface of RAW-SIGNR1 cells. The cells were first treated with Fc-block for 30 min and then with biotin-labeled anti-SIGNR1 or anti-FLAG rabbit antibody. Expression of SIGNR1 and FLAG epitopes was visualized by treatment with FITC-labeled streptavidin or FITC-labeled anti-rabbit antibody. Open and shaded peaks indicate RAW-SIGNR1 cells and RAW-mock cells, respectively. (B) RAW-SIGNR1 cells were treated with anti-FLAG rabbit antibody on ice followed by treatment with an anti-rabbit Fc antibody at 37 °C for the indicated time (min). N.S. indicates no stimulation of cells. Total protein was isolated, separated by SDS–PAGE (10 μg/lane), and analyzed by immunoblotting for phospho-JNK (p-JNK), phospho-p38-MAPK (p-P38), phospho-ERK (p-ERK), phospho-Akt (p-Akt), or phospho-Src family kinases (p-SFK). Tubulin was used as a control for protein loading in the lanes.

tion of MAPKs by cross-linking of SIGNR1–FLAG molecules on the surface of RAW-SIGNR1 cells. Cross-linking of SIGNR1 molecules was performed by treatment of the cells with an anti-FLAG rabbit IgG followed by a secondary anti-rabbit Fc goat IgG. As shown in Fig. 1B, the level of phospho-JNK was significantly increased within 10 min after cross-linking of SIGNR1 on RAW-SIGNR1 cells, and gradually increased over 60 min. In contrast, the levels of phosphorylation of other MAPKs such as p38 and ERK did not change after treatment. Similar treatment of RAW-mock cells did not induce phosphorylation of any MAPKs (data not shown). We then examined phosphorylation of Akt and Src family kinases (SFKs) (Fig. 1B). The level of phospho-Akt in RAW-SIGNR1 cells was also increased by cross-linking of SIGNR1–FLAG molecules. SIGNR1-cross-linking also led to transient induction of phosphorylation of SFKs in RAW-SIGNR1 cells.

Lipid rafts are specialized areas in the plasma membrane that serve as membrane signaling platforms to facilitate outside-in signaling in association with signaling molecules such as kinases and adaptors [14,15]. To address whether SIGNR1 is localized in such specialized areas, RAW-SIGNR1 cells were lysed with 0.5% Brij 58-containing buffer and the resulting cell lysates were separated on sucrose density gradients. About half of the SIGNR1–FLAG molecules expressed in RAW-SIGNR1 cells were detected in detergent-insoluble fractions (fractions 4 and 5 in Fig. 2A), with the other half of these molecules detected in detergent-soluble fractions.

In macrophages and neutrophils, Hck, Fgr, and Lyn among the SFKs play a primary role in integrin-signaling pathways [16–18], and are often found in lipid rafts [18]. Therefore, to address whether SIGNR1 is associated with SFKs in lipid rafts in RAW-SIGNR1 cells, we examined the cellular distributions of these molecules (Fig. 2A). About half of the Lyn molecules and most Fgr were found in detergent-insoluble fractions. Some Hck also appeared in low-density fractions, but most was found in soluble fractions. These results suggest that SIGNR1 might co-localize with these signaling molecules in lipid rafts in the cells. To examine this association more closely, SIGNR1–FLAG molecules were immunoprecipitated using the anti-FLAG antibody. In fractions containing lipid rafts, Lyn, Fgr, and Hck were all co-immunoprecipitated with SIGNR1–FLAG with this antibody, but not with the isotype control, indicating that these tyrosine kinases are associated with SIGNR1 in lipid rafts (Fig. 2B).

When the membrane distribution of phosphorylated SFKs was examined 10 min after cross-linking of SIGNR1 with the antibodies on RAW-SIGNR1 cells, most phosphorylated SFKs were detected in detergent-insoluble fractions, whereas a high percentage of SFKs appeared in detergent-soluble fractions (Fig. 2C). Therefore, SFKs localized in lipid rafts from RAW-SIGNR1 cells were preferentially phosphorylated by the cross-linking, although SFKs and SIGNR1 also appeared in detergent-soluble fractions. These results suggest that only SIGNR1 molecules in specific membrane microdomains activate co-localized SFKs following the cross-linking.

To determine whether activation of SFKs or PI3 K by SIGNR1-cross-linking has regulatory effects on activation of JNK in RAW-SIGNR1 cells, the cells were pretreated with a series of specific inhibitors directed against the signaling proteins. They were then treated with an anti-FLAG antibody for 10 min to ligate SIGNR1 before assessment of JNK phosphorylation (Fig. 3A). The JNK inhibitor, but not the p38 inhibitor or the ERK inhibitor, inhibited JNK phosphorylation, as described previously [19]. Inhibition of SFKs by PP2 and inhibition of protein tyrosine kinases using genistein resulted in significant blocking of JNK phosphorylation, indicating a requirement for at least one Src-related tyrosine kinase, which might be associated with SIGNR1. In contrast, treatment of cells with a PI3 K inhibitor (LY294002) and a Syk inhibitor (piceatannol) did not affect JNK phosphorylation, suggesting that the PI3 K/Akt pathway and Syk pathway are not involved in activation of the JNK pathway in these cells.

Since MAPKs have been demonstrated to be involved in activation of a large array of pro- and anti-inflammatory cytokine genes [19,20], we next examined the release of cytokines from the cells. Cross-linking of SIGNR1 led to the production of significant levels of TNF-α from RAW-SIGNR1 cells (Fig. 3B). Production of TNF-α

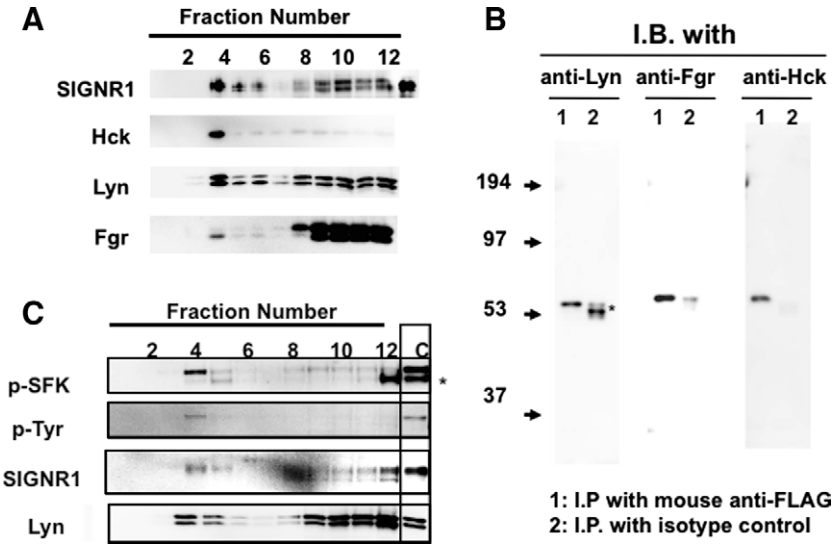

Fig. 2. SIGNR1 is localized in lipid raft fractions in the membrane of RAW-SIGNR1 cells and associate with Src family kinases. (A) RAW-SIGNR1 cells were harvested and homogenized on ice with 0.5% Brij 58-containing lysis buffer. The lysates were fractionated on a sucrose density gradient. Twelve fractions were harvested from the top of the gradient and numbered 1–12 from top to bottom. Aliquots (10 μl) of each fraction were analyzed by Western blotting using antibodies directed against SIGNR1, Lyn, Fgr, and Hck. (B) Detergent-insoluble fractions (fractions 4 and 5 in Fig. 2) were concentrated and incubated with an anti-FLAG antibody at 4 °C for overnight. The immune complexes were recovered by addition of protein G-Sepharose for 3 h at 4 °C (lane 1). As a control, a rabbit IgG was used instead of the anti-FLAG antibody (lane 2). The resin was recovered by centrifugation. Proteins bound to resin were separated by SDS–PAGE, transferred to a PVDF membrane, and analyzed by Western blotting using anti-Lyn, anti-Fgr, and anti-Hck antibodies. Asterisks indicate rabbit IgG. (C) Cells (1×10^7) were ligated with an anti-FLAG antibody (rabbit IgG) and an anti-rabbit Fc antibody. Ten minutes after the cross-linking, the cells were lysed in 0.5% Brij 58-containing lysis buffer, and the resulting cell lysates were fractionated on a sucrose density gradient. Aliquots (10 μl) of each fraction and the concentrated detergent-insoluble fractions (C) were analyzed by Western blotting using antibodies directed against phospho-SFKs, phosphotyrosine (4G10), SIGNR1, and Lyn, respectively. Asterisks indicate rabbit IgG.

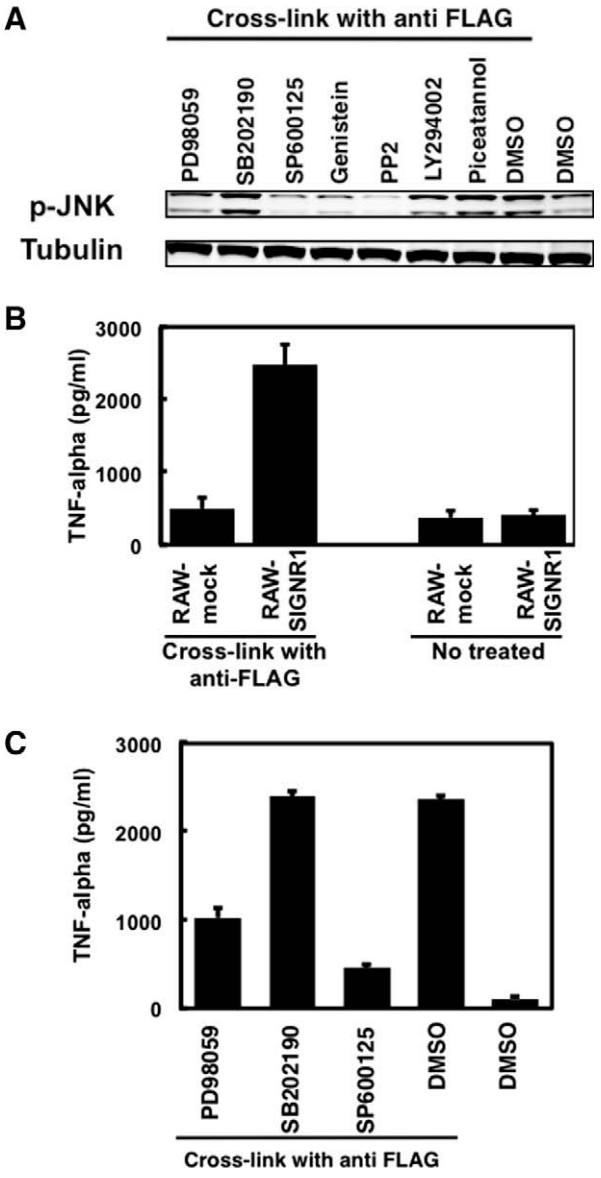

Fig. 3. Effects of signal transduction inhibitors on phosphorylation of JNK and production of TNF-α in SIGNR1-ligated RAW-SIGNR1 cells. (A) Fresh medium containing inhibitors of ERK (PD98059), p38-MAPK (SB202190), JNK (SP600125), PI3 K (LY294002), SFKs (PP2), Syk (piceatannol), or protein tyrosine kinases (genistein) was added to RAW-SIGNR1 cell cultures 30 min prior to cross-linking of SIGNR1. As a control, cells were treated with medium containing 0.1% DMSO. The cells were stimulated by cross-linking for 10 min, and total protein was isolated and analyzed by immunoblotting for phospho-JNK. Tubulin was used as a control for protein loading. (B) Production of TNF-α from RAW-SIGNR1 cells. RAW-SIGNR1 or RAW-mock cells (1×10^6) suspended in 400 μl of medium were treated with an anti-FLAG antibody followed by an anti-rabbit Fc antibody, incubated overnight at 37 °C, and TNF-α in the culture supernatants was quantified by ELISA. Each bar represents the mean ± SD for three experiments. (C) RAW-SIGNR1 cells were treated with inhibitors for MAPKs (PD98059, SB202190, and SP600125) for 30 min, treated with the antibodies to cross-link SIGNR1 molecules, and incubated overnight at 37 °C. TNF-α secreted in culture supernatants was analyzed by ELISA. Each bar represents the mean ± SD for three experiments.

from the cells in response to the cross-linking was inhibited strongly by SP600125 and weakly by PD98059 but not by SB203580 (Fig. 3C), suggesting that the antibody-induced activation of JNK pathway leads to production of TNF-α.

We have previously shown that OMLs are preferentially ingested into PEMs and that the OML-ingested macrophages mature to APCs that produce IL-12 [7]. In addition, we have shown that OMLs are recognized and captured in part by SIGNR1 expressed on PEMs [9]. Molecular structures containing terminal mannose play a role as PAMPs, and thus the characterization of the signaling route triggered by engagement of mannose receptors is an important issue. However, only a few studies have shown that members of the C-type lectin family participate in signal transduction events, leading either to cell activation or inhibition through interference with TLR-mediated signaling [1]. Recently, ligation of DC-SIGN, a mannose-binding C-type lectin expressed on DCs, by specific antibodies has been shown to induce preferential activation of the ERK pathway in human DCs [13], leading to evocation of Th2/tolerogenic responses [21]. Thus, the main aim in this study was to define whether SIGNR1 could directly activate a signaling pathway in macrophages and lead to subsequent cytokine production. Our results show that engagement of SIGNR1 molecules on SIGNR1-expressing murine macrophage-like cells by a specific antibody preferentially induces activation of one of the major MAPKs, JNK, without concomitant activation of p38 and ERK and that SIGNR1-mediate JNK activation leads to TNF-α production from the cells.

In the current study, we showed that SIGNR1 molecules expressed in transfectants are located partly in lipid rafts and are associated with SFKs such as Lyn, Fgr, and Hck in these rafts. Our results also revealed that cross-linking of SIGNR1 led to preferential activation of these SFKs in the lipid rafts, and that at least one of the SFKs associated with SIGNR1 acts as an upstream molecule in the JNK signaling pathway. Recently, JNK has been demonstrated to mediate production of TNF-α, IFN-γ, and IL-12 from macrophages and to be involved in maturation of APCs [19,22]. In addition, Lyn has been found to modulate maturation of murine DCs [23], and has been shown to positively regulate IL-12 production and been proposed to act as a negative regulator of Th2 immune responses in mice [24]. We have shown that OMLs captured in part by SIGNR1 expressed on PEMs induce maturation of these cells and production of IL-12, with subsequent induction of Th1 immune responses specific for OML-entrapped antigens [7–10]. Thus, our current results are consistent with our previous findings, although most were obtained using SIGNR1-transfected macrophage-like cells. Mice deficient in SIGNR1 exhibit increased susceptibility to *Streptococcus pneumoniae* [6], indicating that intercellular signaling initiated by SIGNR1 might result in effector functions that increase inflammatory or Th1 responses. Further studies using PEMs are required to determine the functional significance of SIGNR1 molecules, as well as SFKs and MAPKs, in induction of IL-12 production and cell maturation.

Acknowledgments

This work was supported by the Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN), and in part by a Grant-in-Aid for Special Research from The Promotion and Mutual Aid Corporation for Private Schools of Japan.

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