Cooperation of specific ICAM-3 grabbing nonintegrin-related 1 (SIGNR1) and complement receptor type 3 (CR3) in the uptake of oligomannose-coated liposomes by macrophages

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Introduction

Molecular structures containing terminal mannose are unusual components of mammalian tissues, but are abundant in the walls of a variety of microorganisms and are recognized as highly conserved molecular signatures of pathogens, so-called pathogen-associated molecular patterns. Lectin-like receptors on the surface of antigen-presenting cells (APCs) facilitate binding and endocytosis of ligands with a terminal sugar, such as mannose, fucose, and N-acetylgalcosamine, and are thought to participate in capture of pathogens (Avraméas et al. 1996; Turner 1996). This activity was first detected in the macrophage mannose receptor (MR, CD206), but candidates have since been expanded to the family of dendritic cell-specific ICAM-3-grabbing nonintegrins (DC-SIGN, CD209) (Geijtenbeek et al. 2000; Soilleux et al. 2000; Park et al. 2001), which are now well established as mannose-binding proteins (Mitchell et al. 2001). The mouse genome encodes several DC-SIGN homologs that are referred to as DC-SIGN (mDC-SIGN, SIGNR5) and SIGN-related-1 (SIGNR1) -2, -3, -4, -6, -7, and -8 (Park et al. 2001). Expression studies suggest that these molecules are differentially expressed in various tissues, indicating that they may have tissue-specific roles (Park et al. 2001). SIGNR1 is primarily expressed on a subset of macrophages in the spleen marginal zone and lymph node medulla and may be involved in the uptake of fluorescence-labeled dextran and capsular polysaccharides of Streptococcus pneumoniae (Park et al. 2001; Geijtenbeek et al. 2002). SIGNR1 expression has also been shown on resident peritoneal macrophages (PEMs), which are strategically located to play an important role in protection against bacterial infection through phagocytic action and production of proinflammatory cytokines (Takahara et al. 2004). SIGNR1 is also a major mannose receptor for fungi (Taylor et al. 2004), but little is known regarding the biological function of SIGN molecules.

In previous studies, we have demonstrated that liposomes coated with a neoglycolipid constructed from mannnotriose and dipalmitoylphosphatidylethanolamine (Man3-DPPE) are preferentially and rapidly taken up by PEMs following administration of Man3-DPPE-coated liposomes (hereafter referred to as oligomannose-coated liposomes, OMLs) into the peritoneal cavity (Ikehara et al. 2006; Ikehara and Kojima 2007). In addition, PEMs mature together with the upregulation of MHC class II and co-stimulatory molecules and produce IL-12 in response to the intraperitoneal uptake of OMLs (Takagi et al. 2007; Ikehara et al. 2008). Furthermore, intraperitoneal or subcutaneous administration of antigen-encasing OMLs induces antigen-specific cell-mediated immunity that is sufficient to reject tumors or parasites (Shimizu et al. 2007; Ikehara et al. 2008; Kojima et al. 2008), indicating that preferential carbohydrate-dependent recognition and incorporation of OMLs into PEMs are essential steps for induction of effective cellular immunity. Recently, we have shown that complement component C3 and complement...
receptor type 3 (CR3, CD11b/CD18) contribute to the effective uptake of OMLs by PEMs (Abe et al. 2008). However, the mannose-binding lectin-like receptors used by PEMs to ingest OMLs have not been identified. To investigate the possible role of SIGNR1 in the uptake of OMLs, we expressed this receptor in macrophage-like RAW 264.7 cells and compared their responses to OMLs with those of control RAW 264.7 cells, which do not normally express SIGNR1. We report here that SIGNR1-expressing RAW264.7 cells ingest OMLs through SIGNR1 in concert with complement receptor type 3 (CR3, CD11b/CD18). In addition, we also demonstrate the participation of SIGNR1 in OML uptake by PEMs in the peritoneal cavity.

Results

SIGNR1, but not mDC-SIGN and SIGNR3, participates in the uptake of OMLs

We have shown previously that multilamellar liposomes prepared from dipalmitoylphosphatidylcholine (DPPC), cholesterol, and Man3-DPPE at a molar ratio of 10:10:1 (OMLs) can specifically target APCs, deliver antigens in MHC class I pathways and stimulatory signals to APCs, and induce efficient anti-tumor immunity to reject a tumor, whereas OMLs containing a lower ratio of Man3-DPPE failed to reject the tumor (Takagi et al. 2007; Ikehara et al. 2008; Kojima et al. 2008). Therefore, OMLs consisting of DPPC:cholesterol:Man3-DPPE (10:10:1) were used in all experiments unless otherwise indicated. The molar ratio of DPPC, cholesterol, and Man3-DPPE in the OMLs was 1.00:1.03 ± 0.09:0.091 ± 0.012 (mean ± SD from five independent preparations).

CD11c+ dendritic cells (DCs) express SIGNR1, SIGNR3, and mDC-SIGN (Park et al. 2001), and PEMs express significant amounts of SIGNR1 and mDC-SIGN as major mannose receptors (Takahara et al. 2004; Taylor et al. 2004). Therefore, we examined whether these DC-SIGN-related lectins that are potentially expressed in APCs could be involved in capture of OMLs, using CHO cells transiently transfected with cDNA encoding mDC-SIGN, SIGNR1, and SIGNR3, which are tagged with a FLAG epitope at their C-termini. The results are summarized in Figure 1. Expression of these lectins on the cell surface of CHO cells was confirmed by staining with an anti-FLAG antibody. SIGNR1- and SIGNR3-transfected CHO cells ingested FITC-dextran during a 24 h incubation, whereas mDC-SIGN-transfected CHO cells failed to take up FITC-dextran, as reported previously (Takahara et al. 2004), indicating that SIGNR1 and SIGNR3 were expressed on the cell surface as functional molecules. When the cells were incubated with OMLs with encased FITC-BSA (FITC-OMLs), fluorescent signals from FITC-OMLs were detected in SIGNR1-transfected CHO cells during a 24 h incubation. In contrast, fluorescent signals from FITC-OMLs were not seen in mDC-SIGN- and SIGNR3-transfected CHO cells, or in mock-transfected cells. Fluorescent signals from FITC-BSA encased in liposomes without an oligomannose coating (bare liposome; BL) were not detected in cells transfected with any of the molecules (data not shown). Transfection of the constructs without a FLAG epitope tag led to the same results as those described above (data not shown). These results demonstrate that SIGNR1 may play a role in the uptake of OMLs by PEMs.
fluorescence intensity in RAW-SIGNR1 cells incubated with Alexa647-OMLs was also significantly higher than that with incubation of bare liposomes containing Alexa647-BSA. To show OML recognition and uptake of OMLs by RAW-SIGNR1 cells in a prolonged incubation period, the cells were incubated with FITC-OMLs for 1 h or 20 h. Fluorescent signals from FITC-OMLs were detected on the cell surface after 1 h and intracellular FITC signals were observed after 20 h (Figure 2C). No fluorescent signal was detected on the cell surface after 1 h and intracellular FITC signals were observed after 20 h. Fluorescent signals from FITC-OMLs were detected on the cell surface after a short incubation (1 h) and seen in intracellular regions after incubation for 20 h.

Contribution of complement to OML capture by SIGNR1-expressing macrophage-like cells

It has been reported that deposition of the complement C3 component on Streptococcus pneumoniae is initiated by SIGNR1 expressed on APCs by the activation of the classical complement pathway (Kang et al. 2006). In addition, C3 have been shown to contribute the uptake of OMLs by PEMs in peritoneal cavity (Abe et al. 2008). To clarify the contribution of complement to recognition and uptake of OMLs by RAW-SIGNR1 cells, we cultured RAW-SIGNR1 cells with or without 5% mouse serum. The fluorescent intensities of cells cultured with serum increased more rapidly than those cultured without serum (Figure 4A), indicating that OML capture by RAW-SIGNR1 cells was accelerated in the presence of serum. The acceleration of OML capture was not observed in the presence of heat-inactivated serum or serum obtained from C3−/− mice (Figure 4B), indicating that the complement C3 component is involved in capture of OML by SIGNR1-expressing macrophage-like cells under serum-containing conditions. In addition, the acceleration was reduced slightly (about 25%) when serum was pre-treated with mannos-agarose but unaffected in the presence of GalNAc-agarose-treated serum (Figure 4C), suggesting that OML capture by RAW-SIGNR1 cells was directed in part by serum mannos-binding protein under serum-containing conditions. On the other hand, capture of OMLs by RAW-SIGNR1 cells in the presence of serum was significantly (about 50%) inhibited by an ER-TR9 monoclonal antibody and completely abolished by EDTA (Figure 4D), similarly to cells in the absence of serum (Figure 3B). Therefore, SIGNR1 is also involved in recognition and uptake of OMLs even in the presence of serum. Inhibition of binding by mannan and by α-methyl-mannoside but not by galactose was clearly observed (Figure 3B), and inhibition by mannan was more effective than that by α-methyl-mannoside, indicating that multiple mannos residues might be recognized preferentially by SIGNR1. EDTA (10 mM) also inhibited binding almost completely (Figure 3B). Recognition of OMLs by RAW-SIGNR1 cells was significantly inhibited by mannose-agarose but unaffected in the presence of heat-inactivated serum or serum obtained from C3−/− mice (Figure 4B), indicating that OML capture by RA W-SIGNR1 cells is directed in part by SIGNR1 in the RAW-SIGNR1 cells. Inhibition of binding by mannan and by α-methyl-mannoside but not by galactose was clearly observed (Figure 3B), and inhibition by mannan was more effective than that by α-methyl-mannoside, indicating that multiple mannos residues might be recognized preferentially by SIGNR1. EDTA (10 mM) also inhibited binding almost completely (Figure 3B). Recognition of OMLs by PEMs was also significantly inhibited by ER-TR9, mannan, and EDTA (Figure 3C), but treatment with an anti-MR antibody (MR5D3, rat IgG) or an isotype control (rat IgG or IgM) did not affect the recognition. These results suggest that a specific interaction between mannos residues on OMLs and a carbohydrate-recognition domain (CRD) of SIGNR1 trigger OML recognition by PEMs as well as RAW-SIGNR1.
Complement receptor type 3 (CR3, CD11b/CD18) is also involved in OML uptake in RAW-SIGNR1 cells

To clarify whether complement receptor type 3 (CR3, CD11b/CD18), a receptor for C3-deposited particles, participates in OML capture, we next examined the effect of an anti-CR3 (anti-CD11b) antibody on OML capture by RAW-SIGNR1 cells in the presence of serum. Similarly to inhibition by ER-TR9, the anti-CR3 monoclonal antibody, M1/70, partially (about 40%) but significantly inhibited OML capture in the presence of serum (Figure 5A). Interestingly, OML capture by RAW-SIGNR1 cells in the presence of serum was almost completely inhibited by a combination of ER-TR9 and M1/70 (Figure 6A). The CR3 antibody, M1/70, also inhibited OML capture by RAW-SIGNR1 cells in the absence of serum (Figure 5B), indicating that nonopsonized OMLs are also recognized in part by CR3 expressed on RAW-SIGNR1 cells. Furthermore, a combination of ER-TR9 and M1/70 led to complete inhibition of OML capture in the absence of serum (Figure 5B). These results suggest that CR3 acts as an additional receptor for recognition and uptake of OMLs, and that CR3 and SIGNR1 cooperate in an additive way in the recognition of OMLs in RAW-SIGNR1 cells in either the absence or presence of serum.

SIGNR1 and CR3 contributes to capture of OMLs by resident peritoneal macrophages in the peritoneal cavity

To clarify whether SIGNR1 participates in recognition and uptake of OMLs by PEMs, ER-TR9 and M1/70 (5 µg) were injected into the peritoneal cavity 15 min before administration of Alexa647-OMLs, and then OML capture in the peritoneal cavity was examined 10 min after OML administration. As shown in Figure 6, ER-TR9 treatment led to reduction of OML capture by PEMs in the peritoneal cavity, while the isotype control (rat IgM) did not do so, indicating that SIGNR1 expressed on PEMs is involved in recognition and uptake of OMLs under physiological conditions. OML capture by PEMs was also reduced significantly by M1/70 and much more strongly by a combination of ER-TR9 and M1/70 than by M1/70 or ER-TR9 alone. OML capture by PEMs in the peritoneal cavity was not reduced by an anti-MR antibody (MR5D3, rat IgG) or an isotype control (rat IgG).
Fig. 4. Effect of serum on recognition and uptake of OMLs by RAW-SIGNR1 cells. (A) RAW-SIGNR1 cells were incubated with Alexa647-OMLs in the presence or absence of 5% mouse serum at 37 °C for the indicated times. OML uptake was evaluated by flow cytometry and was shown as histograms and mean fluorescent intensity (MFI). The results are representative of three separate experiments. (B) The cells were incubated with Alexa647-OMLs in the presence of 5% untreated mouse serum, heat-inactivated (65 °C, 30 min) mouse serum, or serum obtained from C3−/− mouse at 37 °C for 30 min, and then OML uptake was evaluated by flow cytometry. For the control, cells were treated without mouse serum. Each bar represents the mean ± SD of four independent experiments. *P < 0.05 compared to the MFI of control cells. (C) A RPMI 1640 medium (1 mL) was added to 100 µL of mouse serum, and then mannose-agarose (Man-Ag, 1 mL of resin) equilibrated with RPMI 1640 was added to the sample and incubated at 4 °C overnight with gentle rotation to deplete mannose-binding molecules from serum. For the control, serum was treated with GalNAc-agarose (GalNAc-Ag). After removing the resin by centrifugation, the supernatant was used as Man-Ag- or GalNAc-Ag-treated serum. Capture of OMLs by RAW-SIGNR1 cells was examined in the presence of untreated mouse serum, Man-Ag-treated serum, or GalNAc-Ag-treated serum as described above. Each bar represents the mean ± SD of three independent experiments. *P < 0.05 compared to the MFI of the cells treated with untreated serum. (D) Cells were treated with antibodies or other reagents at 4 °C for 30 min prior to the addition of OMLs with encased Alexa647-BSA (Alexa647-OMLs), as described in Figure 3. One hour after incubation with Alexa647-OMLs in a medium containing 5% mouse serum, cells were harvested, washed with the medium, and fixed with 2% paraformaldehyde. The uptake of liposomes was evaluated by flow cytometry and data are shown as the relative MFI of Alexa647. Each bar represents the mean ± SD of three independent experiments. *P < 0.05 compared to the MFI of untreated RA W-SIGNR1 cells.

Discussion

We have shown previously that OMLs are preferentially ingested into resident PEMs and that OML-containing PEMs mature to APCs that produce IL-12 (Takagi et al. 2007). However, the receptors that recognize and capture the OMLs remain obscure. In the current study, we demonstrated that SIGNR1, a C-type lectin related to DC-SIGN that is highly expressed on PEMs as a mannose receptor (Takahara et al. 2004; Taylor et al. 2004), participates in recognition and capture of OMLs with exposed mannobiose residues in a carbohydrate-dependent manner, using mouse macrophage-like RAW264.7 cells that stably express SIGNR1 (RAW-SIGNR1 cells). RAW-SIGNR1 cells recognized the OMLs, but control mock-transfected RAW-neo cells did not do so. Furthermore, OML capture by RAW-SIGNR1 cells as well as PEMs was inhibited by an anti-SIGNR1 mAb, EDTA, and mannose-containing glycoconjugates such as mannan, but not by other carbohydrates such as galactose. Finally, precipitation of SIGNR1 on PEMs was confirmed in capture of OMLs under physiological conditions. OML capture by PEMs in the peritoneal cavity was abolished by an anti-SIGNR1 mAb but not by an isotype-matched antibody. Since anti-MR antibody treatment did not affect OML recognition by RAW-SIGNR1 cells and by PEMs, even in the peritoneal cavity, the involvement of MR in OML capture by RAW-SIGNR1 cells and PEMs is unlikely.

Among the tested DC-SIGN-related membrane-bound C-type lectins (mDC-SIGN, SIGNR1 and SIGNR3), only SIGNR1 expressed on CHO cells was able to recognize OMLs. The carbohydrate ligands for SIGNR1 and SIGNR3 have been examined using recombinant soluble forms of the molecules and shown to be oligosaccharides with mannose and fucose termini (Galusstein et al. 2004; Koppel et al. 2005). In addition, both SIGNR1- and SIGNR3-transfected cells ingest dextran and zymosan (Takahara et al. 2004), suggesting that the SIGNR1 and SIGNR3 possess similar carbohydrate-binding properties. We also confirmed the uptake of dextran by both SIGNR1- and SIGNR3-transfected cells (Figure 1). However, our results for neoglycolipid-coated liposomes indicate that carbohydrate structures recognized by SIGNR1 differ from those recognized
In addition to SIGNR1, the complement receptor CR3 (CD11b/CD18) participates in recognition and/or uptake of OMLs in RAW-SIGNR1 cells, and CR3 and SIGNR1 may cooperate in an additive way in the recognition and uptake of OMLs by both RAW-SIGNR1 cells and PEMS, based on the following evidence: (i) OML capture by RAW-SIGNR1 cells was partially inhibited by an anti-CR3 (anti-CD11b) mAb, M1/70; (ii) RAW-neo cells, which express equivalent levels of CR3 compared to RAW-SIGNR1 cells, did not capture OMLs; (iii) treatment of RAW-SIGNR1 cells with a combination of an anti-SIGNR1 mAb and an anti-CR3 mAb almost completely abolished OML capture; and (iv) similar additive inhibitory effects of ER-TR9 and M1/70 were observed on OML capture by PEM in the peritoneal cavity. APCs such as macrophages and DCs express several types of C-type lectins that recognize mannose residues, including MR and SIGNR1, and these lectins are thought to participate in phagocytosis of bacteria, parasites, viruses, and mannose-exposed particles (Lanoue et al. 2004; Takahara et al. 2004). CR3 is also involved in phagocytosis of bacteria and parasites (Drevets and Campbell 1991; Ferguson et al. 2004), and phagocytosis is enhanced by a low concentration of nonimmune serum due to binding of complement protein C3 to the surface of the bacteria (Ferguson et al. 2004). Recently, it has been reported that C3 deposition on microorganisms is initiated by SIGNR1 interactions with polysaccharides on the microorganism and the complement C1 subcomponent, followed by the activation of the classical complement pathway (Kang et al. 2006). Therefore, acceleration of OML uptake and cooperative involvement of SIGNR1 and CR3 in the uptake of OMLs in the presence of serum may be explained by SIGNR1-triggered C3-deposition on OMLs. In fact, serum from C3\(^{-/-}\) mice did not lead to acceleration of uptake. Serum mannose-binding protein may also contribute to the acceleration of uptake via SIGNR1 and CR3 in the presence of serum, since the acceleration was significantly reduced in the presence of mannose-agarose-treated serum.

Cooperation of SIGNR1 and CR3 in recognition of OMLs by RAW-SIGNR1 cells was also seen under conditions without complement components (without serum). CR3 has been characterized as a β-glucan receptor of phagocytes that can bind to polysaccharides containing glucose, mannose, and \(N\)-acetylglucosamine via a lectin-like binding domain in the CD11b subunit (Ross et al. 1997) and functions as a nonopsonic receptor in macrophages (Thornton et al. 1996). In addition, an anti-CD11b mAb M1/70 has been shown to block the lectin-like activity of CD11b (Xia et al. 1999). Therefore, it is possible that CR3 expressed on RAW-SIGNR1 cells can recognize carbohydrate residues expressed on OMLs via a lectin-like binding domain of CR3. However, RAW-neo cells did not recognize or ingest OMLs under conditions with or without serum during the test period of 60 min, even though RAW-neo and RAW-SIGNR1 cells express equivalent surface levels of CR3 (Figure 2A). This indicates that CR3 expressed on RAW-neo cells does not serve as an effective receptor for both C3-opsonized and nonopsonized OMLs. A functional association of SIGNR1 with receptors such as TLR4 and dectin-1, a major β-glucan receptor on macrophages (Brown et al. 2002), has been reported in recognition of nonopsonized microorganisms (Taylor et al. 2004; Nagaoka et al. 2005). SIGNR1 is poorly phagocytic (Taylor et al. 2004) and additional receptors on professional phagocytes, such as CR3, might be required for rapid internalization of OMLs. Therefore, SIGNR1 may associate functionally with CR3
on SIGNR1-expressing cells to capture the mannose-exposed particles.

Human DC-SIGN is known to distribute into lipid rafts and associate with Lyn and Syk tyrosine kinases in human DCs (Caparrós et al. 2006), and CR3 is known to associate with several GPI-anchored receptors in human neutrophils (Zhou and Brown 1994), suggesting that CR3 may also be localized in lipid rafts to internalize nonopsonized microorganisms (Peyron et al. 2000). A recent study indicated that lactocyclceramide-enriched lipid rafts are essential for CR3-mediated neutrophil phagocytosis (Nakayama et al. 2008). Our previous report showing that PEMs mature to APCs in response to OML activation (Takagi et al. 2007) suggests that association of OMLs with cell surface receptors leads to the activation of signaling pathways required for maturation of macrophages. We have also recently shown that OMLs activate the phosphatidylinositol 3-kinase/Akt pathway through phosphorylation of Src family kinases to induce the activation of the mitogen-activated protein kinases in macrophage-like J774 cells (Kato et al. 2008). Thus, SIGNR1 may associate with nonreceptor-type tyrosine kinases in lipid rafts during OML recognition, and complexes of these molecules may facilitate subsequent OML uptake and signal transduction to induce an OML-related Th-1 type immune response.

In summary, we have shown in this study that SIGNR1 expressed on macrophage participates in recognition of OMLs. We also demonstrated an additive cooperative effect of CR3 in recognition of OMLs by SIGNR1. At this time, the direct association of SIGNR1 and CR3 in lipid rafts and the signaling triggering these receptors are unclear and further studies are in progress to address these questions. However, the cooperative recognition of OMLs by SIGNR1 and CR3 on macrophages suggests a more complicated regulation of pattern recognition systems in situ.

Material and methods
Cells, reagents, and antibodies
Chinese hamster ovary (CHO) cells and mouse macrophage-like RAW264.7 cells were maintained in DMEM containing 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. Cholesterol, DPPE, DPPC, and α-methylmannoside were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mannotriose (Man3) with the structure Manα1-6(Manα1-3)Man and lacto-N-tetraose (LNT; Galβ1-3GlcNAcβ1-3Galβ1-4Glc) were purchased from Funakoshi (Tokyo, Japan). Man3-DPPE and LNT-DPPE were prepared in our laboratory by conjugation of these oligosaccharides with DPPE (Ikeda et al. 2006; Shimizu et al. 2007). The structures and purities of the neoglycolipids were confirmed by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (Autoflex, Bruker Daltonics, Bremen, Germany) and high-performance thin-layer chromatography, respectively. The purities of Man3-DPPE and LNT-DPPE were at least 98% and 95%, respectively. Monoclonal antibodies (mAbs) directed against SIGNR1 (ER-TR9, rat IgM), MR (MR5D3, rat IgG2a), and CD11b (M1/70, rat IgG2b) were obtained from BMA Biomedicals (Augst, Switzerland), AbD Serotec (Oxford, UK), and Leinco Technologies (St Louis, MO), respectively. Rat IgG2b and rat IgM isotype controls were obtained from R&D Systems. A Phycoerythrin (PE)-labeled anti-F4/80 antibody was purchased from Caltag Laboratories (Burlingame, CA), and peridinin chlorophyll protein-cyanin 5.5 (PerCP-Cy5.5)-labeled anti-CD11b mAb (clone M1/70) and an Fc-block (anti-mouse CD16/32) antibody were purchased from BD PharMingen (San Diego, CA). Biotin-labeled ER-TR9 and FITC-labeled streptavidin were purchased from BMA Biomedicals and Sigma-Aldrich, respectively. AlexaFluor® 647-labeled BSA (Alexa647-BSA) was prepared using a protein-labeling kit (Invitrogen, Carlsbad, CA). Six- to 8-week-old female Balb/c mice were purchased from Japan SLC (Hamamatsu, Japan) and used 2 days after arrival at the animal facility at Tokai University. Mouse serum samples were prepared from female Balb/c mice. The serum from C3−/− mice was kindly provided by Dr. Naoaki Yokoyama (Obihiro University of Agriculture and Veterinary Medicine). To deplete mannose-binding molecules from serum, an RPMI 1640 medium (1 mL) was added to 100 µL of mouse serum, and then mannose-agarose or GalNac-agarose (1 mL of resin, Sigma-Aldrich) equilibrated with RPMI 1640 was added to the sample and incubated at 4°C overnight with gentle rotation. After removing the resin by centrifugation, the supernatant was used as mannose-binding molecule-depleted serum.

Vector construction and transient and stable expression of lectins
Total cellular RNA was extracted from the isolated PEMs using RNeasy (Qiagen Inc., Valencia, CA). The coding sequences of SIGNR1, SIGNR3, and mDC-SIGN (GenBank accession numbers AF373408, AF373409 and AF373411, respectively) were amplified by primary and nested PCRs with ExTaq™ DNA polymerase (Takara Bio Inc., Otsu, Japan) using a total RNA sample prepared from PEMs as template and the following primers (the underlined sequences indicate restriction sites): SIGNR1, 5′-GTC AAG GCC CAC CAT GAG TGA CTC CAC AGA-3′ (forward) and 5′-GAA GGA GCT GGC ACG AAA G-3′ (reverse) for the primary reaction, and 5′-CTC GAG GCC CAC CAT GAG TGA CTC CAC AGA-3′ (forward) and 5′-CAT GAA GGT ACC GAG GAT GAG GAG-3′ (reverse) for the nested reaction; mDC-SIGN, 5′-GCG ACT GAG AAG TGG CTG TGA A-3′ (forward) and 5′-AAA CTG GCA T AG CT A-3′ (reverse) for the primary reaction, and 5′-ACT GAG AGC TAG CTG TGA A-3′ (reverse) and 5′-AGC TAT GGT CTA CTT CTT GCT GC-3′ (reverse) for the nested reaction; dectin-1, 5′-ATT CAA GTG CTC TCT GTG GTC AA-3′ (reverse primer) for the primary reaction, and 5′-ACT GAG AGC TAG CTG TGA AAC ATG AG-3′ (forward) and 5′-AGC TAT GGT CTA CTT CTT GCT GC-3′ (reverse) for the nested reaction; dectin-1, 5′-ATT CAA GTG CTC TCT GTG CC-3′ (reverse) for the primary reaction, and 5′-CCT CGA GCC CTG TGA AGC AAT GAA A-3′ (forward) and 5′-ATT CAA GTG CTC TCT GTG CC-3′ (reverse) for the nested reaction; dectin-1, 5′-ATT CAA GTG CTC TCT GTG CC-3′ (reverse) for the primary reaction, and 5′-CCT CGA GCC CTG TGA AGC AAT GAA A-3′ (forward) and 5′-ATT CAA GTG CTC TCT GTG CC-3′ (reverse) for the nested reaction. To enable cell surface detection, a FLAG epitope tag was inserted at the 3′-end of the coding sequences. The resulting constructs with or without the C-terminal FLAG epitope tag were inserted into a pCMV expression vector (Clontech, Palo Alto, CA). Cloned cDNAs (1.0 µg) were transfected into CHO cells that had been seeded on a Lab-Tek® Chamber Slide
(8 chamber, Electron Microscopy Sciences, Hatfield, PA) using Lipofectamine 2000. Twenty-four hours after transfection, OMLs with encased FITC-BSA (FITC-OMLs) (10 µg/mL of cholesterol) or FITC-dextran (100 µg/mL) were added to the cultures and incubated for another 24 h. Cells were washed and fixed with 2% paraformaldehyde, and then fluorescent signals were detected using fluorescent microscopy (BZ-9000, Keyence, Osaka, Japan). For establishment of SIGNR1 stable transfectants, RAW264.7 cells were harvested and seeded at 1 × 10⁶ cells/35-mm dish. The cells were transfected with 4 µg of CMV-SIGNR1 or mock vector using Lipofectamine 2000 and selected in the presence of 1 mg/mL G418.

Preparation of liposomes
Liposomes were prepared as described previously (Shimizu et al. 2007; Kojima et al. 2008), with some modifications. Briefly, a chloroform-methanol (2:1, v/v) solution containing 1.5 µmol of DPPC, 1.5 µmol of cholesterol, and 0.15 µmol of neoglycolipid was added to a flask and evaporated to prepare a lipid film containing neoglycolipid. PBS (150 µL) containing 2 mg/mL of FITC-BSA or Alexa647-BSA was added to the dried lipid film and multilamellar vesicles were prepared by intense vortex dispersion. The vesicles were extruded 10 times through a 1 µm pore polycarbonate membrane (Nucleopore, Pleasanton, CA). Liposomes with entrapped antigens were separated from free soluble proteins by three rounds of washing in PBS with centrifugation (20,000 × g, 30 min at 4°C). Molar ratios of the lipid components of the liposomes were determined using HPLC (Shimizu et al. 2001).

Evaluation of recognition and uptake of OMLs by macrophages
Recognition and uptake of liposomes into macrophage-like RAW264.7 cells transfected with SIGNR1 or PEMs were evaluated by flow cytometry (FACSCalibur, BD Biosciences, San Diego, CA) and data analysis was conducted using FlowJo software (Tree Star, Inc., San Carlos, CA). The peritoneal cells (PECs) were harvested from peritoneal cavities of Balb/c mice by lavage using 5 mL of ice-cold PBS. OMLs (10 µg/mL of cholesterol) with encased Alexa647-BSA (Alexa647-OMLs) were added to 1 × 10⁶/mL of RAW-SIGNR1, RAW-neo cells, or peritoneal cells (PECs) suspended in a serum-free DMEM medium in a 1.5 mL siliconized tube (Sumitomo Bakelite Co. Ltd., Tokyo, Japan) and the cells were incubated at 37°C with gentle rotation. One hour after incubation, the RAW-SIGNR1 or RAW-neo cells were harvested, washed with the medium, and fixed with 2% paraformaldehyde. For detection of fluorescent signals by fluorescent microscopy, FITC-OMLs were used instead of Alexa647-OMLs. PECs were harvested after a one-hour incubation, washed twice with PBS, treated with Fc block to reduce nonspecific binding of immunoglobulins, and then stained with a PerCP-Cy5.5-labeled anti-CD11b mAb to detect PEMS. Fluorescent signals from Alexa647 in CD11b-positive cells were analyzed to show specific capture of OMLs by PEMS.

For inhibition of OML recognition by antibodies, cells were treated with 10 µg/mL of ER-TR9 (anti-SIGNR1, rat IgM), 10 µg/mL of M1/70 (anti-CD11b, rat IgG), 10 µg/mL of MR5D3 (anti-MR, rat IgG), or a combination of ER-TR9 and M1/70 for 30 min at 4°C. For the control, 10 µg/mL of isotype control antibodies (rat IgG or rat IgM) were used. For inhibition of OML recognition by glycoconjugates, mannose (100 µg/mL), α-methyl-mannoside (50 mM), or galactose (50 mM) was added to the culture and the cells were incubated for 30 min at 4°C. The cells were incubated with Alexa647-OMLs (10 µg/mL of cholesterol) for 60 min at 37°C and the fluorescence intensity of Alexa647-BSA in the cells was evaluated by flow cytometry.

For the evaluation of OML uptake by PEMs in the peritoneal cavity, OMLs (50 µg of cholesterol) with encased Alexa647-BSA were injected into the peritoneal cavity of Balb/c mice, and PECs were harvested 10 min after the injection. PECs were washed twice with PBS, treated with Fc block, and then stained with a PE-labeled anti-F4/80 mAb to detect PEMS (Abe et al. 2008). Fluorescent signals from Alexa647 in F4/80-positive cells were analyzed to show the specific uptake of OMLs in PEMs. For inhibition, antibodies (5 µg) against SIGNR1(ER-TR9) and CD11b (M1/70) were injected into the peritoneal cavity 15 min prior to administration of OMLs.

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Conflict of interest statement
None declared.

Abbreviations
APCs, antigen-presenting cells; CR, complement receptor; DC, dendritic cell; DC-SIGN, DC-specific ICAM-3-grabbing nonintegrin; DPPC, dipalmitoylphosphatidylcholine; DPPE, dipalmitoylphosphatidylethanolamine; LNT, lacto-N-tetraose; mAb, monoclonal antibody; Man3, mannotriose; Man3-nonintegrin; DPPC, dipalmitoylphosphatidylethanolamine; MR, mannose receptor (CD206); OML, oligomannose-coated liposome; PEM, peritoneal cell; PEM, peritoneal macrophage; SIGNR, SIGNR-related.

References