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Preferences for uptake of carbohydrate-coated liposomes by C-type lectin receptors as antigen-uptake receptors

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Abstract We evaluated the carbohydrate preferences of the C-type lectin receptors (CLRs) SIGNR1, SIGNR3, and Langerin as pathogen-uptake receptors based on uptake of liposomes consisting of cholesterol, DPPC, and various neoglycolipids at molar ratios of 10:10:1 and 10:7:4, respectively, using non-phagocytic CHO cells that express these receptors transiently. SIGNR1-expressing cells ingested liposomes coated with neoglycolipids with terminal mannose residues, such as Man2-, Man3-, and Man5-DPPE, and with a terminal *N*-acetylglucosamine. SIGNR1 mediated uptake of Man3-DPPE-coated liposomes most efficiently. Uptake of liposomes with lower neoglycolipid content by SIGNR3- or Langerin-expressing cells was slight or negligible, but uptake into these cells was detected for liposomes with higher neoglycolipid content. SIGNR1-expressing cells clearly ingested liposomes coated with Lewis X antigen, whereas SIGNR3- or Langerin-expressing cells barely ingested these liposomes, even at the higher neoglycolipid content. In contrast, SIGNR3 or Langerin, but not SIGNR1, mediated uptake of liposomes coated with blood group H antigen. These results indicate that CLRs with similar carbohydrate-recognition characteristics have distinct properties as pathogen-uptake receptors for carbohydrate-decorated particles.

Keywords Langerin · Liposome · Neoglycolipid · Phagocytosis · SIGNR1 · SIGNR3

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Abbreviations

APC	antigen-presenting cell
BNCP	biantennary N-linked core pentasaccharide
CLR	C-type lectin receptor
DC	dendritic cell
DPPE	dipalmitoylphosphatidylethanolamine
LNFP	lacto-N-fucopentaose
LNT	lacto-N-tetraose
Man3	mannotriose
Man3-DPPE	neoglycolipids constructed with mannotriose and dipalmitoylphosphatidylethanolamine
Man5	mannopentaose
SIGN	specific ICAM-3-grabbing nonintegrin
SIGNR	SIGN-related

Introduction

Recognition and capture of pathogens by antigen-presenting cells (APCs) are crucial in the induction of adaptive immunity. Dendritic cells (DCs) are located throughout the body and serve as professional APCs to capture and internalize invading pathogens. APCs have a specific set of pattern-recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) to identify pathogens [1]. Among PAMPs, carbohydrates are universal features presented on the surface of all living cells, including pathogens, making it likely that exposed carbohydrates are the first molecular patterns of pathogens to be sensed by immune cells. Therefore, C-type lectin receptors (CLRs), which recognize carbohydrates to mediate uptake of pathogens and subsequent antigen presentation, are particularly important among the PRRs [2, 3]. It is becoming clear that

these CLRs serve not only as pathogen uptake receptors but also as signaling molecules involved in induction of specific gene expression for activation and regulation of immune responses [3]. Triggering of CLRs induces signaling cascades that initiate or modulate specific cytokine responses for tailoring of T cell polarization to the pathogens.

CLRs with an EPN-motif in their carbohydrate recognition domain facilitate binding and uptake of ligands with terminal mannose, fucose and *N*-acetylglucosamine residues, and are thought to participate in capture of pathogens [4, 5]. This activity is focused on the family of DC-SIGNs (CD209) [6, 7] and Langerin [8, 9], since expression of these CLRs are restricted to professional APCs. Subsets of murine macrophages and dendritic cells (DCs) express SIGNR1 and/or SIGNR3 as mannose receptors [7, 10–12]. Langerhans cells, which are a subset of DCs and are resident in an immature state in the skin epidermis and mucosal epithelium, express Langerin as a mannose receptor [13]. Screening of glycan arrays with soluble recombinant CLRs has revealed that SIGNR1, SIGNR3 and Langerin recognize similar carbohydrates such as high-mannose containing structures and fucose containing Lewis antigens (Le^a, Le^x, Le^b, and Le^y) [9, 14, 15]. In a comparison of the functions of SIGNR1, SIGNR3, and Langerin, Takahara *et al.* demonstrated that only SIGNR1 participates in the capture of *Salmonella typhimurium* and *Escherichia coli*, although cells with any of the three CLRs capture yeast and yeast-like zymosan particles, indicating that CLRs with similar carbohydrate specificities have distinct carbohydrate recognition patterns for microbial uptake [10]. However, the detailed preferences of these CLRs in relation to uptake of carbohydrate-decorated particles such as pathogens remain to be elucidated.

We have shown that liposomes coated with a neoglycolipid constructed from mannotriose and dipalmitoylphosphatidylethanolamine (Man3-DPPE) are rapidly and preferentially taken up by resident peritoneal macrophages *in vivo* [16], and induce strong cellular immunity against antigens encased in liposomes to protect against protozoan infections, tumor progression, and allergy [17–20]. We have also shown that SIGNR1 expressed on resident peritoneal macrophages acts as a physiological receptor for Man3-DPPE-coated liposomes [21]. In addition, Man3-DPPE-coated liposomes are recognized and taken up by SIGNR-1-transfected Chinese hamster ovary (CHO) cells, but not by SIGNR3-transfected CHO cells [21], although both SIGNR1- and SIGNR3-transfected cells ingest dextran, a known ligand for SIGNR1 and SIGNR3 [10].

These contrasting results suggest that the preferences for uptake of carbohydrate-decorated particles by membrane-associated CLRs differ from the carbohydrate-recognition specificities of soluble recombinant CLRs. To understand the preferences of CLRs expressed on the cell surface as pathogen uptake receptors, in the present study we evaluated uptake of carbohydrate-decorated particles by CLRs using neoglycolipid-coated liposomes and CLR-transfected non-phagocytic CHO cells. Our results indicate that the preferences of membrane-associated CLRs as pathogen uptake receptors differ from those of CLRs as carbohydrate recognition molecules. In addition, SIGNR1 may be a more efficient pathogen uptake receptor than SIGNR3 or Langerin for mannose-decorated particles.

Materials and methods

Reagents and cells

CHO cells were maintained in RPMI1640 containing 10 % heat-inactivated fetal calf serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cholesterol, dipalmitoylphosphatidylethanolamine (DPPE), dipalmitoylphosphatidylcholine (DPPC), and bovine serum albumin conjugated with fluoroscein isothiocyanate (FITC-BSA) were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Mannobiose (Man2; Manα1-6Man), mannotriose (Man3; Manα1-6(Manα1-3)Man, mannopentaose (Man5; Manα1-6(Manα1-3)Manα1-6(Manα1-3)Man, biantennary N-linked core pentasaccharide (BNCP; GlcNAcβ1-3Manα1-6(GlcNAcβ1-3Manα1-3)Man), lacto-N-fucopentaose I (LNFP-I; Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc), lacto-N-fucopentaose III (LNFP-III; Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc) and lacto-N-tetraose (LNT; Galβ1-3GlcNAcβ1-3Galβ1-4Glc) were purchased from Funakoshi (Tokyo, Japan). A biotin-conjugated monoclonal anti-FLAG antibody and phycoerythrin (PE)-conjugated streptavidin were obtained from Rockland (Gilbertsville, PA) and BD PharMingen (San Diego, CA), respectively.

Preparation of neoglycolipids

Neoglycolipids (Man2-DPPE, Man3-DPPE, Man5-DPPE, LNFP-I-DPPE, LNFP-III-DPPE, BNCP-DPPE, and LNT-DPPE) were prepared in our laboratory by conjugation of the oligosaccharides with DPPE by reductive amination, as described previously [22] with several modifications. In brief, 20 μmol of the oligosaccharide and 70 μmol of DPPE were dissolved in 5 ml of chloroform/methanol/water (10:10:1, v/v/v) in a reaction vial and the mixture was sonicated at 37°C for 2 h. Then 300 μmol of sodium cyanoborohydride dissolved in methanol (1 ml) was added and the reaction mixture was kept at 80°C for 5 h. After removing the solvent, the residue was suspended in 10 ml of chloroform/methanol/water (4:50:50, v/v/v) and applied to a C18 column (Bond Elute, MEGA BE-C18, Varian, Harbor City, CA) equilibrated with the same solvent. The column

was washed with 50 ml of chloroform/methanol/water (4:50:50, v/v/v) and total lipids were eluted from the column with 30 ml of chloroform/methanol/water (10:10:3, v/v/v). Purification of the neoglycolipid from the total lipid sample was performed by high-performance liquid chromatography (HPLC) on a silica column (Wakosil 5SIL-120, 0.75×30 cm, Wako Pure Chemical, Tokyo, Japan) with a linear gradient of chloroform/methanol/water from 65:30:5 to 50:55:18. 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 (HPTLC) on silica gel 60 plates (Merck, Darmstadt, Germany), respectively. The purities of all neoglycolipids were at least 95 %. The purified neoglycolipid was quantified by determination of total phosphate and hexose. The neoglycolipids used in this paper are summarized in Fig. 1.

Preparation of neoglycolipid-coated liposomes

Neoglycolipid-coated liposomes consisting of cholesterol, DPPC, and a neoglycolipid at a molar ratio of 10:10:1 were prepared as described previously [23] with some modifications. Briefly, a chloroform-methanol (1:1, 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 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 FITC-BSA were separated from free soluble proteins by three rounds of washing in PBS with centrifugation (20,000×g, 30 min at 4 °C). Neoglycolipid-coated liposomes consisting of cholesterol, DPPC, and a neoglycolipid at a molar ratio of 10:7:4 were prepared from 1.5 μmol of cholesterol, 1.05 μmol of DPPC, and 0.6 μmol of neoglycolipid. The amounts of cholesterol and DPPE in each liposome preparation were determined using HPLC [24] and that of the neoglycolipid was determined by the phenol-sulfuric acid method using glucose as the standard. The concentration and fluorescent intensity of FITC-BSA entrapped in each liposome was determined by spectrofluorophotometry (RF-5300PC, Shimadzu, Kyoto, Japan) in the presence of 0.3 % (w/v) sodium dodecyl sulfate, using FITC-BSA as the standard. The particle sizes of the liposomes were determined by dynamic light scattering using a particle size analyzer (LB-550, Horiba, Kyoto, Japan).

Vector construction and transient and stable expression of lectins

Total cellular RNA was extracted from isolated peritoneal macrophages or dermal dendritic cells using RNeasy

Oligosaccharide-DPPE	Structure
Man2	Manα1→6 Man
Man3	Manα1→6 Man Manα1→3 Man
Man5	Manα1→6 Manα1→6 Man Manα1→3 Manα1→3 Man
BNCP	GlcNAcβ1→3Manα1→6 Man GlcNAcβ1→3Manα1→3 Man
LNFP-I (Blood group H)	Fucα1→2Galβ1→3GlcNAcβ1→3Galβ1→4Glc
LNFP-III (Lewis X)	Galβ1→4GlcNAcβ1→3Galβ1→4Glc Fucα1→3 GlcNAc
LNT	Galβ1→3GlcNAcβ1→3Galβ1→4Glc

Fig. 1 Schematic diagram of structures of the oligosaccharides in neoglycolipids used in this study. *Gal* D-galactose; *Glc* D-glucose; *Fuc* L-fucose; *Man* D-mannose; *GlcNAc N*-acetyl-D-glucosamine

(Qiagen Inc., Valencia, CA). The coding sequences of SIGNR1, SIGNR3, Langerin, and dectin-1 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 a template, as described previously [21]. 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 a C-terminal FLAG epitope tag were inserted into a pCMV expression vector (BD Biosciences Clontech, Palo Alto, CA).

Evaluation of liposome uptake

CHO cells (1×10^6) were transfected with cloned cDNAs encoding SIGNR1, SIGNR3, Langerin, or dectin-1 (1.0 μg) using Lipofectamine 2000 (Invitrogen) in a 1.5-ml siliconized tube. At 24 h after transfection, neoglycolipid-coated liposomes with encased FITC-BSA (10 μg of cholesterol) were added to the cultures and incubated for another 16 h with gentle rotation. For the positive control, FITC-labeled zymosan (Molecular Probes, 10 μg/ml) was added to the cultures and incubated for 16 h. After washing the cells with PBS containing 1 mM EDTA twice to eliminate liposomes bound to cell surfaces, the cells were stained with a biotin-conjugated anti-FLAG antibody followed by PE-conjugated streptavidin to detect the FLAG-tag on CLRs expressed on the cell surfaces. The cells were then fixed with 2 % paraformaldehyde and the fluorescent signals of FITC in the FLAG-tag-positive CHO cells were analyzed by flow cytometry using FACSCalibur (BD Bioscience) with data analysis using FlowJo software (Tree Star).

Results

Cells expressing SIGNR1 preferentially take up Man3-DPPE-coated liposomes

We first examined whether CLRs are involved in capture of neoglycolipid-coated liposomes. CHO cells were transfected with cDNAs encoding SIGNR1, SIGNR3 or Langerin tagged with a FLAG epitope at the C-terminus. At 24 h after transfection, FITC-labeled zymosan particles or liposomes (DPPC:cholesterol:Man3-DPPE, 10:10:1) with encased FITC-BSA were added to the culture medium and incubated overnight. Fluorescent signals were subsequently detected using fluorescent microscopy. Cells transfected with SIGNR1, SIGNR3, or Langerin all captured zymosan particles, indicating that these CLRs were expressed on the cell surface as functional molecules (Fig. 2a, upper panels). For cells incubated with Man3-DPPE-coated liposomes, fluorescent signals from encased FITC-BSA were detected in SIGNR1-transfected CHOs (Fig. 2a, lower panels), but were undetectable in SIGNR3- and Langerin-transfected CHO cells. Mock transfectants did not show fluorescent signals for cells incubated with zymosan or liposomes. Most of the FLAG epitope was distributed on the cell surface, but fluorescent signals from FITC-BSA were also detected in cytoplasm (Fig. 2b), indicating that the liposomes had been incorporated into the cells.

Liposome uptake by CLR-expressing CHO cells was evaluated quantitatively based on fluorescent intensity using flow cytometry after the cells were treated with a biotin-conjugated anti-FLAG antibody followed by PE-labeled streptavidin to determine CLR expression. To compare the liposome uptake mediated by three different CLRs quantitatively, the expression levels of SIGNR1, SIGNR3, and

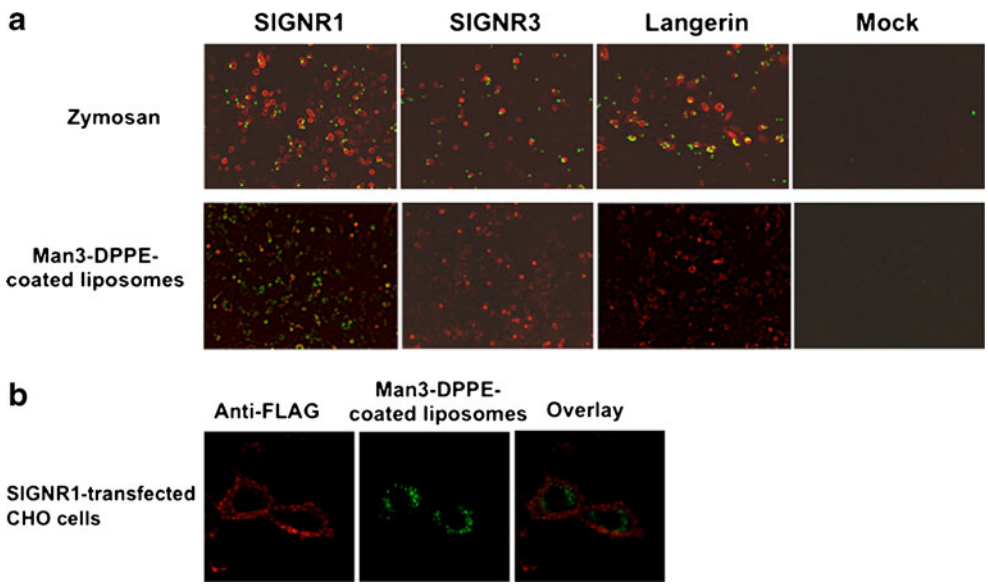

Fig. 2 Uptake of Man3-DPPE-coated liposomes into CLR-transfected CHO cells. CHO cells were transfected with cDNAs encoding SIGNR1, SIGNR3, or Langerin tagged with C-terminal FLAG. FITC-labeled zymosan particles (*upper panel*) or Man3-DPPE-coated liposomes with encased FITC-BSA (*lower panel*) were added to cell cultures at 24 h after transfection. CLR expression determined by an anti-FLAG antibody (*red*) and particle uptake (*green*) were detected by fluorescent microscopy

Langerin were evaluated after transfection of CHO cells with FLAG-tagged CLRs. About 15–20 % of the CHO cells expressed the FLAG epitope after transfection and the FLAG epitope-positive cells (R1 in Fig. 3) showed similar mean fluorescent intensities regardless of which CLR was transfected (Fig. 3 upper panel). In addition, ingestion of zymosan was observed in 89.1±10.1, 85.7±7.3, and 87.5± 8.9 % of SIGNR1, SIGNR3, and Langerin-expressing cells, respectively. Therefore, the CLR-expressing CHO cells expressed equivalent levels of functional CLRs on the cell surface.

Most SIGNR1-expressing cells (R1 in Fig. 3a) had fluorescent signals from Man3-DPPE-coated liposomes with encased FITC-BSA (Fig. 3a, lower panel). In contrast, a negligible signal was detected in SIGNR1-expressing cells after treatment with uncoated bare liposomes with encased FITC-BSA. No FITC-BSA signal was observed in mock transfected cells after treatment with Man3-DPPE-coated or uncoated liposomes (data not shown). Thus, uptake of neoglycolipid-coated liposomes was mediated by SIGNR1 expressed on CHO cells. In contrast, only about 30 % of SIGNR3-expressing cells and 15 % of Langerin-expressing cells showed fluorescent signals in similar experiments (Fig. 3b, c). In the following sections, we evaluated uptake of liposomes mediated by CLRs based on the percentage of FITC-positive cells among CLR-positive cells, as shown in Fig. 3.

Cells expressing SIGNR1 preferentially ingest Man3-DPPE-coated liposomes

Next, we examined the preference of SIGNR1-expressing cells for uptake of neoglycolipid-coated liposomes containing DPPC, cholesterol, and a neoglycolipid at a molar ratio of 10:10:1. The FITC-BSA concentrations in Man2-DPPE-, Man3-DPPE-, Man5-DPPE-, LNFP-I-DPPE-, LNFP-III-DPPE-, BNCP-DPPE-, and LNT-DPPE-coated and uncoated bare liposomes were 209, 210, 225, 189, 197, 201, 207, and 254 μg/mg of cholesterol, respectively. The mean (± SD) molar ratio of DPPC, cholesterol, and neoglycolipid in the

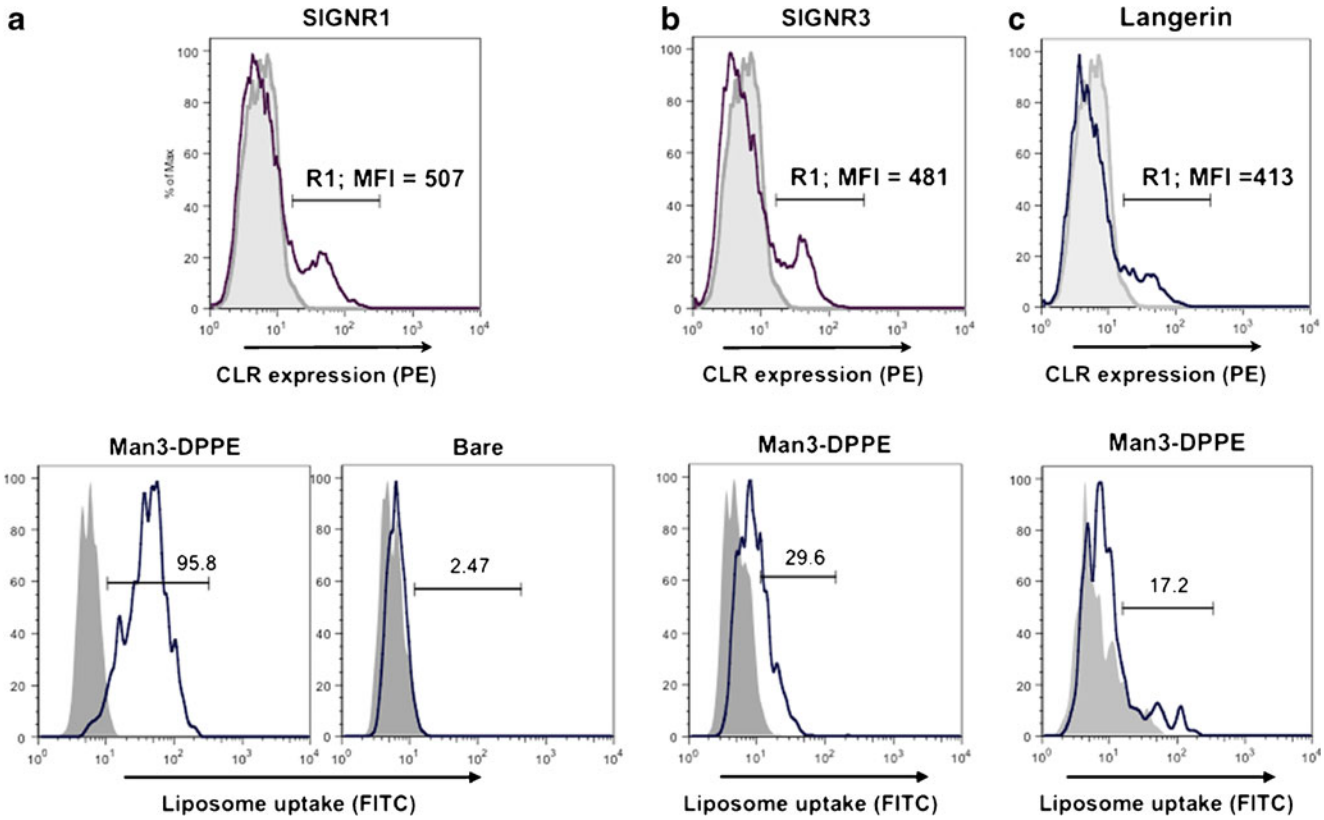

Fig. 3 Uptake of Man3-DPPE-coated liposomes by CHO cells expressing SIGNR1, SIGNR3 or Langerin. CHO cells were transfected with FLAG-tagged SIGNR1 (**a**), SIGNR3 (**b**) or Langerin (**c**) and incubated with FITC-labeled liposomes coated with Man3-DPPE (Man3-DPPE) or uncoated liposomes (Bare) for 18 h. The cells were then stained with a biotin-conjugated anti-FLAG antibody followed by PE-conjugated streptavidin, and expression levels of CLRs were evaluated by FACS (*upper panels*). White and gray peaks indicate SIGNR1-transfected and mock-transfected CHO cells, respectively. Each R1 region of the CLR-transfected cells was gated and the fluorescent signals from FITC-BSA encased in the liposomes in the gated cells were analyzed (*lower panels*). The mean fluorescent intensity (MFI) of cells in each R1 region is indicated in each upper panel. White and gray peaks indicate cells incubated with and without liposomes, respectively. The percentage of FITC-positive cells is shown in each panel

liposomes was 1.00: 1.09±0.21: 0.11±0.03. The median particle sizes of the liposomes ranged from 1065 to 1442 nm, with a mean of 1265 nm. These analytical data show that the neoglycolipid-coated liposomes had similar FITC-BSA levels (and thus similar potential fluorescent intensity), oligosaccharide surface densities, and particle sizes.

SIGNR1-expressing CHO cells ingested liposomes coated with Man2-, Man3-, and Man5-DPPE (Fig. 4a), with Man3-DPPE-coated liposomes having the highest uptake. Thus, uptake of mannose-decorated particles by these cells did not increase with a greater number of mannose residues, whereas binding of soluble SIGNR1 to mannose-containing neoglycolipids is correlated with the number of mannose residues [14].

Le^x (LNFP-III)-DPPE- and BNCP-DPPE-coated liposomes, but not blood group H (LNFP-I)-DPPE-coated liposomes, are ingested by SIGNR1-expressing cells

SIGNR1-expressing CHO cells ingested liposomes coated with LNFP-III-DPPE and BNCP-DPPE, which have a terminal fucose and *N*-acetylglucosamine, respectively (Fig. 4a). However, the uptake efficiency of these liposomes was lower than that of Man3-DPPE-coated liposomes and almost the same as Man5-DPPE-coated liposomes. Uptake of liposomes coated with a neoglycolipid containing LNT with a terminal galactose into SIGNR1-expressing cells was not observed. SIGNR1-expressing CHO cells were also unable to ingest liposomes coated with neoglycolipids containing LNFP-I (blood group H type oligosaccharide) with a terminal fucose. This result is consistent with the finding that recombinant soluble SIGNR1 does not bind to a neoglycolipid containing blood group H type oligosaccharide [14]. Collectively, our results show preferred uptake of Man3-DPPE-coated liposomes mediated by SIGNR1 as a pathogen-uptake receptor in CHO cells.

Preferences for uptake of neoglycolipid-coated liposomes by cells expressing SIGNR3 and langerin differ from those of cells expressing SIGNR1

SIGNR3-expressing cells took up Man2-, Man3-, and Man5-DPPE-coated liposomes (Fig. 4b), but the uptake efficiencies were significantly lower than those of SIGNR1-expressing cells. Uptake by SIGNR3-expressing cells also correlated with the number of mannose residues in the neoglycolipids; *i.e.*, Man5-DPPE-coated liposomes were ingested most effectively among the liposomes. In contrast to SIGNR1-expressing CHO cells, the cells expressing SIGNR3 did not exhibit clear uptake of liposomes coated with LNFP-III-DPPE and BNCP-DPPE (Fig. 4b). Thus, the uptake preferences of SIGNR3 for neoglycolipid-coated liposomes seem to differ from those of SIGNR1, although the oligosaccharide binding preferences of SIGNR3 are similar to those of SIGNR1 [10, 11]. Weak uptake of Man2-, Man3-, and Man5-DPPE-coated liposomes was seen in cells expressing dectin-1 (Fig. 4d). Since dectin-1 is a β-glucan receptor [25], the cells ingest these liposomes nonspecifically. Based on these results, Langerin-expressing cells did not exhibit clear uptake of any neoglycolipid-coated liposomes with a 10:10:1 molar ratio of DPPC, cholesterol, and neoglycolipid (Fig. 4c).

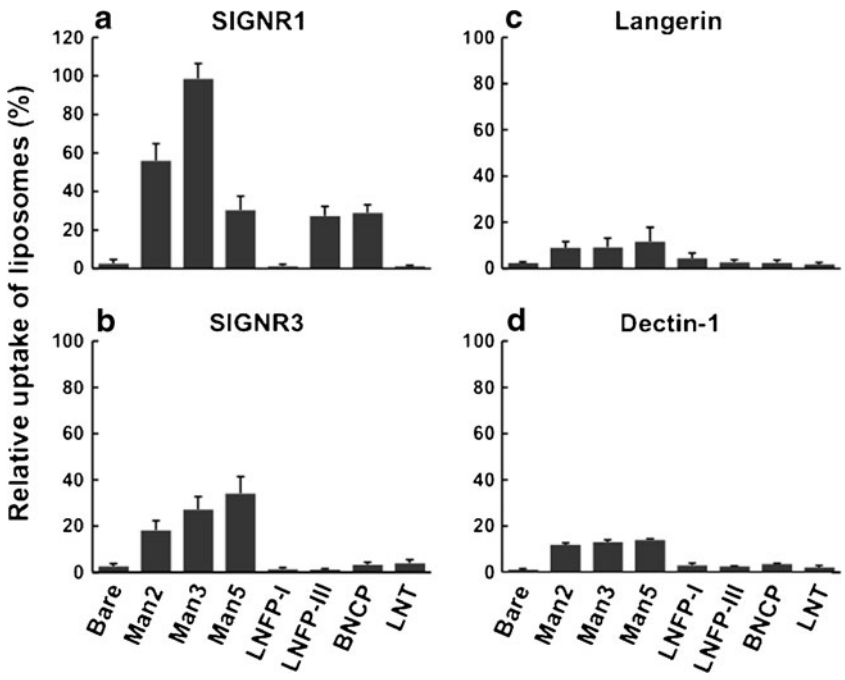

Fig. 4 Efficiency of uptake of liposomes by CLR-expressing CHO cells. FLAG-tagged SIGNR1 (**a**), SIGNR3 (**b**), Langerin (**c**), or dectin-1(**d**) transfected cells were incubated with FITC-BSA-containing liposomes consisting of DPPC, cholesterol, and various neoglycolipids (indicated on abscissa) at a molar ratio of 10:10:1 for 24 h. The cells were then stained with a biotin-conjugated anti-FLAG antibody followed by PE-conjugated streptavidin, and analyzed by FACS. The percentage of FITC-positive cells in PE-positive CLR-expressing cells is shown on the vertical axis. Each bar shows the mean ± SD for 5 independent experiments

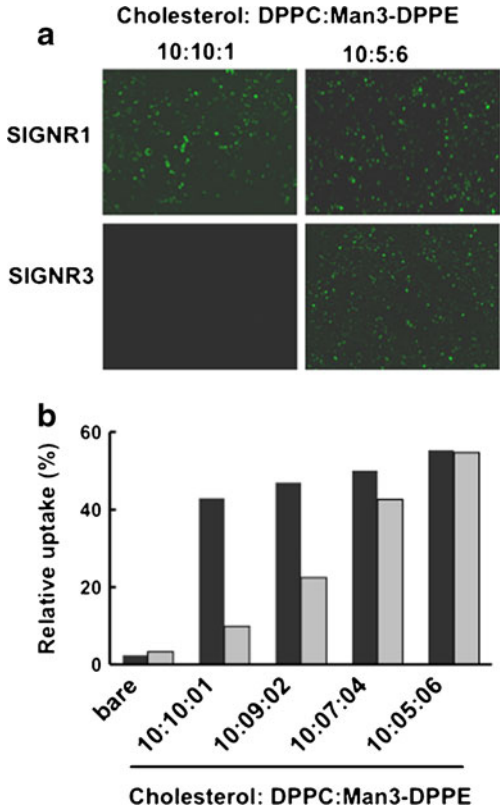

Fig. 5 Effect of neoglycolipid content on liposome uptake by CLR-expressing cells. **a** CHO cells transfected with FLAG-tagged SIGNR1 or SIGNR3 were incubated with liposomes consisting of cholesterol, DPPC, and Man3-DPPE at molar ratios of 10:10:1 and 10:5:6, respectively. Liposome uptake was detected by fluorescent microscopy. **b** Cells transfected with SIGNR1 (*black bars*) or SIGNR3 (*gray bars*) were incubated with liposomes containing different ratios of Man3-DPPE, as indicated in the figure, and the uptake of each liposome was analyzed as described in Fig. 2. Data are expressed as the mean percentage of 2 independent experiments

Uptake by cells expressing SIGNR3 requires a higher carbohydrate density on particles compared to uptake by cells expressing SIGNR1

Since it is accepted that multivalent carbohydrates are required for recognition by lectins [26], the relatively low or negligible liposome uptake by SIGNR3- and Langerin-expressing cells might be due to the low density of carbohydrates on the liposome surface. To examine this possibility, liposomes containing different amounts of neoglycolipids were prepared and their uptake into CLR-expressing cells was determined. Fluorescent signals from liposomes constructed from DPPC, cholesterol, and Man3-DPPE (10:10:1) were detected in SIGNR1-expressing cells, but not in SIGNR3-expressing cells. However, strong signals were detected in SIGNR3-expressing cells when liposomes consisting of DPPC, cholesterol, and Man3-DPPE (5:10:6) were used (Fig. 5a). Uptake of Man3-DPPE-coated liposomes into SIGNR3-expressing cells increased with an increased percentage of Man3-DPPE in the liposomes (Fig. 5b). These results suggest that SIGNR3 requires a higher carbohydrate density than SIGNR1 to act as a pathogen-uptake receptor.

Uptake of liposomes coated with a higher neoglycolipid density by CLR-expressing cells

The efficiency of uptake of neoglycolipid-coated liposomes consisting of cholesterol, DPPC, and neoglycolipid (10:7:4) was analyzed. The mean (±SD) molar ratios of DPPC, cholesterol, and neoglycolipid in these liposome preparations were 1.00: 0.66±0.12: 0.31±0.06, indicating a higher density of carbohydrates on the liposomes. As shown in Fig. 6a, SIGNR1-expressing cells ingested liposomes coated with the higher levels of Man2-, Man3-, and Man5-DPPE to an almost equal extent. Liposomes coated with higher levels of LNFP-III-DPPE and BNCP-DPPE were also taken up by

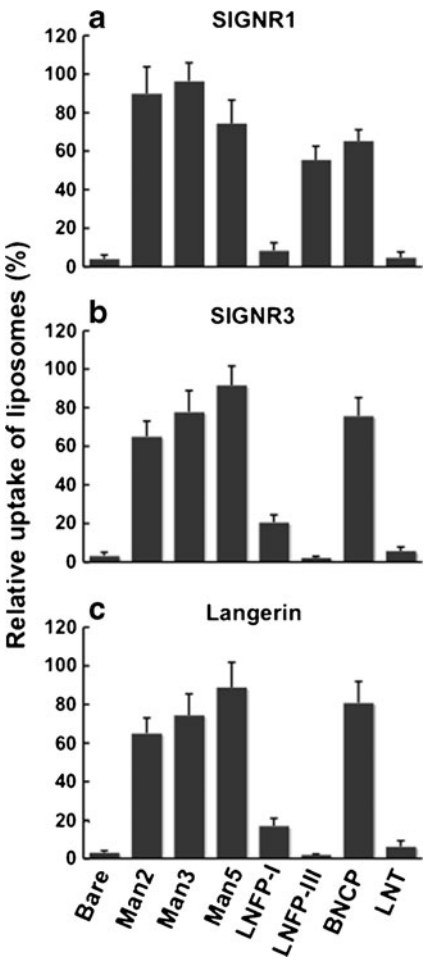

Fig. 6 CLR-mediated uptake of liposomes with a higher density of neoglycolipids. CHO cells transfected with FLAG-tagged SIGNR1 (**a**), SIGNR3 (**b**), and Langerin (**c**) were incubated with neoglycolipid-coated liposomes containing cholesterol, DPPC, and various neoglycolipids at a molar ratio of 10:7:4. Liposome uptake was analyzed as described in Fig. 2. Each bar shows the mean ± SD of 3 independent experiments

SIGNR1-expressing cells. However, these cells did not ingest liposomes coated with LNFP-I, even with the higher neoglycolipid level. The uptake selectivity of SIGNR1-expressing CHO cells for liposomes coated with a higher level of neoglycolipid decreased in comparison with that for liposomes with less neoglycolipid, but these cells still ingested Man3-DPPE-coated liposomes preferentially among the tested liposomes.

SIGNR3- and Langerin-expressing cells showed significant uptake of liposomes with higher levels of neoglycolipids (Fig. 6b,c), in contrast to their uptake of liposomes with lower neoglycolipid content (Fig. 4). The order of uptake by these cells was Man5-DPPE>Man3-DPPE>Man2-DPPE. Both cell types also ingested liposomes coated with a higher level of BNCP-DPPE, again in contrast to the lack of uptake with a lower neoglycolipid level (Fig. 4). Uptake of BNCP-DPPE-coated liposomes by SIGNR3- and Langerin-expressing cells was of the same order as that of mannose-coated liposomes. In contrast, LNFP-III-DPPE-coated liposomes were barely ingested by these cells, even with the higher neoglycolipid content (Fig. 6b,c), whereas uptake of these liposomes by SIGNR1-expressing cells was significant (Fig. 6a). SIGNR3- and Langerin-expressing cells showed weak, but clear, uptake of LNFP-I-DPPE-coated liposomes with an increased neoglycolipid content.

Discussion

In this study, we demonstrated distinctive uptake of carbohydrate-decorated particles mediated by membrane-associated SIGNR1, SIGNR3 and Langerin, which share an EPN-motif in their carbohydrate recognition domain that is postulated to recognize mannose residues. Our results using liposomes coated with neoglycolipids with definite carbohydrate structures revealed that the carbohydrate preferences of these CLRs for ingestion of liposomes differed from the carbohydrate binding preferences of soluble forms of the CLRs determined using glycan arrays [9, 14, 15]. In Table 1, the carbohydrate preferences of the membrane-associated CLRs examined in this study are compared to those of soluble forms determined in a previous report [14].

Murine SIGNR1, SIGNR3 and Langerin mediated uptake of liposomes coated with neoglycolipids containing terminal mannose residues (Man2-, Man3-, and Man5-DPPE) and acted as uptake receptors for oligosaccharides with terminal *N*-acetylglucosamine (BNCP-DPPE), which have also been shown to be ligands for human DC-SIGN-related lectins [27]. However, uptake *via* SIGNR3 and Langerin required a much higher carbohydrate density on the liposomes, compared to uptake *via* SIGNR1. This kind of multivalent effect for efficient carbohydrate recognition by lectins has been observed in binding of monomeric soluble form of DC-SIGN related CLRs to immobilized neoglycoproteins [28]. SIGNR1 contains a juxta-membrane extended neck region and a carbohydrate recognition domain in the extracellular domain [7], and SIGNR1 and human DC-SIGN form oligomers on the cell surface through the extended neck region [29–31]. In contrast, SIGNR3 and Langerin are present in a monomeric state on the cell surface because of their short neck regions [15]. Oligomerization of lectin domains has been shown to alter the affinity and specificity of carbohydrate recognition [32]. Thus, the more efficient uptake of liposomes with a lower density of carbohydrates by SIGNR1 may be due to oligomer formation on the cell surface.

Discrepancies between binding of soluble CLRs to solid phase carbohydrates and uptake of carbohydrate-decorated particles mediated by membrane-associated CLRs have been shown for mannose- and fucose-containing

Table 1 Semi-quantitative evaluation of carbohydrate specificities of membrane-associated and soluble CLRs

Carbohydrates	Membrane-associated form[1)]			Soluble form[2)]		
	SIGNR1	SIGNR3	Langerin	SIGNR1	SIGNR3	Langerin
Man2	++++	+++	+++	ND[4)]	ND	ND
Man3	+++++	++++	+++	+	–	–
Man5	+++	+++++	++++	++	+	–
Lex (LNFP-III)	+++	+/–	+/–	++	+	+/–
H (LNFP-I)	+/–	+	+	–	–	+/–
Terminal GlcNAc[3)]	+++	++++	++++	+	–	+/–
LNT	–	–	–	–	–	–

[1)] Semi-quantitative scores based on results shown in Fig. 6

[2)] Semi-quantitative scores based on results from Ref. 14

[3)] BNCP and chitotriose were used as oligosaccharides with a terminal GlcNAc as membrane-associated and soluble CLRs, respectively

[4)] ND indicates not determined

oligosaccharides (Table 1). Membrane-associated SIGNR1 mediated uptake of liposomes coated with Man3-DPPE is more effectively than those coated with Man2-DPPE or Man5-DPPE, particularly with a low carbohydrate density on the liposome surface. In contrast, the strength of recognition of mannose-containing neoglycolipids by soluble forms of SIGNR1 is clearly correlated with the number of mannose residues [14]. Previous studies have shown that soluble forms of SIGNR3 and Langerin, as well as SIGNR1, can bind to the Lewis X oligosaccharide [9, 14, 15]. However, the results presented here indicated that membrane-associated SIGNR3 and Langerin barely mediate uptake of liposomes coated with a Lewis X oligosaccharide (LNFP-III-DPPE-coated liposomes), even at a high carbohydrate density, while significant uptake of these liposomes was mediated by SIGNR1 at lower carbohydrate density (Table 1). In contrast, membrane-associated SIGNR3 and Langerin, but not SIGNR1, mediated uptake of liposomes coated with the blood group H oligosaccharide, although soluble forms of these CLRs did not bind to this oligosaccharide (Table1).

These discrepancies may be explained by functional differences between the soluble forms of CLRs and the membrane-associated forms of the receptors. For example, interaction of soluble forms of SIGNR1 and SIGNR3 with dextran cannot be detected, while the membrane-associated forms of these CLRs mediate dextran binding and uptake [11, 14]. Oligomer formation of SIGNR1 on the cell surface may explain the different carbohydrate preference of the membrane-associated protein compared to the soluble protein [32]. Since lectins recognize the same carbohydrates on different carriers in distinct manners [33, 34], differences in presentation patterns of carbohydrates on the lipid bilayer and in the solid phase may also affect recognition.

It is noteworthy that the uptake selectivity of SIGNR1 for liposomes coated with a lower density of neoglycolipids was greater than that for liposomes with a higher neoglycolipid density. Since the densities of carbohydrates and particle sizes of neoglycolipid-coated liposomes can be strictly controlled, it may be possible to design ligand-decorated particles that are preferred by individual lectin receptors using neoglycolipid-coated liposomes, even if the carbohydrate binding specificities of these receptors overlap. The neoglycolipids can be easily constructed from oligosaccharides and DPPE, and a wide variety of neoglycolipid-coated liposomes with different densities of carbohydrates can be prepared simply using a neoglycolipid library. Liposomes coated with neoglycolipids with definite carbohydrate structures may be useful for functional analysis of lectin receptors such as CLRs and sialic acid binding Ig-like lectins (siglecs). Neoglycolipid-coated liposomes with high affinity for a particular lectin receptor may also be useful as ligands for targeting to particular types of cells [35, 36], because these receptors exhibit restricted expression on a few cell types, and CLRs restricted to DCs have been evaluated as potential targets in development of DC-based vaccines [37]. Investigation of cellular responses toward these neoglycolipid-coated liposomes will be required for application of the liposomes in cell-targeted therapies. Carbohydrate-decorated particles with definite carbohydrate structures and particle sizes might also be useful for investigation of CLR-mediated signaling to understand plasticity in tailoring adaptive immunity to pathogens, since distinct DC-SIGN signaling pathways are induced by mannose- and fucose-expressing pathogens such as HIV-1 and *Helicobacter pylori* [38, 39], and *Neisseria meningitidis* can induce Th1 differentiation targeted by DC-SIGN through β1-6-*N*-acetylglucosamine structures on the bacteria [40].

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