

# Functional comparison of the mouse DC-SIGN, SIGNR1, SIGNR3 and Langerin, C-type lectins

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## Abstract

The mouse (m) DC-SIGN family consists of several homologous type II transmembrane proteins located in close proximity on chromosome 8 and having a single carboxyl terminal carbohydrate recognition domain. We first used transfected non-macrophage cell lines to compare the polysaccharide and microbial uptake capacities of three of these lectins—DC-SIGN, SIGNR1 and SIGNR3—to another homologue mLangerin. Each molecule shares a potential mannose-recognition EPN-motif in its carbohydrate recognition domain. Using an anti-Tag antibody to follow Tag-labeled transfectants, we found that each molecule could be internalized, although the rates differed. However, mDC-SIGN was unable to take up FITC-dextran, FITC-ovalbumin, zymosan or heat-killed *Candida albicans*. The other three lectins showed distinct carbohydrate recognition properties, assessed by blocking FITC-dextran uptake at 37°C and by mannan binding activity at 4°C. Furthermore, only SIGNR1 was efficient in mediating the capture by transfected cells of Gram-negative bacteria, such as *Escherichia coli* and *Salmonella typhimurium*, while none of the lectins tested were competent to capture Gram-positive bacteria, *Staphylococcus aureus*. Interestingly, transfectants with SIGNR1 lacking the cytoplasmic domain were capable of binding FITC-zymosan in a manner that was abolished by EDTA or mannan, but not laminarin. In addition, resident peritoneal CD11b<sup>+</sup> cells expressing SIGNR1 bound zymosan at 4°C in concert with a laminarin-sensitive receptor. Therefore these homologous C-type lectins have distinct recognition patterns for microbes despite similarities in the carbohydrate recognition domains.

## Introduction

C-type lectins are expressed in most cell types including macrophages and dendritic cells (DCs), which must internalize various glycoproteins and microbes for the purposes of clearance and antigen presentation to T lymphocytes (1). Human (h) DC-SIGN (CD209) is of special interest because it is involved in the interaction of certain DCs with several different pathogens including several viruses [HIV-1 (2), HCV (3), dengue virus (4,5), CMV (6), ebola virus (7)] and other microbes [mycobacteria (8,9), Leishmania (10) and candida

species (11)]. This type II transmembrane protein has a single C-type lectin domain and is expressed on immature monocyte-derived DCs.

Previously, we have cloned five mouse hDC-SIGN homologues that form a gene cluster in an ~210 kb region on chromosome 8 (12). Only one of these named mDC-SIGN was expressed in a restricted fashion in DCs, while the others had a different tissue distribution and were termed SIGNR1, SIGNR2, SIGNR3 and SIGNR4 for SIGN-related proteins.

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Despite similarities in the carbohydrate recognition domains (CRDs) of the mouse homologues to hDC-SIGN, the membrane proximal neck domains were much shorter.

It will be important to begin to identify the natural ligands for this new family of C-type lectins. We and Geijtenbeek *et al.* have recently reported that SIGNR1 is primarily expressed on a subset of macrophages in spleen marginal zone and lymph node medulla, and that SIGNR1 is involved in the uptake of fluorescence-labeled dextran (FITC-dextran) and capsular polysaccharides of *Streptococcus pneumoniae* (13–15). SIGNR1 dependent uptake was observed for both splenic marginal zone macrophages *in vivo* and by cell lines transfected with the SIGNR1 gene. Except for SIGNR4, the mouse DC-SIGN family has an EPN motif that is postulated to recognize mannose residues (16,17) (SIGNR4 contains a QPN motif).

We now report on the recognition of polysaccharides and microbes as well as endocytic capabilities of mDC-SIGN and SIGNRs using transfected non-macrophage cell lines, so that we could distinguish the function of the SIGN family from the many other C-type lectins expressed by macrophages and DCs. We were also interested in comparing mDC-SIGN, SIGNR1 and SIGNR3 with mLangerin, another homologous C-type lectin that has an EPN motif in its CRD but is expressed on a distinct subset of DCs (12,18). We further show that SIGNR1 is expressed by resident peritoneal CD11b<sup>+</sup> cells and recognizes zymosan in cooperation with laminarin-sensitive receptor. Our results reveal differences in polysaccharide recognition, endocytic capacities and microbe capture among these lectins.

## Methods

### *Cells and cultures*

Human embryonic kidney HEK293T cells, African green monkey kidney COS-7 cells, mouse fibroblast NIH3T3 cells and Chinese hamster ovary CHO cells were maintained in DMEM containing 10% FCS, 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. For CHO transfectants, 1 mg/ml of G418 (Sigma-Aldrich, Irvine, CA) was added to the medium.

### *Vector constructions and transient expression of lectins*

The coding sequences of mDC-SIGN, SIGNR1, SIGNR3 (12) and mLangerin (18,19) were amplified with KlenTaq DNA polymerase (Sigma-Aldrich), and then cloned into pEF6/V5-His (Invitrogen, Carlsbad, CA) and into pcDNA4/HisMax (Invitrogen) to add V5 and Xpress Tag sequence at C-terminus and N-terminus, respectively, according to the manufacturer's protocol. Cloned cDNAs were transfected with Lipofectamine 2000 reagent (Invitrogen) into HEK293T and COS-7 or with TransFast transfection reagent (Promega, Madison, WI) into NIH3T3. Following 24 h culture, cells were collected, seeded either into tissue culture plates or onto poly L-lysine (Sigma-Aldrich)-coated cover slips placed in culture vessels at an appropriate cell density, and cultured for another 24 h. Transfection efficiencies were over 80% for HEK293T cells, 25–30% for NIH3T3 cells and 35–45% for COS-7 cells, as assessed by staining with anti-Tag mAbs.

### *Kinetic and microscopic analyses of internalization of C-type lectins*

Single cell suspensions of HEK293T cell transfectants were incubated with 1 µg/ml of anti-V5 monoclonal antibody (mAb) (mouse IgG2a; Invitrogen) for 30 min on ice, washed with cold PBS and then incubated for various periods at 37°C to allow internalization. At the end of the incubation, the cells were stained with biotin-donkey F(ab')<sub>2</sub> anti-mouse IgG antibody (Jackson Immuno Research, West Grove, PA) and phycoerythrin-labeled (PE-) streptavidin (Jackson Immuno Research), and then acquired with a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA). The relative amounts of remaining Ab on the cell surface were analyzed with Flowjo (Tree Star, Inc., San Carlos, CA) or CELLQuest (Becton Dickinson). In order to facilitate internalization, transfectants were first labeled with anti-V5 mAb and biotin-donkey F(ab')<sub>2</sub> anti-mouse IgG antibody before the incubation at 37°C. Cells incubated at 4°C served as controls.

To detect the internalized lectins by immunostaining, NIH3T3 transfectants on cover slips were first treated with 1 µg/ml of anti-V5 antibody at 4°C for 30 min. After extensive washes, the cells were incubated at 37°C for 60 min, fixed with ice-cold 3.7% formalin in phosphate-buffered saline (PBS) followed by permeabilization by 0.01% saponin-PBS supplemented with 1% FCS. Then, cells were stained with biotin-donkey F(ab')<sub>2</sub> anti-mouse IgG antibody + Texas Red-streptavidin (Dupont NEN, Boston, MA) in combination with rat anti-transferrin receptor (TIB219) or LAMP-2 (ABL-93) mAb + FITC-labeled donkey F(ab')<sub>2</sub> anti-rat IgG (Jackson Immuno Research) antibody. After mounting with 1% propyl gallate (Wako, Osaka, Japan) in 50% glycerol, internalization of lectins was analyzed with a deconvolution microscope (BX51-FL: Olympus, Tokyo, Japan) using imaging software (Openlab: Improvision, Coventry, UK). Images were stored digitally and exported to Adobe Photoshop 7.0 for reproduction.

### *Capture of FITC-dextran and FITC-ovalbumin (OVA) by transfectants*

HEK293T cell transfectants expressing lectins with N-terminal Xpress Tag were cultured in the presence of neutral FITC-dextran (α-1,6-glucan) with molecular weight of 4.3 (FD-4), 40 (FD-40S), 250 (FD-250S), 500 (FD-500S) or 2000 kDa (FD-2000S) (all from Sigma-Aldrich) at 16 µg/ml for 1 h or 1.6 µg/ml for 12 h. FITC-OVA (Sigma-Aldrich) containing mainly (Man)<sub>n</sub>GlcNAcGlcNAc-Asn was also added to the culture at 12.5 µg/ml for 12 h. Controls were HEK293T cells transfected with pcDNA4/HisMax. After washing with PBS containing 5 mM EDTA to detach surface bound materials, cells were analyzed with flow cytometry. For inhibition assays, transfectants were incubated with graded doses of β-glucan (from barley: G-6513), mannan (from *Saccharomyces cerevisiae*: M-7504), N-acetylglucosamine (GlcNAc: A-8625), N-acetylgalactosamine (GalNAc: A-2795) or Fucose (F-2252) (all from Sigma-Aldrich) for 15 min at room temperature prior to the addition of 16 µg/ml of FITC-dextran (molecular weight 2000 kDa) for 1 h at 37°C. Blocking efficacies were indicated as percent inhibition according to the formula: (% FITC<sup>+</sup> cells in the control group without blocking agents – that in

experimental groups) / that in control group  $\times 100$ . To examine affinity of lectin molecules for 2000 kDa FITC-dextran, the transfectants were preincubated with graded doses of mannan for 20 min at room temperature prior to the addition of 16  $\mu\text{g/ml}$  FITC-dextran and then cultured for 1 h at 37°C.

#### *Binding assay using mannan agarose*

Binding of the lectins to mannan agarose was tested as described previously (18). Briefly, cell lysates in lysis buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 25 mM  $\text{CaCl}_2$ , 0.5% Triton X-100, 1 mM PMSF and 20  $\mu\text{g/ml}$  aprotinin) were prepared from HEK293T cells transiently transfected with the pcDNA4/HisMax constructs and then incubated with mannan agarose beads (Sigma-Aldrich: M9917) for 12 h at 4°C. Then, the agarose beads were washed with binding buffer (lysis buffer including 0.05% Triton X-100) five times with or without 25 mM EDTA. Proteins bound to the agarose beads were separated on 12% SDS-PAGE under denaturing conditions and transferred to Immobilon-P membrane (Millipore, Bedford, MA). The membrane was probed with HRP-labeled anti-Xpress antibody (Invitrogen) for Langerin, mDC-SIGN and SIGNR3 or rabbit anti-SIGNR1 Ab (13) followed by HRP-anti-rabbit IgG, then visualized by LumiGLO chemiluminescent substrate (Cell Signaling Technology, Beverly, MA) according to the manufacturer's protocols.

#### *Uptake of microbes including zymosan*

COS-7 transfectants with pcDNA4/HisMax constructs on cover slips were cultured with Texas Red-zymosan A (four particles/cell; Molecular Probes, Eugene, OR) or Texas Red-*E. coli* (20 particles/cell; Molecular Probes) for 12 h in  $\text{CO}_2$  incubator. *Salmonella typhimurium* (strain GIFU5001) obtained from Dr M. Mitsuyama (Kyoto University) was labeled with FITC after heat treatment at 60°C for 30 min as described (20), and added to the cell culture at 20 particles/cell for 6 h. *Candida albicans* at the yeast stage was killed at 80°C for 30 min and added to the cell culture at two particles/cell for 4 h. Bound and internalized *C. albicans* was visualized by staining with FITC-anti-*C. albicans* antibody (Biogenesis, Kingston, NH). Following extensive washes, cells were fixed-permeabilized and stained with anti-Xpress mAb (mouse IgG1; Invitrogen) followed sequentially by biotin-donkey F(ab')<sub>2</sub> anti-mouse IgG antibody (Jackson Immuno Research) and FITC- or Texas Red-streptavidin to visualize transfectants expressing lectins. After mounting with 1% propyl gallate in 50% glycerol, cells were analyzed with fluorescence microscopy. In order to determine the internalization of FITC-zymosan A (Molecular Probes), cells capturing zymosan were treated with 0.05% trypan blue with 13 mM potassium dihydrogen citrate in saline at pH 4.5 for a few minutes before observations by fluorescence microscopy (21). Inhibition assays were performed by preincubating COS-7 transfectants with  $\beta$ -glucan, 2000 kDa dextran (both at 100  $\mu\text{g/ml}$ ) or mannan (at 100, 500 or 1000  $\mu\text{g/ml}$ ) for 15 min at 4°C, and then continuing the culture by adding Texas Red-zymosan for another 3 h. The number of captured zymosan particles was enumerated under the fluorescence microscope after visualizing cells expressing lectins by staining with Abs as described above. At least 200 FITC<sup>+</sup> transfectants were examined, and cells capturing more than two particles were taken as positive.

The index was calculated as the average number of captured zymosan particles per FITC<sup>+</sup> cell.

#### *Zymosan-binding assay of CHO-SIGNR1 transfectants and peritoneal cells*

For binding assays with transfectants at 4°C, CHO cells that stably expressed whole SIGNR1 or SIGNR1 lacking 47 N-terminal amino acids including putative adopter protein-binding sites (called #48) (13) were used, because cell membrane of the transiently transfected cells was fragile for binding assay. The stable transfectants were mixed at  $2 \times 10^5$ /well with  $8 \times 10^5$ /well FITC-zymosan in 100  $\mu\text{l}$  of Hanks'-balanced saline solution (HBSS) containing 1% FCS and 0.1%  $\text{NaN}_3$  in V-bottomed 96-well plates and centrifuged at 250 g for 5 min. After incubation for 4 h on ice, the cells were suspended and analyzed with flow cytometry. For inhibition assay, 1 mg/ml of mannan, laminarin (Sigma-Aldrich: #L-9634) or 20 mM EDTA was added and incubated for 1 h prior to the addition of FITC-zymosan.

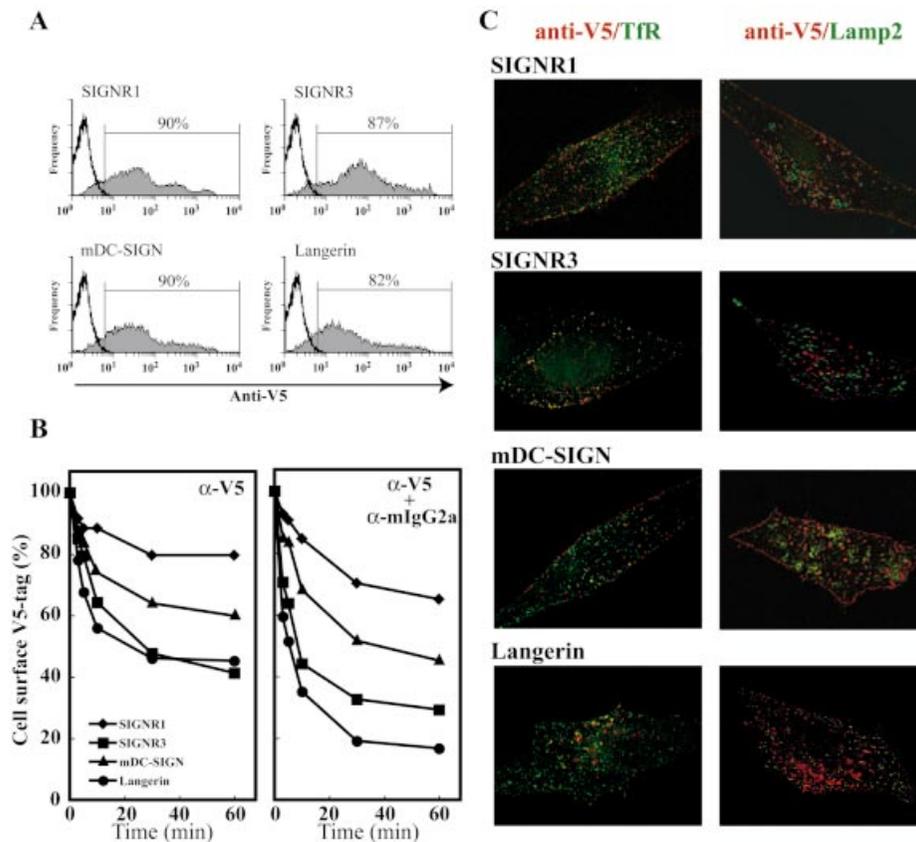
Resident peritoneal cells were obtained by injecting ice-cold PBS containing 5 mM EDTA. After washing, cells were preincubated with anti-CD16/32 mAb for 30 min for Fc-block and stained with PE-CD11b (clone M1/70; BD PharMingen, San Diego, CA) and biotin-ER-TR9 (BMA Biomedicals, Augst, Switzerland) (22) followed by streptavidin-Cy-Chrome (BD PharMingen) to detect SIGNR1 expression. Biotin-DX5 (CD49b; BD PharMingen) was used as an isotype-matched control. For binding assays, bulk resident peritoneal cells were first blocked for Fc-binding and stained with PE-labeled anti-CD11b. Then the cells were mixed with FITC-zymosan at four particles/cells and spun in V-bottomed 96-well plates as above. Inhibition assay was also performed by preincubating the cells in the presence of 1 mg/ml of mannan or laminarin, 25 mg/ml of ER-TR9 (Bachem Bioscience, King of Prussia, PA) or rat IgM (clone R4-22; BD PharMingen) on ice for 1 h before the assay.

## Results

#### *Internalization by antibody cross-linking*

We first studied the internalization of mouse DC-SIGN, SIGNR1, SIGNR3 and Langerin molecules using HEK293T transfectants with C-terminus Tag. As shown in Fig. 1(A), nearly 90% of cells expressed lectins at comparable level. Ligation of the lectins with the antibody resulted in the reduction of the cell surface lectins, demonstrating the internalization. However, the efficiency was greatest for SIGNR3 and Langerin, which were taken up more rapidly than mDC-SIGN and SIGNR1 (Fig. 1B, left panel). When secondary Ab was added to facilitate cross-linking, more rapid and efficient internalization was observed for all lectins, with Langerin being a little more efficient than SIGNR3 (Fig. 1B, right panel).

By deconvolution microscopy, the internalized anti-Tag mAbs in NIH3T3 transfectants were observed in numerous vesicles, of which some were double labeled with anti-transferrin receptor mAb to mark early endosomes (Fig. 1C, left panels). Consistent with the results of Fig. 1(B), relatively large amounts of SIGNR1 and mDC-SIGN remained on the cell



**Fig. 1.** Internalization of mouse DC-SIGN homologues and Langerin by antibody cross-linking. (A) HEK293T cells were transfected with V5 C-terminal tagged-lectins. Cell surface expression of the lectins was checked using anti-V5 tag antibody. (B) The transfectants were treated with (left panel) anti-V5 antibody or (right panel) anti-V5 antibody followed by biotin-donkey F(ab')<sub>2</sub> anti-mouse IgG antibody. Following washing, cells were incubated for the indicated time points at 37°C, and the antibodies remaining on the cell surface were visualized by staining with biotin-donkey F(ab')<sub>2</sub> anti-mouse IgG antibody and PE-streptavidin (left panel) or PE-streptavidin (right panel). Internalization is given by the percentage of MFI in experimental groups relative to control samples at 4°C. The MFIs in the control groups of (left panel) and (right panel) were 86 and 92 for SIGNR1, 62 and 55 for SIGNR3, 86 and 82 for mDC-SIGN and 46 and 43 for Langerin, respectively. (C) NIH3T3 transfectants expressing C-terminal tagged-lectins were treated with anti-V5 antibody. After 60 min incubation at 37°C, the anti-V5 antibodies were visualized with biotin-donkey F(ab')<sub>2</sub> anti-mouse IgG antibody followed by Texas Red-streptavidin. To localize the internalized antibodies, the transfectants were also stained with anti-transferrin receptor (TfR) or anti-LAMP-2.

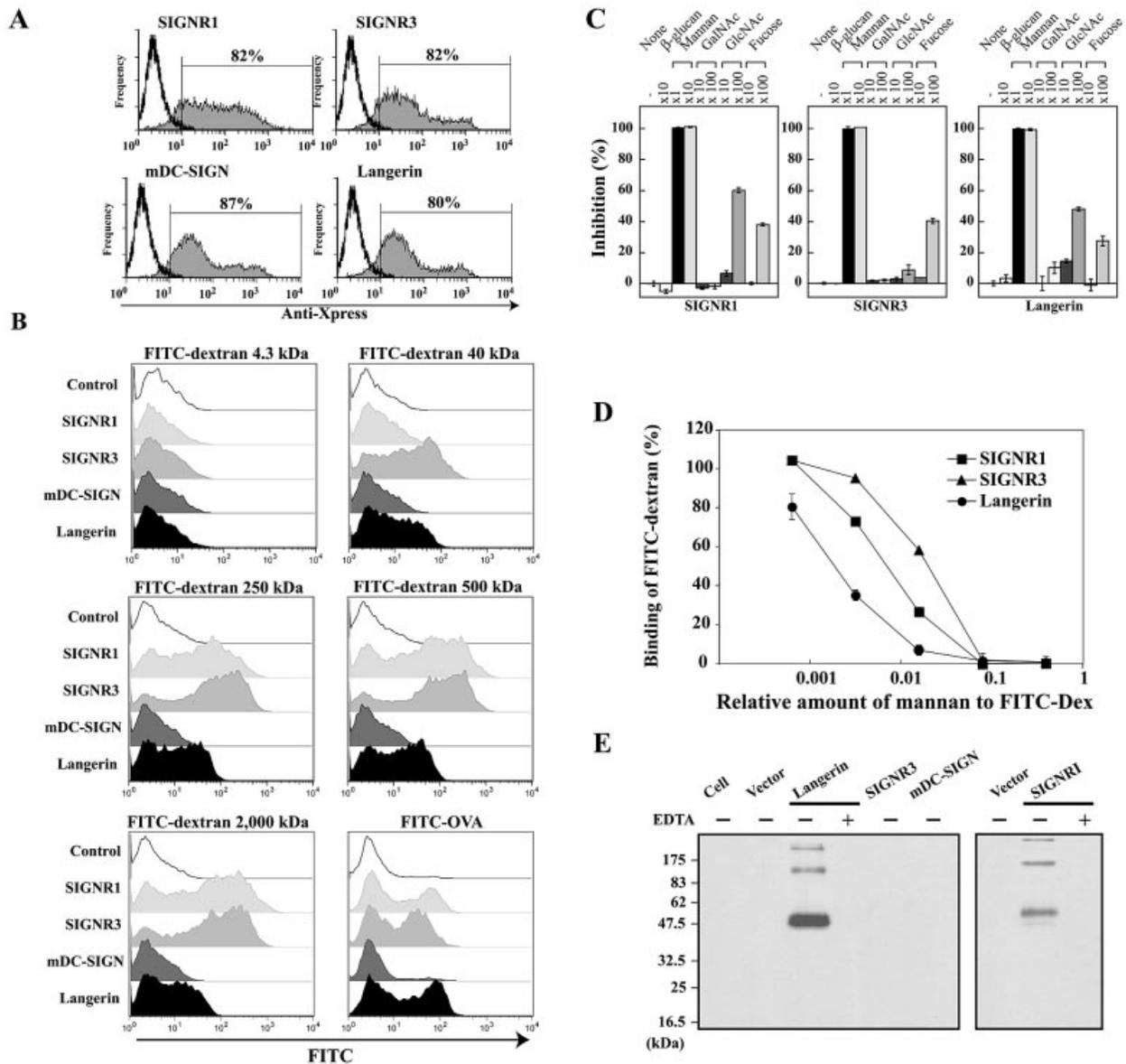
surface (Fig. 1C), even after 1 h incubation. All of the lectins were localized in LAMP-2 negative non-lysosomal compartments (Fig. 1C, right panels), as reported for hDC-SIGN (23,24).

#### Different sugar recognition by the lectins

We next examined the endocytosis of FITC-dextran and FITC-OVA using HEK293T cell transfectants expressing the N-terminal-tagged lectins, since both dextran and OVA bind to macrophage mannose receptors via an EPN motif in one of the CRDs (16,17). Total expression levels of the lectins in transfectants were tested by intracellular staining with anti-tag antibody (Fig. 2A). As shown in Fig. 2(B), none of the transfectants showed uptake for small 4.3 kDa FITC-dextran. mDC-SIGN was also unable to endocytose any of the larger FITC-dextran, even though mDC-SIGN was internalized following cross-linking with the monoclonal antibody (Fig. 1B). Likewise, mDC-SIGN failed to take up FITC-OVA. In contrast, SIGNR3 and Langerin took up 40 kDa FITC-dextran, the form that is often used for endocytosis assays.

However, SIGNR1 endocytosed only the larger FITC-dextran, 250 kDa or more. Similar results were also obtained when the transfectants were incubated at 1/10 of concentration of 40 or 2000 kDa FITC-dextran but for 12 h (data not shown). Cells transfected with control plasmid were negative for the uptake of any size of FITC-dextran. FITC-OVA, a glycosylated protein, was also taken up by SIGNR1, SIGNR3 and Langerin, but not by mDC-SIGN (Fig. 2B, bottom-right panel).

The effects of sugar inhibitors on FITC-dextran uptake were also investigated (Fig. 2C). Mannan completely blocked FITC-dextran uptake via any the lectins tested, even at the same concentration as FITC-dextran. In contrast, excess amounts of  $\beta$ -glucan and GalNAc were unable to inhibit endocytosis of FITC-dextran. A large dose (~1.6 mg/ml) of fucose substantially reduced their FITC-dextran uptake, while GlcNAc was only inhibitory for SIGNR1 ( $57.8 \pm 1.7\%$ ) and Langerin ( $43.6 \pm 2.6\%$ ), but not for SIGNR3. These results for Langerin were comparable to competition data using radio-labeled mannose-bound BSA by Stambach *et al.* (25). In addition, the

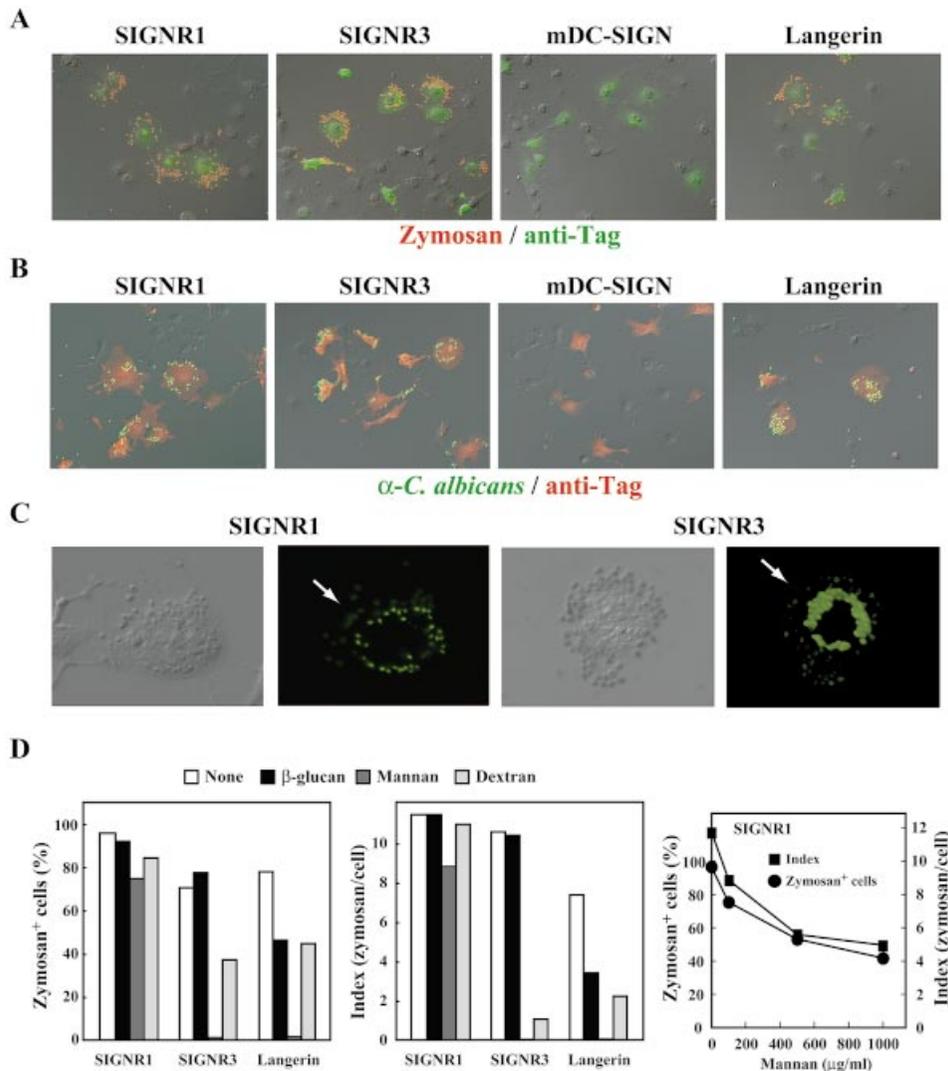


**Fig. 2.** Comparison of internalization capacities for FITC-dextran and FITC-OVA as well as binding affinity for mannan. (A) Expression of SIGNR1, SIGNR3, mDC-SIGN and Langerin in transfectants were checked using anti-Xpress tag antibody. Open histogram indicates a control staining of transfectants with the parental plasmid. (B) The transfectants were incubated with various molecular weights of FITC-dextran for 1 h or FITC-OVA for 12 h (bottom right panel). Open histograms indicate background uptake by transfectants with the parental plasmid. The results shown are representative of five independent experiments. (C) Inhibition of FITC-dextran (molecular weight 2000 kDa) uptake by soluble carbohydrates in HEK293T cell transfectants. Cells were incubated with 1–100 $\times$  higher concentrations of soluble  $\beta$ -glucan, mannan, GalNAc and GlcNAc for 15 min before incubation with 16  $\mu$ g/ml FITC-dextran for 1 h. Inhibition is indicated as the percent decrease of FITC<sup>+</sup> cells in experimental groups compared to respective controls without blocking sugars. FITC<sup>+</sup> cells in control groups were 72% for SIGNR1, 74% for SIGNR3 and 32% for Langerin. A representative result from four independent experiments is shown. (D) Titration of the inhibitory effect by mannan. The FITC-dextran uptake was inhibited by graded amounts of mannan. The relative amount shown as '1' indicates 16  $\mu$ g/ml mannan. (E) Mannan binding properties of the lectins. Cell lysates of the HEK293T transfectants were incubated with mannan-agarose. Bound protein was detected by western blotting using anti-Xpress or anti-SIGNR1 antibody with/without EDTA.

monosaccharide specificity for SIGNR1 and Langerin was similar to those for other C-type lectins, such as mannose receptor, serum mannose-binding protein and hDC-SIGN (26–28). Thus, these results indicate that SIGNR3 has distinct specificity from SIGNR1 and Langerin. The oligosaccharide sequences recognized by these three lectins have been investigated in detail (C. Galustian, C. G. Park, W. Chai,

M. Kiso, S. A. Bruening, Y. S. Kang, R. M. Steinman and T. Feizi, submitted for publication).

More precise analysis was performed for the inhibition by mannan of FITC-dextran uptake via SIGNR1, SIGNR3 and Langerin (Fig. 2D). FITC-dextran was most efficiently blocked by mannan in the case of Langerin, followed by SIGNR1 and SIGNR3. To extend these data, lectin binding assays for



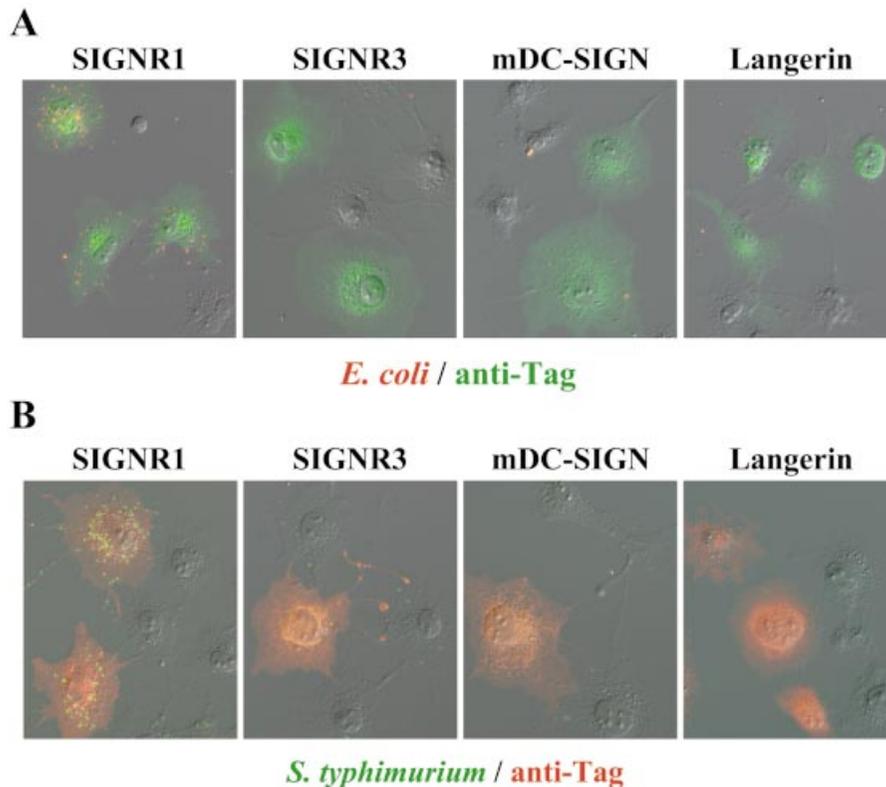
**Fig. 3.** Capture of yeast and yeast-like particles zymosan. COS-7 transfectants of N-terminal tagged-SIGNR1, SIGNR3, DC-SIGN and Langerin were incubated with Texas Red-zymosan (A) for 12 h and *C. albicans* (B) for 4 h. Lectins expressed by the transfectants were visualized with anti-Xpress Tag antibody. Internalization of FITC-zymosan was confirmed by quenching fluorescence using treatment with trypan blue (arrows). (C) Intracellular zymosan particles stayed brightly positive for FITC, whereas extracellular ones largely lost fluorescence by trypan blue (arrows). (D) COS-7 transfectants of SIGNR1, SIGNR3 and Langerin were preincubated with  $\beta$ -glucan, dextran or mannan before adding zymosan particles. After staining with anti-Xpress mouse IgG antibody followed by biotin-donkey F(ab')<sub>2</sub> anti-mouse IgG antibody + FITC-streptavidin, cells capturing more than two zymosan particles were regarded as positive cells, and only the numbers of particles in positive cells were counted. Index denotes the number of particles per cell in more than 200 transfectants. Left and middle panels show results with the inhibitors at 100  $\mu$ g/ml. Right panel indicates that a higher amount of mannan is required for inhibition in the case of SIGNR1 transfectants. Result shown is representative of three separate experiments.

mannan-agarose were carried out using cell lysates of the transfectants. We found that Langerin and SIGNR1, but not SIGNR3, bound mannan in a Ca<sup>2+</sup>-dependent manner (Fig. 2E). Although SIGNR1 appeared to capture mannan less efficiently than Langerin, this result was not conclusive since the amounts of lectins were not adjusted.

#### *SIGNR1, SIGNR3 and Langerin capture zymosan via its mannan moiety*

Mannan is the major constituent of zymosan and is recognized by mannose-binding receptors (29,30). Thus the recognition and uptake of FITC-zymosan was studied. For this

experiment, we used COS-7 cells because their large size permits easier observation of zymosan binding/internalization under the microscope. Similar to the data with 2000 kDa FITC-dextran and FITC-OVA, transfectants of SIGNR1, SIGNR3 and Langerin were capable of capturing a large number of zymosan particles (Fig. 3A) and heat-killed *C. albicans* (Fig. 3B). For DC-SIGN, only a small number of the transfectants, if any, could bind a few yeast particles (data not shown). The internalization function of SIGNR1 and SIGNR3 was also confirmed using trypan blue quenching method for FITC-zymosan (Fig. 3C). This treatment reduced the percentages of FITC<sup>+</sup> zymosan-bearing SIGNR1- and



**Fig. 4.** Capture of Gram-negative bacteria. COS-7 transfectants were incubated with Texas Red-*E. coli* (A) for 12 h or FITC-*S. typhimurium* (B) for 6 h. Lectins expressed by the transfectants were visualized as in Fig. 3.

SIGNR3-transfected cells from 75–85% to 15–30%. Control transfectants with the parental plasmid were negative for capture of zymosan. Therefore, SIGNR1 and SIGNR3 can internalize zymosan, although uptake was moderate in our transfectants.

The blocking of zymosan uptake was also tested using mannan and  $\beta$ -glucan as inhibitors, since a large part of the yeast cell wall is composed of mannan,  $\beta$ -glucan and chitin (29). Although mannan at 100  $\mu$ g/ml completely abrogated FITC-dextran uptake via SIGNR1 (Fig. 2C), an only slight inhibition of zymosan uptake was detected at the same dose of mannan (Fig. 3D, left and middle panels). However, a large excess of mannan (500 and 1000  $\mu$ g/ml) significantly reduced zymosan capture by SIGNR1 (Fig. 3D, right panel). In contrast to SIGNR1, mannan was still a potent inhibitor for zymosan uptake by SIGNR3 and Langerin.  $\beta$ -glucan had no effect on SIGNR1 and SIGNR3, but substantially inhibited the activity of Langerin. On the other hand, dextran significantly inhibited SIGNR3- and Langerin-mediated capture of zymosan.

#### *Capture of Gram-negative bacterial by SIGNR1, but not the other lectins tested*

In striking contrast to the data with FITC-zymosan, Gram-negative bacteria, such as *E. coli* and *S. typhimurium*, were captured, but only by SIGNR1 transfectants, and not by the other C-type lectin transfectants (Fig. 4A and B). On the other hand, the Gram-positive bacterium *Staphylococcus aureus*

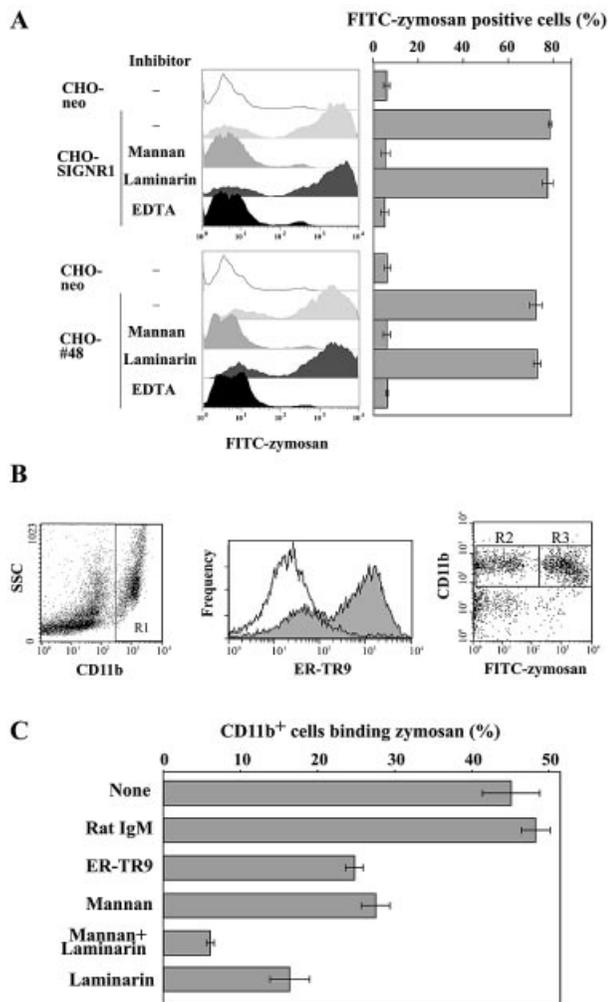
was not captured by any type of transfectants examined here (data not shown).

#### *Zymosan capture by SIGNR1-transfectant is mediated via the recognition for mannan moiety*

Because of the broad specificity of SIGNR1 for microbes, we attempted to detect direct binding of zymosan by SIGNR1 transfectants at 4°C. For this purpose, CHO cells stably expressing intact or truncated SIGNR1 (#48) were utilized. As shown in Fig. 5(A), both transfectants effectively and comparably bound FITC-zymosan (72–78%), and the treatment with mannan and EDTA, but not laminarin, resulted in a complete block of zymosan binding to control levels (~6%).

#### *Expression of SIGNR1 and zymosan binding by a subpopulation of resident peritoneal cells*

As reported previously, SIGNR1 protein is expressed by macrophages in spleen and lymph nodes (13). SIGNR1<sup>+</sup> marginal zone macrophages play a role in capturing *Streptococcus pneumoniae* (15). Although we are able to obtain such SIGNR1<sup>+</sup> macrophages from spleen, it is difficult to prepare a large number of SIGNR1<sup>+</sup> macrophages using conventional methods. We therefore sought another source of SIGNR1<sup>+</sup> cells using monoclonal antibody ER-TR9 (22), and found that 75% or more of CD11b<sup>+</sup> cells in the resident peritoneal cavity express SIGNR1 (Fig. 5B, left and center panels). This made it possible to examine a role of SIGNR1 zymosan recognition by direct binding assays at 4°C. About



**Fig. 5.** Direct binding of stably SIGNR1-transfected CHO cells and of resident peritoneal cells expressing SIGNR1. (A) SIGNR1 itself bound FITC-zymosan in a mannan and EDTA-sensitive manner. CHO-SIGNR1 and CHO-#48 cells lacking the cytoplasmic tail of SIGNR1 were incubated with FITC-zymosan on ice with/without inhibitors described. Reduction of FITC-zymosan capture by inhibitors is depicted in the right panel. (B) Expression of SIGNR1 on resident peritoneal cells was analyzed by flow cytometry. A majority of CD11b<sup>+</sup> cells were stained with ER-TR9, anti-SIGNR1 mAb (left and center panels). Open histogram represents control staining with DX-5 antibody. The CD11b<sup>+</sup> cells were capable of binding FITC-zymosan at 4°C (right panel). (C) Cooperation in zymosan binding by SIGNR1 and laminarin-sensitive receptor. Peritoneal cells were pretreated with inhibitors represented for 1 h. Binding (%) indicates the percentage of FITC<sup>+</sup> cells in CD11b<sup>+</sup> cells.

45% of CD11b<sup>+</sup> peritoneal cells were able to bind FITC-zymosan (Fig. 5B, right panel), and this binding was significantly inhibited by SIGNR1-specific ER-TR9 mAb (Fig. 5C). An excess of mannan was effective, although both ER-TR9 and mannan did not completely inhibit, as in Fig. 5(A). Since macrophages are known to express other C-type lectins with different carbohydrate specificity, such as Dectin-1 recognizing  $\beta$ -glucan on zymosan, laminarin, a known ligand for Dectin-1 was used as an inhibitor (31). Laminarin itself blocked zymosan binding to SIGNR1<sup>+</sup> peritoneal cells, and the

blocking was augmented by co-addition of mannan (Fig. 5C). Similar results were also obtained using heat-killed *C. albicans* (data not shown). Therefore, it is likely that SIGNR1 and a laminarin-sensitive receptor, possibly Dectin-1, on a population of peritoneal cells cooperate to bind zymosan by recognizing different carbohydrate moieties.

## Discussion

In the present study, we demonstrate the distinctive uptake of polymerized dextran, glycosylated protein and microbes, as well as the binding capacity to mannan and zymosan, by C-type lectins in the mouse DC-SIGN family (DC-SIGN, SIGNR1 and SIGNR3) and Langerin, of which all share an EPN-motif in their CRD. Results are summarized in Table 1.

For the presentation of exogenous antigens, it is necessary to internalize and deliver them to LAMP-2-positive late endosome/lysosome compartments, where processed antigens are loaded onto MHC class II molecules. These four lectins on the cell surface of transfectants could each be internalized by cross-linking with specific antibody in HEK293 T cell and NIH3T3 transfectants. However, the internalized antibodies were detected in cytoplasmic compartments lacking LAMP-2 in NIH3T3 transfectants. As for SIGNR1, Geijtenbeek *et al.* (14) recently suggested a rapid sorting of FITC-dextran captured by SIGNR1 into lysosome-like compartments using an erythroleukemic cell line K562, although they did not double label with a lysosomal marker. In contrast, we find that internalized FITC-dextran is delivered to compartments lacking LAMP-1/CD107a, pan-lysosomal marker, using stably transfected RAW264.7 SIGN-R1 transfectants (13). These discrepancies may depend on cell types tested. In human DCs, however, Langerin is also reported to deliver bound mAb to non-lysosomal compartments (32), while DC-SIGN sorts captured mycobacteria and specific mAb into lysosomal compartments (9). Therefore, it is also possible that adaptor proteins for each lectin are involved in internalization and intracellular sorting.

Our results show that the efficacy of uptake of bound mAb varies amongst the lectins. The results of Fig. 1(B) are consistent with those of Fig. 1(C) where DC-SIGN and SIGNR1 are less efficient for internalization than SIGNR3 and Langerin. These distinctions may reflect differences in the cytoplasmic tails. It has been shown that mDC-SIGN and SIGNR1 have a single dileucine motif (DEELL and DDELM, respectively), which binds adaptor proteins for the clathrin-coated pit, while SIGNR3 has both a dileucine motif and tyrosine internalization motif (DEECLM and YSDI) (12). Langerin has a distinct type of proline-rich sequence (PREPPP) that may bind a signal protein containing a src homology 3 (SH3) domain (33), leading to a rapid internalization by cross-linking with anti-Langerin mAb (32). Examination of internalization efficiency of these lectins using transfectants of other cell types remains to be conducted. Regarding uptake of polymerized FITC-dextran, mDC-SIGN was found to be inactive even for high molecular weight forms (although mDC-SIGN was capable of internalizing a ligated mAb as mentioned). Furthermore, none of the microbes tested were captured by mDC-SIGN. In contrast, SIGNR3 and Langerin were capable of efficiently taking up FITC-dextran even with a

**Table 1.** Summary of carbohydrate recognition abilities of SIGNR, SIGNR3, mDC-SIGN and Langerin

		Lectins			
		SIGNR1	SIGNR3	mDC-SIGN	Langerin
Internalization					
Ligation with anti-Tag mAb		+	+++	++	+++
Capture of antigen					
FITC-OVA		++	++	±	+++
FITC-dextran					
4.3 kDa		-	-	-	-
40 kDa		-	++	-	+
250 kDa		++	+++	-	++
2000 kDa		+++	+++	-	++
Inhibition by	Mannan	***	***	N.T.	***
	GalNAc	*	*	N.T.	*
	GlcNAc	**	*	N.T.	**
	Fucose	**	**	N.T.	**
Capture of zymosan					
Binding		+++	+++	N.T.	++
Inhibition by	Mannan	**	***	N.T.	***
	Dextran	**	**	N.T.	**
	β-glucan	*	*	N.T.	*
Uptake		++	++	N.T.	N.T.
Capture of bacteria					
<i>E. coli</i>		+++	-	N.T.	-
<i>S. typhimurium</i>		+++	-	N.T.	-

-, negative; ±, very weak but not negative; + → +++, positive → strongly positive; \* no obvious effect; \*\* moderate inhibition; \*\*\* strong inhibition; N.T., not tested.

small molecular weight. SIGNR1, however, could only take up highly polymerized dextran. Moreover, uptake of high molecular weight FITC-dextran was blocked by mannan with an efficacy order of Langerin > SIGNR1 > SIGNR3, and direct binding to mannan-agarose was only detected with Langerin and SIGNR1, but not DC-SIGN and SIGNR3, in a Ca<sup>2+</sup>-dependent manner. These observations and the results showing distinctive blocking efficiency with free monosaccharides for FITC-dextran uptake, indicate that the lectins tested here have characteristic sugar recognition abilities, even though they share a common EPN motif in their single CRD domains.

SIGNR1, SIGNR3 as well as Langerin could also capture zymosan and *C. albicans*. However, capture via Langerin was much more efficiently inhibited not only by mannan but also by β-glucan and polymerized dextran, than that via SIGNR1 and SIGNR3. Furthermore, Langerin-mediated capture was the lowest among these three lectins, in terms of the number of zymosan particles captured per transfectant. On the other hand, the capture via SIGNR1 was not much affected by mannan, even though CRDs of SIGNR1 and SIGNR3 are homologous; 69.9% in amino acid sequence. This might be ascribed to the difference in their avidity for mannan, but this possibility is unlikely, since mannan was more potent in blocking the uptake of FITC-dextran by SIGNR1 than that by SIGNR3. Another possibility is that SIGNR1 and SIGNR3 recognize different target moieties on zymosan particles, since dextran was more effective in inhibiting the capture of zymosan by SIGNR3. Alternatively, SIGNR1 might recognize a

particular spatial arrangement or pattern of target sugars on the zymosan particles. It has been reported that the binding specificities of hDC-SIGN and L-SIGN might be generated by the spatial arrangement of target sugars in high mannose oligosaccharides (27,34). We also demonstrated that SIGNR1 molecules primarily exist in aggregated form *in situ* (13). Furthermore, the potency to bind zymosan through the recognition of mannan was also shown in SIGNR1-transfected CHO cells. This binding was dependent on Ca<sup>2+</sup>, but did not require the cytoplasmic tail. Therefore, SIGNR1 may have a higher avidity to multivalent/repetitive target sugars spaced on zymosan.

Interestingly, SIGNR1 was able to capture Gram-negative bacteria, such as *E. coli* and *S. typhimurium*, but not Gram-positive bacteria; *S. aureus* in the present study. However, we previously reported that capsular polysaccharide of *Streptococcus pneumoniae*, Gram-positive bacteria, can be recognized by SIGNR1 (15). This discrepancy has yet to be solved, but may be ascribed to the loss or damage of capsule of fluorescence-labeled *S. aureus* available on the market.

At present, we cannot exclude the possibility that polysaccharide and microbial uptake could be somehow influenced by intracellular tag. However, SIGNR1, SIGNR3 and Langerin with intracellular tag are competent for uptake of dextran and OVA, and their endocytic capacity is dependent on ligand and its structure. Therefore, we suspect the effect of the expression tag on endocytic activity.

Recently, Dectin-1, a β-glucan receptor, has been reported to collaborate with Toll-like receptor (TLR) 2 to recognize

microbes for inflammatory response (35,36). Therefore, it will be of interest to elucidate the involvement of other pattern recognition receptors, such as TLRs, for the recognition of Gram-negative bacteria in collaboration with SIGNR1. In addition, results showing cooperative recognition of yeast by SIGNR1 and laminarin-sensitive receptor (possibly Dectin-1) on peritoneal cells may lead us to anticipate more complicated regulation in pattern recognition systems *in situ*. During revision of this manuscript, similar results demonstrating participation of both SIGNR1 and Dectin-1 for recognizing zymosan were reported by Taylor *et al.* (37). The different properties of mouse DC-SIGN, SIGNR1, SIGNR3 and Langerin indicate that distinct microbial components are recognized by lectins expressed by cells of the innate immune system.

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### Abbreviations

CRD	carbohydrate recognition domain
DC	dendritic cell
DC-SIGN	DC-specific ICAM-3 grabbing non-integrin
LAMP	lysosomal-associated membrane protein
SIGNR	SIGN-related gene
TLR	Toll-like receptor

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