Difference in fine specificity to polysaccharides of *C. albicans* mannoprotein between mouse SIGNR1 and human DC-SIGN

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Running title: *Recognition of C. albicans polysaccharides by SIGNR1*

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21 **Abbreviations used in this paper:** antibody, Ab; carbohydrate recognition domain, CRD;
22 fluorescein isothiocyanate, FITC; human DC-SIGN, hDC-SIGN; monoclonal antibody, mAb;
23 phycoerythrin, PE; phosphorylated mannose, PM; RAW264.7 cells expressing human DC-SIGN,
24 RAW-hDC-SIGN; RAW264.7 cells expressing hDC-SIGN of which CRD is replaced with
25 SIGNR1 CRD, RAW-chimera; RAW264.7 cells expressing SIGNR1, RAW-SIGNR1; soluble
26 lectin, sLectin; soluble form SIGNR1, sSIGNR1; soluble form human DC-SIGN, shDC-SIGN;
27 toll-like receptors, TLRs.

28

Abstract

C-type lectin SIGNR1 directly recognizes *Candida albicans* and zymosan, and has been considered to share properties of polysaccharide recognition with human (h)DC-SIGN. However, the precise specificity of SIGNR1 and difference from that of hDC-SIGN remains to be elucidated. We prepared soluble forms of SIGNR1 and hDC-SIGN and conducted experiments to examine their respective specificities.

Soluble SIGNR1 (sSIGNR1) bound several types of live clinical isolate *C. albicans* strains in an EDTA-sensitive manner. Inhibition analyses of sSIGNR1 binding by glycans from various yeast strains demonstrated that SIGNR1 preferentially recognizes N-glycan α-mannose side chains in Candida mannoproteins, as reported in hDC-SIGN. Unlike shDC-SIGN, however, sSIGNR1 recognized not only *S. cerevisiae* but also *C. albicans* J-1012 glycan even after α-mannosidase treatment that leaves only β1,2-mannose capped α-mannose side chains. In addition, the glyco-microarray analyses showed that sSIGNR1 binds mannans from *C. albicans* and *S. cerevisiae*, but does not recognize Lewis$^{a/b/x/y}$ antigen polysaccharides as in shDC-SIGN. Consistent with these results, RAW264.7 cells expressing hDC-SIGN of which the carbohydrate recognition domain (CRD) was replaced with that of SIGNR1 (RAW-chimera) produced comparable amounts of IL-10 in response to glycans from *C. albicans* and *S. cerevisiae*, but those expressing hDC-SIGN produced less IL-10 to *S. cerevisiae* than *C. albicans*. Furthermore, RAW-hDC-SIGN cells remarkably reduced IL-10 production after α-mannosidase treatment

compared with RAW-chimera.

These results indicate that SIGNR1 recognizes *C. albicans*/yeast through a partly distinct specificity from its homologue hDC-SIGN.

Introduction

Numerous microbes are covered with the polysaccharides. Recognition of the polysaccharides by pattern recognition receptors (PRRs), including lectins, is vital in order to recognize pathogens, since recognition of the outermost components is the first interactive step with immune cells during infection to evoke innate and adaptive immune responses.

Candida albicans is an opportunistic agent of infection in immune compromised patients. In the host innate immune system, several types of receptors for sensing ligands on the microbe have been defined, *e.g.*, C-type lectins and toll-like receptors (TLRs) (18). Ligands for these receptors are present in the outer structure of microbes. However, some ligands are sequestered by the outermost polysaccharides, which consist of mannoproteins, as reported in the case of β-glucan, a ligand for Dectin-1 (6). Mannoproteins are rich in polysaccharides composed of mostly α- and β-mannose and recognized by mannose/mannan type lectins.

C-type lectin human (h)DC-SIGN (CD209) has been shown to interact with a wide range of pathogens, including microbes, viruses and protozoa (11) *via* mannose and fucose moieties on the surface of the pathogens. Microbes such as *Mycobacterium tuberculosis* and *C. albicans* are endocytosed and processed for antigen presentation to induce the subsequent T cell-mediated immune responses. However, the recognition also induces immunosuppressive responses in cooperation with TLRs (8).

Mice have eight hDC-SIGN homologues (19, 20). One of these homologues, SIGNR1,

69 is structurally related to hDC-SIGN based on its long neck domain. SIGNR1 is expressed on
70 particular subsets of macrophages (Mϕ)/dendritic cells (DC) in the marginal zones of the spleen,
71 resident peritoneal cavity, medulla of lymph nodes, skin and lamina propria (4, 10, 17, 30).
72 SIGNR1 on these cells plays a role as a sentinel in the recognition of pathogens through capsular
73 polysaccharides. In fact, SIGNR1 on marginal zone Mϕ recognizes *Streptococcus pneumoniae*
74 (10), leading to efficient activation of the complement system *in situ* (9).

75 Previously, we reported that SIGNR1 recognizes Gram-negative bacteria (*Salmonella*
76 *typhimurium* and *Escherichia coli*) and *C. albicans* (27). The former are recognized through their
77 non-reductive end of the lipopolysaccharide core sequence by SIGNR1 (17). This is also the case
78 of hDC-SIGN in recognition of *E. coli* (13). Recently, hDC-SIGN has been reported to strongly
79 recognize the α-mannose structure of N-glycan side chains of *C. albicans*, but weakly that of *S.*
80 *cerevisiae* (2). However, the recognition motif on *C. albicans* by SIGNR1 is not clear at present.

81 Therefore, we aimed to elucidate the properties of SIGNR1 in the recognition of
82 polysaccharide on *C. albicans*. To this end, we prepared soluble forms of SIGNR1 (sSIGNR1)
83 and hDC-SIGN (shDC-SIGN) and used structurally distinguished glycans purified from various
84 types of *C. albicans* and *S. cerevisiae* as well as respective microbes. The results indicate that
85 sSIGNR1 binds equally well to glycans from *S. cerevisiae* as to *C. albicans.* Furthermore,
86 sSIGNR1, but not shDC-SIGN, was shown to readily recognize *C. albicans* glycan treated with
87 α-mannosidase. In addition, a glyco-microarray based on an evanescent-field fluorescence

88 detection method clearly revealed that sSIGNR1 binds α-mannose monosaccharide and mannans
89 from *C. albicans* and *S. cerevisiae*, but dose not recognize Lewis$^{a/b/x/y}$ antigen polysaccharides as
90 in shDC-SIGN. Different properties in recognition of yeast glycans between SIGNR1 and
91 hDC-SIGN CRD were also observed in induction of IL-10 from RAW264.7 cells.
92

92 **Materials and methods**

93 **Cells and cultures.** Maintenance of human embryonic kidney (HEK) 293T cells and
94 macrophage-like RAW264.7 cells and preparation of RAW264.7 expressing SIGNR1
95 (RAW-SIGNR1) were as described previously (27). In order to prepare RAW-hDC-SIGN,
96 cDNA encoded hDC-SIGN (kindly provided by Dr. R.M. Steinman, Rockefeller University) was
97 cloned into pMX-IRES-puromycin (12). RAW264.7 cells were transfected by the plasmid with
98 Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's protocol.
99 RAW264.7 cells expressing hDC-SIGN (RAW-hDC-SIGN) were maintained in the presence of
100 4 μg/ml puromycin (InvivoGen, San Diego, CA). To prepare RAW264.7 cells expressing
101 chimeric lectin consisting of hDC-SIGN and SIGNR1 (RAW-chimera), cDNA fragments
102 encoding amino acids 1 - 253 (corresponding to cytosolic-neck domain of hDC-SIGN) and 193 -
103 325 (corresponding to CRD of SIGNR1) were amplified using KOD polymerase (Toyobo,
104 Tokyo, Japan) using primer pairs (5'-ggtggtacgggaattcatgagtgactccaaggaaccaagac-3',
105 5'-ggcacaggcgttccac-3') and (5'-tggaacgcctgtgccgactctgcccctgggactggacattc-3',
106 5'-atttacgtagcggccgcctagccttcagtgcatggggttgc-3'), respectively. These were introduced into *Eco*
107 RI - *Not* I site of pMX-IRES-puromycin using the In-Fusion PCR Cloning System (Clontech,
108 Mountain View, CA). RAW264.7 cells were transfected by the plasmid as described above.
109
110 **Reagents and yeast strains.** Alexa647-coupled hamster anti-SIGNR1 mAb 22D1 and rabbit

111 anti-hDC-SIGN Ab (H-200) were purchased from eBioscience (San Diego, CA) and Santa Cruz
112 Biotechnology (Santa Cruz, CA), respectively. Anti-hDC-SIGN mAb (DCS-8C1; eBioscience)
113 was labeled with Alexa-555 (Invitrogen) in accordance with the manufacturer's protocol.
114 Glycogen of bovine liver type IX (G0885) and Jack bean (*Canavalia ensiformis*) α-mannosidase
115 (EC 3.2.1.24) were from Sigma-Aldrich (Irvine, CA). *C. albicans* (J-1012: serotype A,
116 NBRC1060 and NIH B-792: serotype B, NBRC10108), *C. lusitaniae* (NBRC1019) and *S.*
117 *cerevisiae* X2180-1A (WT) (BY21559) were obtained from the Biological Resource Center of
118 the National Institute of Technology and Evaluation (Tokyo, Japan). *C. albicans* (JCM1542) was
119 from the Japan Collection of Microorganisms, RIKEN (Saitama, Japan).
120
121 **Preparation of mannan from yeast strains.** Glycan was prepared from mannoprotein of the
122 blastospore (yeast) form using Fehling's solution, as previously described (24). Glycans used in
123 this study were purified from strains of *C. albicans* J-1012 (25), *C. albicans* NIH B-792 (22), *C.*
124 *stellatoidea* (24), *C. parapsilosis* (22), *C. lusitaniae* (23), *S. cerevisiae* (WT) (1), *S. cerevisiae*
125 (*mnn1/mnn4*) (1) and *S. cerevisiae* (*mnn2*) (21). The α-mannosidase treatment of *C. albicans*
126 J-1012 mannan was carried out in 50 mM sodium acetate buffer (pH 4.6) containing 20 units of
127 α-mannosidase at 37°C for 48 h.
128
129 **Preparation of sSIGNR1 and shDC-SIGN and binding analyses to microbes.** Soluble lectin

(sLectin) tetramers, such as sSIGNR1 and shDC-SIGN, were prepared as described (26). Briefly, cDNA fragments encoding their extracellular domains were cloned into pEXPR-IBA44 (IBA, Göttingen, Germany) to add N-terminal BM40 secretion signal and *Strep*-Tag II sequences, followed by the transfer into pEF6/V5-His (Invitrogen). HEK293T cells were then transfected with each plasmid using the calcium phosphate method (3) and cultured in serum-free medium 293 SFM II (Invitrogen) for the last 48 hr. sSIGNR1 and shDC-SIGN in the supernatant were purified using *Strep*-Tactin Sepharose (IBA) in accordance with the manufacturer's protocol (> 95% of purity by SDS-PAGE).

Purified sLectins (2.5 μg/ml) were incubated with PE-labeled *Strep*-Tactin (7.5 μg/ml) in 18 μl of Hanks' balanced salt solution (pH 8.3) (binding buffer) for 2 h at 4°C and for a further 10 min at 37°C. The tetramers thus formed were incubated with 5 x 10^6 live microbes for 4 h at 4°C in the presence of 1% BSA (total volume 25 μl). After washing with the binding buffer, the amount of bound PE-*Strep*-Tactin was measured by Gemini EM (Molecular Devices, Sunnyvale, CA). The direct binding of sSIGNR1 is shown as an arbitrary unit of fluorescence intensity. The % inhibition was calculated using the following formula: [1 - (fluorescence intensity of *C. albicans* by the staining with sLectin plus inhibitor - that without sLectin) / (that with sLectin without inhibitor - that without sLectin)] x 100.

Lectin ELISA. sSIGNR1 and shDC-SIGN were formed by incubating sLectins (62.5 ng) with

149 HRP-*Strep*-Tactin (12.5 ng) in 20 μl of the solution as above. Microtiter plates were coated with
150 50 μl of mannan/glycan (5 mg/ml) in 50 mM sodium bicarbonate buffer (pH 9.6) for 12 h at 4°C,
151 followed by the incubation with 2.5% BSA at room temperature for 2 h after washing with 25
152 mM Tris-HCl pH8.3 + 150 mM NaCl. The plates were then incubated with sSIGNR1 or
153 shDC-SIGN in the presence of 1% BSA for 2 h at 4°C. For inhibition experiments, sLectin
154 tetramer was pre-incubated with inhibitors for 1 h at 4°C before adding to plates. After washing,
155 binding of sLectin tetramer was measured as the absorbance of TMB (eBioscience) at 450 nm by
156 VERSAmax (Molecular Devices). The % inhibition was calculated as above.
157
158 **Inhibition of FITC-dextran binding to RAW-SIGNR1 with glycans.** RAW-SIGNR1 cells (2
159 x 10^5 cells) were pre-incubated with various types of glycan and EDTA (25 mM) for 30 min at
160 4ºC and then mixed with 80 μg/ml of FITC-dextran (2,000 kDa; Sigma-Aldrich) for 4 h at 4ºC.
161 Binding of FITC-dextran was analyzed by a flow cytometer. The % inhibition was calculated
162 using the following formula: [1 - (mean fluorescence intensity (MFI) of RWA-SIGNR1 cells
163 with FITC-dextran plus inhibitor- that without FITC-dextran) / (that with FITC-dextran without
164 inhibitor - that without FITC-dextran)] x 100.
165
166 **Glyco-microarray analyses of sLectins by evanescent-field fluorescence detection.** The
167 glyco-microarray analysis was performed as described (28). To form immune complex,

sSIGNR1 and shDC-SIGN (10 μg/ml) were pre-incubated with Alexa647-anti-SIGNR1 (22D1) and Alexa555–anti-hDC-SIGN (DCS-8C1; 1 μg/ml) for 15 min at room temperature in 25 mM Tris-HCl buffer (pH 7.4) containing 0.8% NaCl, 1% (v/v) Triton-X100, and 2 mM $CaCl_2$ with or without 10 mM EDTA. This complex was directly added to the array immobilized with multivalent glycan ligands (Supplementary Fig. S1), followed by incubation overnight at 20°C. Binding was then detected using an evanescent-field fluorescence-assisted scanner. Data were analyzed with the Array Pro analyzer ver. 4.5 (Media Cybernetics, Bethesda, MD).

IL-10 production of RAW264.7 transfectants by stimulation in microplates coated with glycan. Non-treated plates were pre-coated with 600 μg/ml glycan in PBS for 12 h. After blocking with RPMI1640 containing 10% FCS for 30 min, RAW264.7 transfectants (5×10^4 cells) were cultured in the presence of 100 ng/ml ultra pure LPS (Invitrogen) for 24 h. IL-10 in the supernatants was analyzed using the Cytometric Bead Array (CBA) for mouse inflammation kit (BD Biosciences, Franklin Lakes, NJ).

Statistical analysis. Data are expressed as the means ± SD of triplicate assays. Statistical significance was determined by the two-tailed Student's *t*-test or multiple comparisons with Tukey's multiple range test. All experiments were performed two or more times and representative results are shown.

Results and Discussion

SIGNR1 recognizes various types of Candida strains. Each Candida strain has a unique set of oligomannose side chains, generating a great diversity of N-glycans (see Fig. 1A) compared with that of O-glycans. Moreover, N-glycans account for more than 95% of glycans in the surface mannoproteins.

Therefore, we first examined the direct binding of sSIGNR1 to several Candida strains (Fig. 1B). We used sSIGNR1 tetramerized with *Strep*-Tactin, because the affinity of sSIGNR1 monomer is weak (20). This method not only helps to increase the affinity of sSIGNR1 but also helps to avoid the formation of large complexes using Fc-fusion lectins polymerized with anti-Fc polyclonal Ab. Before using this sSIGNR1-tetramer for experiments, we confirmed that sSIGNR1-tetramer bound to mannan-agarose was eluted with EDTA (data not shown), although the yield was less than half the amounts applied. sSIGNR1 bound clinical isolate live *C. albicans* JCM1542 (serotype A) in an EDTA-sensitive manner, indicating that sSIGNR1 binding occurs *via* the carbohydrate recognition domain (CRD). The other clinical isolate strains, *C. albicans* J-1012 (serotype A) and NIH B-792 (serotype B), were also recognized by sSIGNR1, although sSIGNR1 binding was much less to nosocomial strain *C. lusitaniae*.

It is of note that sSIGNR1 binds to *S. cerevisiae* comparably to clinical isolate *C. albicans* (Fig. 1B), and its binding to *C. albicans* J-1012 microbes was equally inhibited by N-glycans from *S. cerevisiae* (WT and *mnn1/mnn4*) and *C. albicans* NIH B-792 as *C. albicans*

206 J-1012 (Fig. 1C).

207

208 **SIGNR1 possibly recognizes α-mannose in side chains of *C. albicans* N-glycan.** To delineate
209 the polysaccharide structure recognized by SIGNR1, inhibition experiments with lectin ELISA
210 were conducted using N-glycans from the various yeast strains listed (Fig. 1A and Table I).
211 The direct binding of sSIGNR1 to *C. albicans* J-1012 N-glycan, which is composed of
212 complexed side chains, was inhibited by glycans from several types of microbes: *C. albicans*
213 NIH B-792 and *C. stellatoidea* glycans lacking β1,2-mannose, *C. parapsilosis* glycan
214 lacking β1,2-mannose and α1,6-branched mannose, and *S. cerevisiae* WT glycan composed of
215 short side chains lacking β1,2-mannose and α1,6-branched mannose (Fig. 2A). These glycans
216 share the α1,2-mannose side chain structure, suggesting that SIGNR1 recognizes similar moiety
217 in N-glycan as hDC-SIGN (2). Moreover, *S. cerevisiae* 4484-24D-1 (*mnn1/mnn4*) glycan, which
218 lacks phosphorylated mannose (PM), β1,2-mannose, α1,3-mannose, and α1,6-branched mannose
219 but possesses the short (mono- or di-) mannose side chain structure, appeared to be recognized
220 by SIGNR1. However, *S. cerevisiae* X2180-1A-5 (*mnn2*) glycan, which lacks all side chains,
221 wasn't effective (Figs. 2A and B), suggesting the crucial involvement of the side chain structure.
222 In addition, *C. lusitaniae* glycan, the side chain of which is composed of more than 75% of
223 β1,2-mannose (mono- ~ tri-β-mannose)-capped side chain (23), was also ineffective in inhibiting
224 the sSIGNR1 binding (Figs. 2A and B), implying that these β1,2- mannoses disturb the access of

225 SIGNR1 to the α-di-mannose to some extent. Glycogen itself had no effect. As in the case of
226 sSIGNR1 binding to microbes, that to *C. albicans* J-1012–derived N-glycan was EDTA-sensitive
227 (Fig. 2A).

228 Interestingly, treatment of *C. albicans* J-1012 mannan with α-mannosidase, which
229 removed the α-mannose side chains other than the β1,2-mannose-capped side chains (see Fig.
230 1B) (14), did not affect the inhibitory activity (Fig. 2A). This result also indicates that
231 β1,2-mannose capped α-mannose side chains in the N-glycan are sufficient to be recognized by
232 SIGNR1. We also obtained similar results when mannan from *S. cerevisiae* (M7504;
233 Sigma-Aldrich) was employed to coat plastic plates in lectin ELISA (data not shown). Therefore,
234 it is feasible that SIGNR1 recognizes long internal α-mannose (tri- or more-mannose) capped
235 with β1,2-mannose in addition to α-mannose side chains.

236 Since RAW-SIGNR1 cells effectively bind and endocytose high molecular weight
237 FITC-dextran in an EDTA-sensitive manner (27), we carried out the inhibition analysis using
238 RAW-SIGNR1 cells to bind FITC-dextran instead of lectin ELISA (Fig. 2C). The inhibition
239 activities of glycans from *C. albicans* J-1012 and NIH B-792, and *S. cerevisiae* and its mutant
240 (*mnn1/mnn4*) were again comparably effective. The treatment of *C. albicans* J-1012 glycan with
241 α-mannosidase was also ineffective in reducing the inhibitory activity. In addition, low
242 efficiencies of glycans from *S. cerevisiae* (*mnn2*) and *C. lusitaniae* were also confirmed in this
243 experimental system. When resident peritoneal Mϕ that express SIGNR1 (27) were used, similar

244 results were obtained (data not shown). Together with the results using N-glycan only consisting
245 of α-mannose in side chain, these results strengthen the possibility that SIGNR1 recognizes both
246 α-mannose side chain and β1,2-mannose capped α-mannose side chains composed of more than
247 tri-mannoses in N-glycan.
248
249 **Specificity of N-glycan recognition by hDC-SIGN.** Cambi *et al*. previously reported that
250 hDC-SIGN recognizes glycans of *S. cerevisiae* strains less efficiently than those of Candida
251 strains (2). In contrast, SIGNR1 equally recognized glycans from both wild type and *mnn1/mnn4*
252 mutant of *S. cerevisiae* as *C. albicans*, suggesting that the specificities of hDC-SIGN and
253 SIGNR1 are somehow different from each other. In order to examine this possibility, we
254 prepared shDC-SIGN and compared the sugar specificity with that of sSIGNR1.
255 In lectin ELISA, the binding of shDC-SIGN to *C. albicans* J-1012 glycan was more
256 sensitive to fucose than mannose, and less sensitive to glucose and GlcNAc (Fig. 3A), as
257 reported (16). Results using this probe showed that shDC-SGIN bound comparably well to *C.*
258 *albicans* J-1012 and NIH B-792 (Fig. 3B). Unlike sSIGNR1, shDC-SIGN bound less to *S.*
259 *cerevisiae* microbes, as reported (2).
260 In addition, shDC-SIGN binding to *C. albicans* J-1012 glycan was not efficiently
261 blocked by glycans from *S. cerevisiae* and *mnn1/mnn4* mutants (Fig. 3C), indicating different
262 specificity in N-glycan recognition between hDC-SIGN and SIGNR1. It is worth noting that

263 treating *C. albicans* J-1012 glycan with α-mannosidase dramatically reduced its inhibitory
264 activity (Fig. 3C). We further confirmed the abrogation of inhibitory activity of N-glycan from *C.*
265 *albicans* J-1012 by α-mannosidase treatment by titrating its dose (Fig. 3D), showing that the
266 reduced activity is nearly comparable to that of *S. cerevisiae*. These results suggested that
267 hDC-SIGN only recognizes the α1,2-mannose at the non-reductive end of α-mannose side
268 chains, but not the internal α1,2-mannose capped with β1,2-mannose and short α-mannose
269 chains that are recognized by SIGNR1, of the N-glycan side chains in mannoprotein.

270 It has been reported that another SIGNR lectin, SIGNR3, also recognizes *C. albicans*
271 (27) and that its saccharide specificity resembles that of hDC-SIGN (20). Based on the inhibition
272 assay using lectin ELISA, SINGR3 was shown to bind a little more efficiently to wild type and
273 the *mnn1/mnn4* mutant of *S. cerevisiae* than hDC-SIGN but less so than SIGNR1
274 (Supplementary Fig. S2).

275

276 **Glyco-microarray analyses of SIGNR1 and hDC-SIGN.** Previously, Powlesland *et al.*
277 reported that sSIGNR1 did not bind any ligand on array using the regular method (20),
278 suggesting a weak affinity of SIGNR1. However, a sensitive glyco-microarray, based on an
279 evanescent-field fluorescence-assisted detection, has recently been developed (28). This method,
280 in which analysis was performed in the presence of lectin probe without washing, enabled us to
281 detect weak glycan-lectin interactions in the equilibrium state (15, 29), possibly representing

282 genuine interactions of ligand and cellular lectin.

283 An array plate immobilized with the glycans indicated (Fig. 4A and Supplementary Fig.
284 S1) was visualized by staining with Alexa647-coupled sSIGNR1/anti-SIGNR1 mAb complex
285 (Fig. 4B). The results clearly show that SIGNR1 binds to α-mannose and yeast mannans from *C.*
286 *albicans* and *S. cerevisiae*, but not β-mannose, in an EDTA-sensitive manner (Figs. 4B and C).
287 Of note, SIGNR1 did not recognize fucose-containing moieties, such as Fucα2Gal and
288 Lewis$^{a/b/x/y}$ antigen. The binding of sSIGNR1 to heparin is likely to be false positive, because of
289 the insensitivity to EDTA.

290 We previously performed array analyses using shDC-SIGN-Fc fusion as a probe (28).
291 However, structural forms between SIGNR1 and shDC-SIGN-Fc were different and this made it
292 difficult to compare the precise sugar specificities. Therefore, we again performed glyco-array
293 analysis using shDC-SIGN dimer as in sSIGNR1. The results clearly showed the recognition of
294 fucose-containing glycans and *C. albicans*, but not *S. cerevisiae*, mannan by shDC-SIGN (Fig.
295 4D), being consistent with our results in lectin ELISA and direct binding to microbes.

296 However, there are some discrepancies in our current observation and previous array
297 results. It has been shown that SIGNR1 can recognize fucose-containing moiety using
298 glyco-array and solid phase competition binding assays (5). One of our previous microarray
299 analyses also demonstrated that hDC-SIGN-Fc fusion was able to bind to glycans from *C.*
300 *albicans* and *S. cerevisiae* (28). In both reports, lectin CRD was fused with the Fc portion of IgG

and polymerized with anti-Fc polyclonal Ab, giving rise to a very large and multi-valent complexed probe. In the current glyco-microarray analyses, we utilized dimerized lectin probes, which possibly have lower avidity than those used in previous studies, enabling us to uncover the difference in binding activity of shDC-SIGN to *S. cerevisiae* and *C. albicans* glycan.

Biological significance of the different sugar specificity between SIGNR1 and hDC-SIGN in IL-10 production using RAW264.7 transfectants. To examine the biological significance of distinct saccharide recognition between SIGNR1 and hDC-SIGN, we measured IL-10 production by RAW-SIGNR1 and RAW-hDC-SIGN cells, because hDC-SIGN on DCs is known to induce IL-10 production by recognizing *C. albicans* (7), and lamina propria DCs expressing SIGNR1 are also capable of producing IL-10 (30). After stimulation with *C. albicans* J-1012 microbe and glycan coated on plastic plate, RAW-hDC-SIGN, but not RAW-SIGNR1, produced IL-10 (data not shown). Therefore, we prepared RAW264.7 cells expressing the chimeric hDC-SIGN molecule (RAW-chimera), of which CRD was replaced with SIGNR1 CRD, to compare glycan recognition specificity in terms of the induction of IL-10 production. The RAW-chimera cells expressed comparable levels of lectin to that of RAW-hDC-SIGN cells (Fig. 5A). Upon stimulation with *S. cerevisiae* X2180-1A (WT) glycan, RAW-hDC-SIGN and RAW-chimera cells produced equivalent amounts of IL-10 (Fig. 5B, *upper panel*). RAW-hDC-SIGN produced much less IL-10 in response to *S. cerevisiae* than *C. albicans* glycan in comparison with

RAW-chimera (Fig. 5B, *lower panel*). Interestingly, in the case of *C. albicans* J-1012 glycan, RAW-hDC-SIGN produced more IL-10 than RAW-chimera (Fig. 5B, *upper panel*). However, the former significantly reduced IL-10 production after α-mannosidase treatment compared with the latter (Fig. 5C), being consistent with the results showing that SIGNR1, but not hDC-SING, recognizes β-mannose capped α-mannose side chains in *C. albicans* glycan.

It is feasible that *C. albicans* induces higher IL-10 production than *S. cerevisiae via* hDC-SIGN on DCs in humans, leading to the immunosuppressive milieu at the site of infection. This may explain why *C. albicans* is more virulent than *S. cerevisiae*. Regarding the sugar specificity of SIGNR1, there were no significant differences in the binding specificity to mannose moieties between *C. albicans* and *S. cerevisiae* at the molecular level. However, IL-10 production was slightly but significantly higher in response to *C. albicans* than *S. cerevisiae,* implying that cellular responses by the recognition through lectin receptors is affected by some other unknown factors. In this study, we used glycans prepared from the blastospore (yeast) form of each yeast strain. However, it should also be kept in mind that the difference in the growth form between *C. albicans* and *S. cerevisiae* might modulate cellular activity against microbes *in situ*. hDC-SIGN in the mouse cells prepared in this study properly transduce signals for IL-10 production. Although it is unknown how hDC-SIGN activates the mouse Src family and subsequent Raf-1 kinase in this situation, hDC-SIGN likely has a certain motif that is lacking in SIGNR1, to work in both human and mouse cells for IL-10 production.

339

340 Collectively, SIGNR1 and hDC-SIGN bind polysaccharides in surface mannoprotein
341 on live *C. albicans*. However, SIGNR1 recognizes both α-mannose and β1,2-mannose capped
342 α-mannose side chains composed of more than tri-mannoses, whereas hDC-SIGN recognizes
343 only α-mannose at the non-reductive end of the side chains, but not internal α-mannose capped
344 with β1,2-mannose and short α-mannose chains, of N-glycan side chains in mannoprotein.
345 Differential recognition of yeast strains by SIGNR1 and hDC-SIGN may be relevant to the
346 differences in cellular responsiveness.

347

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Figure legends

Fig. 1. sSIGNR1 binds various types of yeast strains.

(A) Structural diagrams of N-glycan used in this study. Structures of N-glycan of *C. albicans* J-1012 (25), *C. albicans* NIH B-792 (22), *C. stellatoidea* (24), *C. parapsilosis* (22) and *C. lusitaniae* (23) are adopted from our structural analyses using NMR. Structure of *S. cerevisiae* wild type, *S. cerevisiae* 4484-24D-1 (*mnn1*/*mnn4*) and *S. cerevisiae* X2180-1-A-5 (*mnn2*) are based on previous reports (1, 21). Side chains that are digested by treatment with α-mannosidase in *C. albicans* J-1012 N-glycan are *shaded*. The side chain sequence is not specified. (B) Binding of sSIGNR1 to Candida and *S. cerevisiae* strains. PE-*Strep*-Tactin alone (-) or PE-sSIGNR1 (+) were incubated with the indicated live yeast strains with or without EDTA (25 mM). (C) Inhibition of sSIGNR1 binding to *C. albicans* J-1012 by glycans purified from the *C. albicans* and *S. cerevisiae* strains indicated. sSIGNR1 was pre-incubated with 50 μg/ml of glycans before mixing with microbes. Glycogen was used as a negative control. Inhibition is indicated as the percent decrease of fluorescence intensity in experimental groups compared with the control without inhibitor. The results are shown as the mean ± SD of triplicate assays. $*p < 0.05$ on solid line by Tukey's multiple range test. Grey lines show no significant differences.

Fig. 2. Recognition of α-mannose side chains in N-glycan by sSIGNR1.

(A) Inhibition analysis by lectin ELISA. Binding of sSIGNR1 to *C. albicans* J-1012 glycan

471 coated on microtiter plates was analyzed in the presence of glycans (25 μg/ml) purified from
472 various types of yeast strains. Blocking activities of inhibitors are shown as the % inhibition of
473 sSIGNR1 binding. (B) Titration of inhibitory activity of glycans from the indicated yeast strains
474 for sSIGNR1 binding by lectin ELISA. Half of maximal inhibition activity was indicated by
475 dashed line. (C) Inhibition of FITC-binding to RAW-SIGNR1 cells by glycans. Transfectants
476 were incubated with the graded dose of glycans as in (B) prior to FITC-dextran. The results are
477 shown as % inhibition. Results are shown as the mean ± SD of triplicate assays. *$p < 0.05$ on
478 solid line by Tukey's multiple range test. Grey lines show no significant differences.
479
480 Fig. 3. Binding of shDC-SIGN to microbes and inhibition of shDC-SIGN binding by glycans.
481 (A) Inhibition analysis of shDC-SIGN binding by monosaccharides (50 mM) and glycan from *C.*
482 *albicans* J-1012 using lectin ELISA. (B) Binding of shDC-SIGN to yeast strains was analyzed as
483 in Fig. 1B. (C) Inhibition analysis using glycans from various yeast strains as in Fig. 2A. (D)
484 Inhibition assay was performed in the presence of graded doses of glycans from the indicated
485 yeast strains. Half of maximal inhibition activity was indicated by dashed line. The results are
486 shown as the mean ± SD of triplicate assays. *$p < 0.05$ on solid line by Tukey's multiple range
487 test. Grey lines show no significant differences.
488
489 Fig. 4. Glyco-microarray analysis of sSIGNR1.

(A) The layout of glyco-microarray. (B) Results of the glyco-microarray analyses. Binding of soluble SIGNR1/Alexa647-anti-SIGNR1 mAb immune complex to the array was performed in the absence (*left panel*) or presence (*right panel*) of 10 mM EDTA and detected by an evanescent-field fluorescence-assisted scanner. (C) Data analyzed with the Array Pro analyzer ver. 4.5. (D) Glyco-array analysis was performed using immune complex of soluble hDC-SIGN/Alexa555-anti-hDC-SIGN mAb as in sSIGNR1.

Fig. 5. IL-10 production of RAW264.7 transfectants upon stimulation with glycan coated on plastic plate.

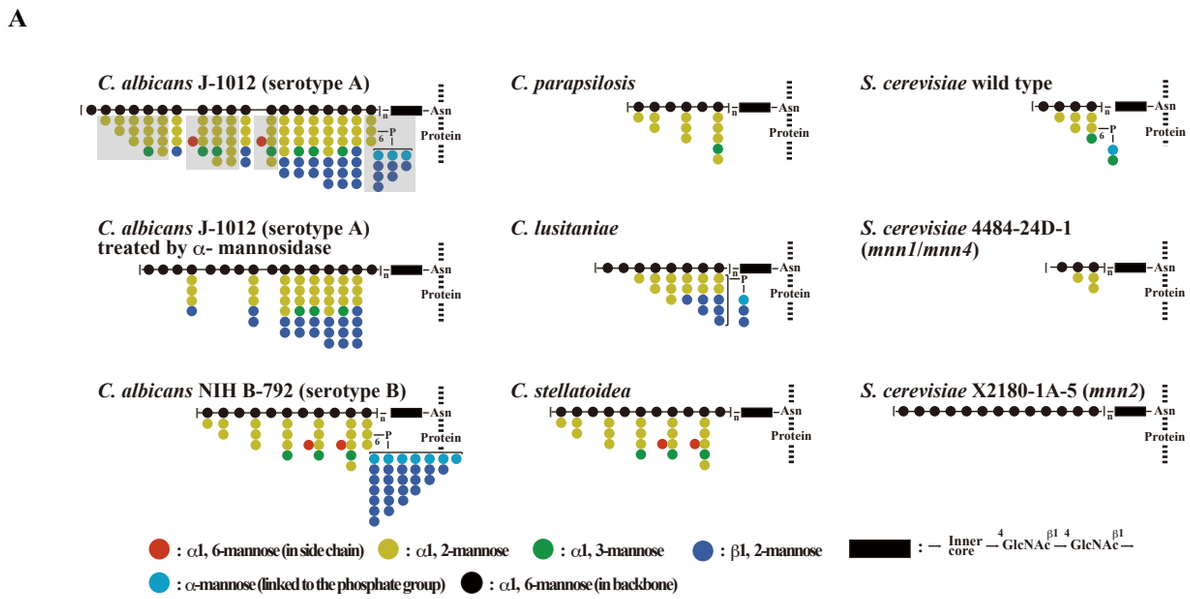
(A) RAW-control, RAW-hDC-SIGN and RAW-chimera cells were analyzed by flowcytometry using polyclonal anti-hDC-SIGN Ab and anti-SIGNR1 mAb (22D1) specific to SIGNR1 CRD. (B) The transfectants (5 x 10^4 cells) were cultured on plates pre-coated with *C. albicans* J-1012 and *S. cerevisiae* X2180-1A (WT) glycan in the presence or absence of LPS (100 ng/ml). After 24 h, IL-10 in the supernatants was analyzed (*upper panel*). IL-10 production against *S. cerevisiae* glycan is shown as a percentage to that against *C. albicans* glycan (*lower panel*). (C) IL-10 production after stimulation with native and α-mannosidase-treated *C. albicans* J-1012 glycan was analyzed as in B. IL-10 production against the treated glycan is shown as a percentage to that against the native glycan. $^{**}p = 0.0067$, $^{***}p = 0.0001$ by Student's *t*-test.

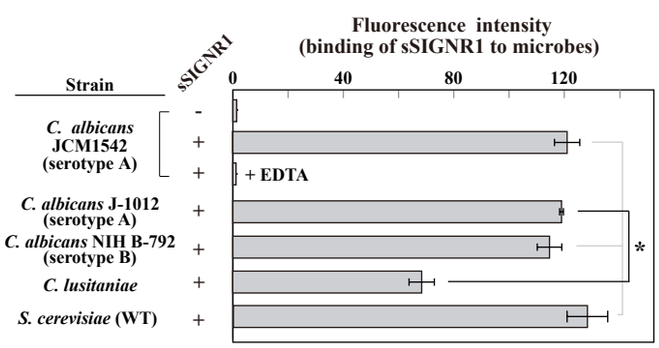
TABLE 1. Composition of side chains and properties of N-glycan used in this study.

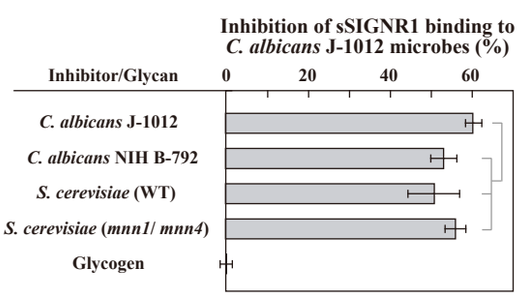
N-glycan	Presence of side chain[a]				
	α1,3-mannose	α1,2-mannose	α1,6-mannose[b]	β1,2-mannose	PM
C. albicans J-1012 (serotype A)	+	+	+	+	+
C. albicans NIH B-792 (serotype B)	+	+	+	+	+
S. cerevisiae (WT)	-	+	-	-	+
S. cerevisiae 4484-24D-1 (*mnn1*/*mnn4*)	-	+	-	-	-
S. cerevisiae X2180-1A-5 (*mnn2*)	-	-	-	-	-
C. stellatoidea	+	+	+	-	-
C. parapsilosis	+	+	-	-	-
C. lusitaniae	-	+	-	+	+
C. albicans J-1012 (α-mannosidase treated)	+	+	-	+	-

[a] PM, phosphorylated mannose. Side chain structure presence and absence in the N-glycan are indicated by + and -, respectively.

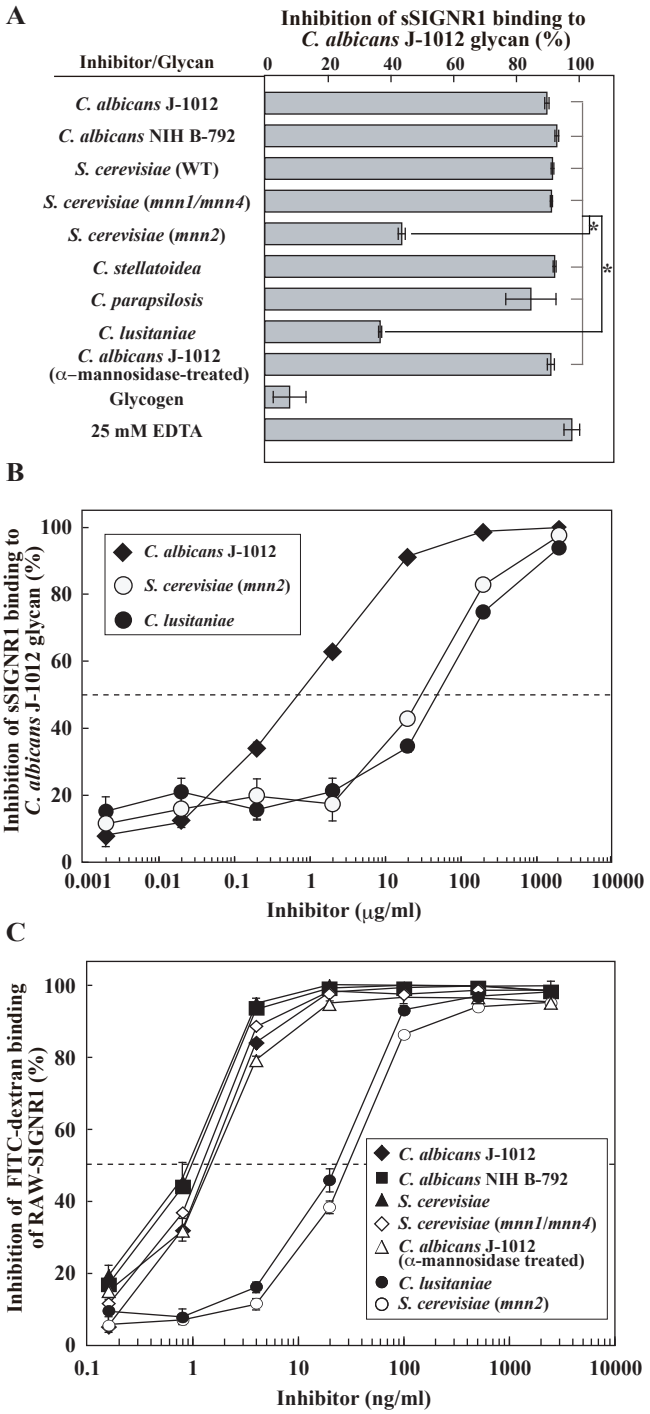
[b] Branching in side chain.



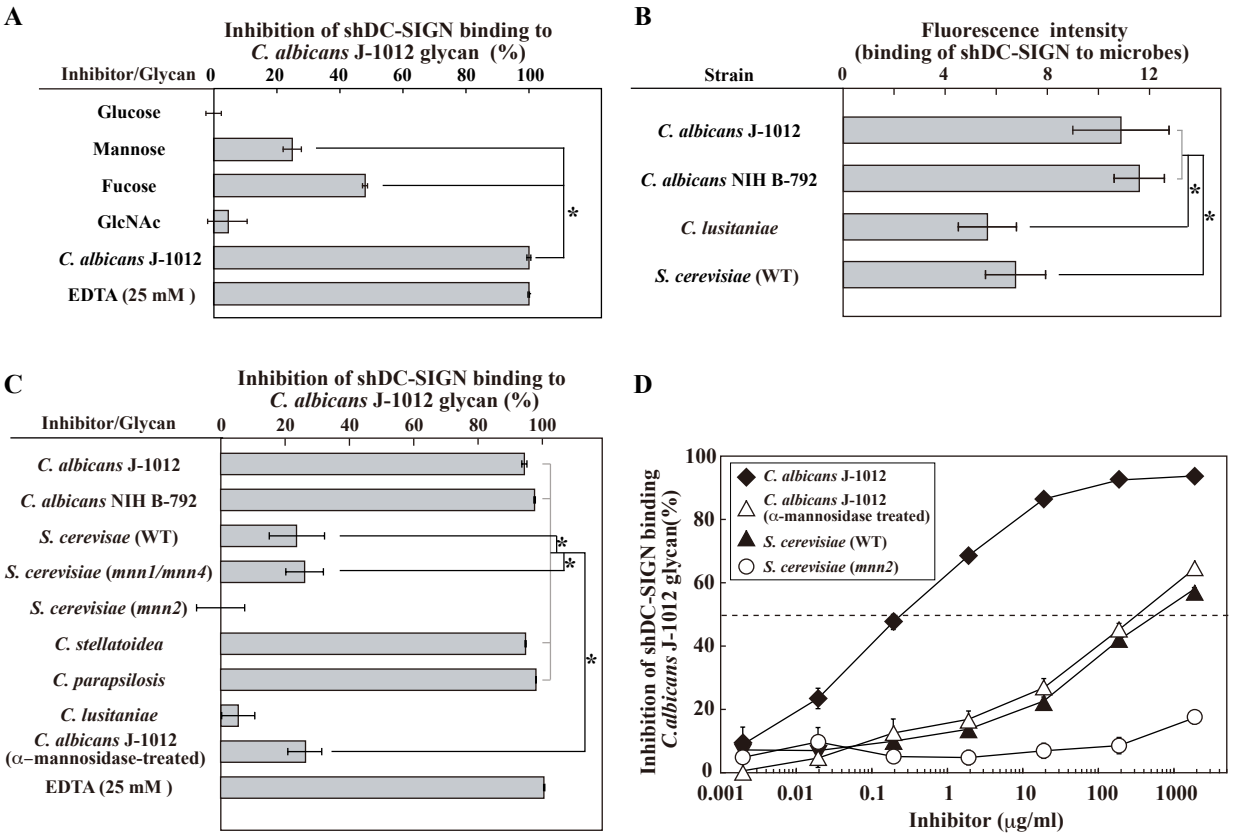




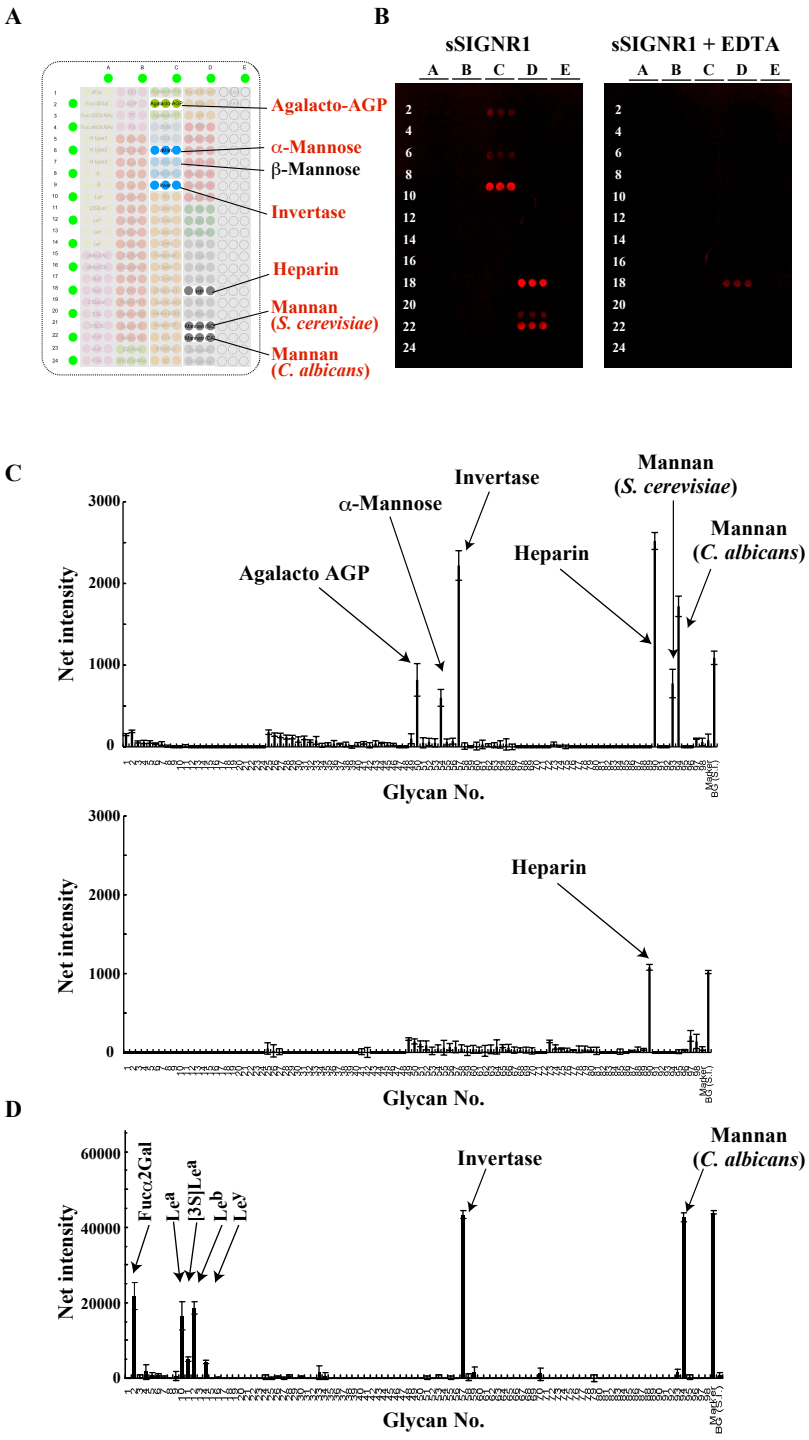
Takahara *et al*. Fig. 1.



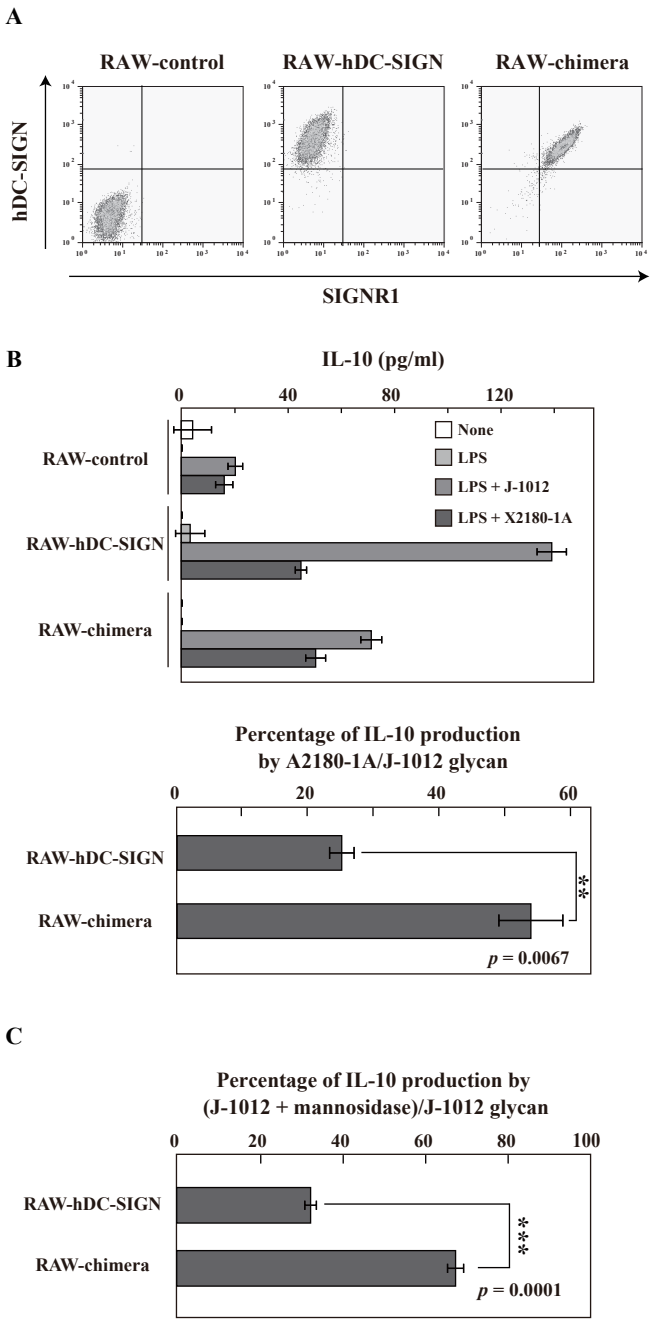
Takahara *et al*. Fig. 2.



Takahara *et al*. Fig. 3.



Takahara *et al*. Fig. 4.



Takahara *et al*. Fig. 5.

Trivial name	Presentation	Glycans	Co.	Cat#
αFuc	PAA	Fucα1-PAA	Glycotech	01-007
Fucα2Gal	PAA	Fucα1-2Galβ1-PAA	Glycotech	01-019
Fucα3GlcNAc	PAA	Fucα1-3GlcNAcβ1-PAA	Glycotech	01-024
Fucα4GlcNAc	PAA	Fucα1-4GlcNAcβ1-PAA	Glycotech	01-025
H type1	PAA	Fucα1-2Galβ1-3GlcNAcβ1-PAA	Glycotech	01-037
H type2	PAA	Fucα1-2Galβ1-4GlcNAcβ1-PAA	Glycotech	08-034
H type3	PAA	Fucα1-2Galβ1-3GalNAcα1-PAA	Glycotech	08-060
A	PAA	GalNAcα1-3(Fucα1-2)Galβ1-4GlcNAcβ1-PAA	Glycotech	08-091
B	PAA	Galα1-3(Fucα1-2)Galβ1-4GlcNAcβ1-PAA	Glycotech	08-092
Le^a	PAA	Galβ1-3(Fucα1-4)GlcNAcβ1-PAA	Glycotech	01-035
[3S]Le^a	PAA	(3OSO$_3$)Galβ1-3(Fucα1-4)GlcNAcβ1-PAA	Glycotech	01-040
Le^b	PAA	Fucα1-2Galβ1-3(Fucα1-3)GlcNAcβ1-PAA	Glycotech	08-042
Le^x	PAA	Galβ1-4(Fucα1-3)GlcNAcβ1-PAA	Glycotech	01-036
Le^y	PAA	Fucα1-2Galβ1-4(Fucα1-3)GlcNAcβ1-PAA	Glycotech	08-043
αNeu5Ac	PAA	Neu5Acα2-PAA	Glycotech	01-012
αNeu5Gc	PAA	Neu5Gcα2-PAA	Glycotech	01-051
Sia2	PAA	Neu5Acα2-8Neu5Acα2-PAA	Glycotech	08-064
Sia3	PAA	Neu5Acα2-8Neu5Acα2-8Neu5Acα2-PAA	Glycotech	01-081
3'SiaLe^c	PAA	Neu5Acα2-3Galβ1-3GlcNAcβ1-PAA	Glycotech	01-078
3'SL	PAA	Neu5Acα2-3Galβ1-4Glcβ1-PAA	Glycotech	01-038
3'SLN	PAA	Neu5Acα2-3Galβ1-4GlcNAcβ1-PAA	Glycotech	01-077
sLe^a	PAA	Neu5Acα2-3Galβ1-3(Fucα1-4)GlcNAcβ1-PAA	Glycotech	08-044
sLe^x	PAA	Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAcβ1-PAA	Glycotech	01-045
6'SL	PAA	Neu5Acα2-6Galβ1-4Glcβ1-PAA	Glycotech	01-039
FET	Glycoprotein	Fetuin (Complex-type N-glycans and O-glycans)	Sigma	F3004
AGP	Glycoprotein	α1-acid glycoprotein (Complex-type N-glycans-)	Sigma	G9885
TF	Glycoprotein	Transferrin (Complex-type N-glycans)	Sigma	T3309
TG	Glycoprotein	Porcine thyroglobulin (Complex and high-mannnose-type N-glycans)	Sigma	T1126
βGal	PAA	Galβ1-PAA	Glycotech	01-004
[3S]βGal	PAA	(3OSO$_3$)Galβ1-PAA	Glycotech	01-015
A-di	PAA	GalNAcα1-3Galβ1-PAA	Glycotech	01-017
Lac	PAA	Galβ1-4Glcβ1-PAA	Glycotech	01-021
Le^c	PAA	Galβ1-3GlcNAcβ1-PAA	Glycotech	01-020
[3'S]Le^c	PAA	(3OSO$_3$)Galβ1-3GlcNAcβ1-PAA	Glycotech	01-062
LN	PAA	Galβ1-4GlcNAcβ1-PAA	Glycotech	01-022
[3'S]LN	PAA	(3OSO$_3$)Galβ1-4GlcNAcβ1-PAA	Glycotech	01-061
[6S]LN	PAA	Galβ1-4(6OSO$_3$)GlcNAcβ1-PAA	Glycotech	01-066
[6'S]LN	PAA	(6OSO$_3$)Galβ1-4GlcNAcβ1-PAA	Glycotech	01-068
βGalNAc	PAA	GalNAcβ1-PAA	Glycotech	01-011
di-GalNAcβ	PAA	GalNAcβ1-3GalNAcβ1-PAA	Glycotech	01-070
LDN	PAA	GalNAcβ1-4GlcNAcβ1-PAA	Glycotech	01-057
GA2	PAA	GalNAcβ1-4Galβ1-4Glcβ1-PAA	Glycotech	08-074
Asialo-FET	Glycoprotein	Asialo fetuin (Desialylated complex-type N- and O-glycans)	Sigma	F3004 (Acid-treated)
Asialo-AGP	Glycoprotein	Asialo α1-acid glycoprotein (Desialylated complex-type N-glycans)	Sigma	G9885 (Acid-treated)
Asialo-TF	Glycoprotein	Asialo transferrin (Desialylated complex-type N-glycans)	Sigma	T3309 (Acid-treated)
Asialo-TG	Glycoprotein	Asialo porcine thyroglobulin (Desialylated complex-type N-glycans, high-mannose-type N-glycans)	Sigma	T1126 (Acid-treated)

βGlcNAc	PAA	GlcNAcβ1-PAA	Glycotech	01-009
[6S]βGlcNAc	PAA	(6OSO$_3$)GlcNAcβ1-PAA	Glycotech	01-016
Agalacto-Fet	Glycoprotein	Agalacto fetuin (Agalactosylated complex-type N- and O-glycans)	Sigma	F3004 (Galactosidase-treated)
Agalacto-AGP	Glycoprotein	Agalacto α1-acid glycoprotein (Agalactosylated complex-type N-glycans)	Sigma	G9885 (Galactosidase-treated)
Agalacto-TF	Glycoprotein	Agalacto transferrin (Agalactosylated complex-type N-glycans, high-mannose-type N-glycans)	Sigma	T3309 (Galactosidase-treated)
OVM	Glycoprotein	Ovomucoid (Complex-type N-glycans)	Sigma	T2011
OVA	Glycoprotein	Ovoalbumin (Hybrid-type N-glycans)	Sigma	A2512
αMan	PAA	Manα1-PAA	Glycotech	01-005
βMan	PAA	Manβ1-PAA	Glycotech	01-050
[6P]Man	PAA	(6OPO$_4$)Manα1-PAA	Glycotech	01-006
INV	Glycoprotein	Yeast invertase (High mannose-type N-glycans)	Sigma	I4504
Tn	PAA	GalNAcα1-PAA	Glycotech	01-010
Core1	PAA	Galβ1-3GalNAcα1-PAA	Glycotech	08-023
Core2	PAA	Galβ1-3(GlcNAcβ1-6)GalNAcα1-PAA	Glycotech	01-083
Core3	PAA	GlcNAcβ1-3GalNAcα1-PAA	Glycotech	01-071
Core4	PAA	GlcNAcβ1-3(GlcNAcβ1-6)GalNAcα1-PAA	Glycotech	01-089
Forssman	PAA	GalNAcα1-3GalNAcβ1-PAA	Glycotech	01-026
Core6	PAA	GlcNAcβ1-6GalNAcα1-PAA	Glycotech	01-072
Core8	PAA	Galα1-3GalNAcα1-PAA	Glycotech	01-028
[3'S]Core1	PAA	(3OSO$_3$)Galβ1-3GalNAcα1-PAA	Glycotech	08-069
Galβ-Core3	PAA	Galβ1-4GlcNAcβ1-3GalNAcα1-PAA	Glycotech	01-116
Asialo-BSM	Glycoprotein	Asialo bovine submaxillary mucin (Tn)	Sigma	M3895 (Acid-treated)
Asialo-GP	Glycoprotein	Asialo human glycophorin MN (T)	Sigma	A9791 (Acid-treated)
STn	PAA	Neu5Acα2-6GalNAcα1-PAA	Glycotech	01-059
STn (Gc)	PAA	Neu5Gcα2-6GalNAcα1-PAA	Glycotech	01-107
ST	PAA	Neu5Acα2-3Galβ1-3GalNAcα1-PAA	Glycotech	01-088
Siaα2-6Core 1	PAA	Galβ1-3(Neu5Acα2-6)GalNAcα1-PAA	Glycotech	01-113
BSM	Glycoprotein	Bovine submaxillary mucin (Sialyl Tn)	Sigma	M3895
GP	Glycoprotein	Human glycophorin (Disialyl T and sialyl Tn)	Sigma	G5017
αGal	PAA	Galα1-PAA	Glycotech	01-003
Galα1-2Gal	PAA	Galα1-2Galβ1-PAA	Glycotech	01-056
Galα1-3Gal	PAA	Galα1-3Galβ1-PAA	Glycotech	01-018
Galα1-3Lac	PAA	Galα1-3Galβ1-4Glcβ1-PAA	Glycotech	01-075
Galα1-3LN	PAA	Galα1-3Galβ1-4GlcNAcβ1-PAA	Glycotech	01-079
Galα1-4LN	PAA	Galα1-4Galβ1-4GlcNAcβ1-PAA	Glycotech	01-110
Melibiose	PAA	Galα1-6Glcβ1-PAA	Glycotech	01-063
αGlc	PAA	Glcα1-PAA	Glycotech	01-001
βGlc	PAA	Glcβ1-PAA	Glycotech	01-002
Maltose	PAA	Glcα1-4Glcβ1-PAA	Glycotech	01-054
HA	BSA	Hyaluronic acid-BSA	Seikagaku	400720
CSA	BSA	Chondoroitin Sulfate A-BSA	Seikagaku	400655
CSB	BSA	Chondoroitin Sulfate B-BSA	Seikagaku	400660
HS	BSA	Heparan Sulfate-BSA	Seikagaku	400700
HP	BSA	Heparin-BSA	Calbiochem	375095
KS	BSA	Keratan Sulfate-BSA	Seikagaku	400760
αRha	PAA	Rhamnoseα1-PAA	Glycotech	01-008
Mannan (SC)	Glycoprotein	*S. cerevisiae* mannan	Sigma	M7504
Mannan (CA)	Glycoprotein	*C.albicans* mannan	Takara	MG001
Zymosan	Glycoprotein	Zymosan	Sigma	Z4250
Chitobiose	PAA	GlcNAcβ1-4GlcNAcβ1-PAA	Glycotech	08-057
BSA	Glycoprotein	-	Sigma	A7638
Negative PAA	PAA	-	Glycotech	01-000

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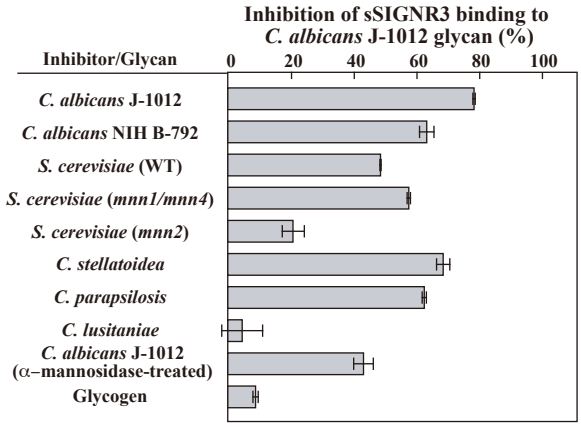
Precise information of ligands on the glyco-microarray.



Takahara *et al.* Fig. S2.

Inhibition analysis by lectin ELISA. Binding of sSIGNR3 to *C. albicans* J-1012 glycan coated on microtiter plates was analyzed as in Fig. 2A.