C-type lectin receptors (CLRs) comprise a large family of immunoreceptors that recognize polysaccharide ligands exposed on pathogen surfaces and are conserved among mammals. However, interspecies differences in their ligand spectra are not fully understood. Dectin-1 is a well-characterized CLR that recognizes β-glucan. We report here that seaweed-derived fucan activates cells expressing human Dectin-1 but not mouse Dectin-1. Low-valency β-glucan components within fucan appeared to be responsible for this activation, as the ligand activity was eliminated by β-glucanase treatment. The low-valency β-glucan laminarin also acted as an agonist for human Dectin-1 but not for mouse Dectin-1, whereas the high-valency β-glucan curdlan activated both human and mouse Dectin-1. Reciprocal mutagenesis analysis revealed that the ligand-binding domain of human Dectin-1 does not determine its unique sensitivity to low-valency β-glucan. Rather, we found that its intracellular domain renders human Dectin-1 reactive to low-valency β-glucan ligand. Substitution with two amino acids, Glu2 and Pro5, located in the human Dectin-1 intracellular domain was sufficient to confer sensitivity to low-valency β-glucan in mouse Dectin-1. Conversely, the introduction of mouse-specific amino acids, Lys2 and Ser5, to human Dectin-1 reduced the reactivity to low-valency β-glucan. Indeed, low-valency ligands induced a set of proinflammatory genes in human but not mouse dendritic cells. These results suggest that the intracellular domain, not ligand-binding domain, of Dectin-1 determines the species-specific ligand profile.

Our bodies are continuously exposed to and infected by various types of pathogens, most of which are directly recognized by pattern recognition receptors such as Toll-like receptors, RIG-I-like receptors, or NOD-like receptors on host cells (1, 2). An additional fourth member of pattern recognition receptors is the emerging C-type lectin receptors (CLRs)3 that senses pathogens or damaged tissues to trigger innate immune responses (3).

Within this family, Dectin-1 is the first immunoreceptor tyrosine-based activation motif (ITAM)-coupled CLR identified and recognizes β-glucans present in the cell wall of fungi (4–6). Dectin-1 is a type II transmembrane protein expressed by myeloid cells and consists of an extracellular carbohydrate recognition domain (CRD) and a cytoplasmic domain containing a binding site for the Syk kinase. The recruited Syk then activates the CARD9-Bcl10-MALT1 and NF-κB pathways to induce inflammatory cytokines, co-stimulatory molecules, and dendritic cell maturation, which promotes Th1 and Th17 responses to orchestrate immunity to pathogens (7, 8). The CRD and hemITAM regions of Dectin-1 are conserved among mammals (5, 9), suggesting the importance of this CLR for promoting acquired immune responses over a wide variety of species. Hence, Dectin-1 agonists hold potential as vaccine adjuvants that may facilitate protective immune responses against pathogens or cancer in mouse models and human patients. Given this potential, it is important to characterize in detail the function of human Dectin-1 in comparison with the evidences accumulating for the more extensively studied mouse Dectin-1 (10, 11).

The ligand potency of β-glucans toward Dectin-1 varies according to their valency. Among several β-glucan-containing polysaccharides, yeast zymosan and bacterial curdlan act as potent agonists, whereas the ligand activity of low valency β-glucans, such as seaweed laminarin, is controversial (12–15).

3 The abbreviations used are: CLR, C-type lectin receptor; hDectin-1, human Dectin-1; mDectin-1, mouse Dectin-1; rDectin-1, rat Dectin-1; CRD, carbohydrate recognition domain; FcR, Fc receptor γ-chain; NFAT, nuclear factor of activated T-cells; hemITAM, hem-immunoreceptor tyrosine-based activation motif; hMoDC, human monocyte-derived dendritic cell; mBMDC, mouse bone marrow-derived dendritic cell; PE, phycoerythrin; PBMC, peripheral blood mononuclear cell.
The inconsistency within studies on the “low-valency” Dectin-1 ligand may be partly due to interspecies difference of Dectin-1. In addition, the function of the cytoplasmic domain, other than its identity as a hemITAM, is not well-characterized.

In this study, we found that low-valency β-glucan can activate cells expressing human Dectin-1, but not mouse Dectin-1. Reciprocal mutagenesis studies revealed that the intracellular domain of human Dectin-1 confers this activity. Furthermore, we found that two intracellular amino acids, which are conserved in primates, play a critical role for enhancing the sensitivity of Dectin-1 independently of the hemITAM.

Results

Fucan activates reporter cells expressing hDectin-1 but not mDectin-1

To search for novel CLRs that recognize natural polysaccharides, we employed nuclear factor of activated T-cells (NFAT)-GFP reporter cells expressing various CLRs. After screening several candidates, we found a seaweed-derived, fucose-containing polysaccharide called fucan activated reporter cells expressing human Dectin-1 (hDectin-1) (Fig. 1). Indeed, hDectin-1 induced reporter activity in response to fucan in a dose-dependent manner (Fig. 2A). A hDectin-1 isoform lacking the stalk region (hDectin-1B) similarly responded to fucan (supplemental Fig. S1). However, fucan did not activate cells expressing either forms of mouse Dectin-1 (mDectin-1) (Fig. 2A and supplemental Fig. S1). In contrast, curdlan, a high-molecular weight β-1,3-glucan, comparably activated the reporter for either hDectin-1 or mDectin-1 (Fig. 2B). From these observations, we initially hypothesized that hDectin-1 but not mDectin-1 binds to fucan.

β-Glucan components contained in fucan activate cells expressing hDectin-1

As β-1,3-glucan is a well-established component recognized by Dectin-1 (4), we next examined whether treatment with Westase, a β-1,3- and β-1,6-glucanase, eliminates the activity of fucan. As expected, Westase treatment completely suppressed the ability of curdlan to activate cells via hDectin-1 and mDectin-1 (Fig. 3A, left). Likewise, fucan treated with this enzyme failed to activate cells expressing hDectin-1 (Fig. 3A, right), suggesting that β-glucan-like components included in fucan are responsible for this activity. Size-exclusion separation revealed that the ligand activity was detected in low-molecular weight fractions (supplemental Fig. S2). We therefore characterized the species-specific ligand activity of laminarin, low-molecular weight β-glucan derived from brown seaweed. As with fucan, laminarin activated the reporter cells expressing hDectin-1, but not mDectin-1, and this activity was sensitive to Westase (Fig. 3B). These data suggest that low-valency, soluble β-glucans may selectively activate cells via hDectin-1 but not mDectin-1.

Non-CRD region of hDectin-1 confers reactivity to low-valency β-glucan

As the CRD of Dectin-1 mediates direct ligand binding (16), we initially suspected that the slightly different amino acid sequences within the CRDs comparing hDectin-1 and mDectin-1 might determine the reactivity to soluble β-glucan. To address this possibility, we generated a chimeric mDectin-1 protein harboring the CRD from hDectin-1 (mDectin-1hCRD chimera) (Fig. 4A). Contrary to our initial assumption, the reporter cells expressing the mDectin-1hCRD chimera were not activated by laminarin similar to mDectin-1-bearing cells (Fig. 4B, mD1hCRD), although they showed substantial activity upon stimulation with high-valency curdlan (Fig. 4C). These results suggest that the direct ligand-binding domain, CRD, is not responsible for determining laminarin sensitivity to hDectin-1. We therefore created a hDectin-1 chimera harboring the CRD.
from mDectin-1 (hDectin-1\textsuperscript{mCRD}) and found that the combination of mouse CRD and human non-CRD acted as a functional activating receptor for laminarin (Fig. 4\textsuperscript{B}, hD1mCRD), suggesting that mouse CRD is capable of recognizing low-valency beta-glucan. Indeed, fluorescence-labeled laminarin did bind to mDectin-1 as well as hDectin-1 (supplemental Fig. S3). Furthermore, soluble Ig-fusion protein derived from mDectin-1 and hDectin-1 showed comparable binding to laminarin (Fig. 4\textsuperscript{D}). Taken together, these findings suggest that the non-CRD region of hDectin-1 confers the sensitivity to laminarin.

**Opposite effect of low-valency beta-glucan on mDectin-1 and hDectin-1**

Given the observation that mDectin-1 binds low-valency beta-glucan but is unable to deliver activating signaling, we next examined whether these ligands act as antagonists for mDectin-1. To assess this, we added a graded amount of fucan or laminarin (Fig. 4\textsuperscript{B}, hD1mCRD), suggesting that mouse CRD is capable of recognizing low-valency beta-glucan. Indeed, fluorescence-labeled laminarin did bind to mDectin-1 as well as hDectin-1 (supplemental Fig. S3). Furthermore, soluble Ig-fusion protein derived from mDectin-1 and hDectin-1 showed comparable binding to laminarin (Fig. 4\textsuperscript{D}). Taken together, these findings suggest that the non-CRD region of hDectin-1 confers the sensitivity to laminarin.

**Cytoplasmic N-terminal region of hDectin-1 determines its sensitivity to low-valency beta-glucan**

As the stalk region of hDectin-1 is dispensable for the reactivity to fucan and laminarin (supplemental Fig. S1A), we next focused on the cytoplasmic domain of hDectin-1. To this end, we generated a series of chimeric mDectin-1 proteins in which each region of the cytoplasmic domain was replaced with the corresponding region of hDectin-1 (Fig. 6\textsuperscript{A}). In contrast to full-length (WT) mDectin-1, mDectin-1 possessing 30 amino acids of the human N-terminal region (mDectin-1hN30) normally responded to laminarin (Fig. 6\textsuperscript{B}) and (C). Further chimeric analysis revealed that as little as a 10-amino acid sequence at the N terminus of hDectin-1 (mDectin-1hN10) was sufficient to confer reactivity to low-valency beta-glucan to mDectin-1 (Fig. 6\textsuperscript{B}).

**Glu\textsuperscript{2} and Pro\textsuperscript{5} of hDectin-1 confer sensitivity to low-valency beta-glucan on mDectin-1**

Within the 10-amino acid N-terminal sequence, hDectin-1 differs from mDectin-1 in four residues: Glu\textsuperscript{2}, Pro\textsuperscript{5}, Asp\textsuperscript{6}, and Leu\textsuperscript{7} (Fig. 7\textsuperscript{A}). We therefore individually substituted these 4 amino acids of mDectin-1 with the corresponding human-specific residues (Fig. 7\textsuperscript{B}). Mutants carrying the combination of K2E and S5P substitutions selectively responded to laminarin (Fig. 7\textsuperscript{C}, mD1\textsuperscript{K2E/S5P/H6D/I7L}, mD1\textsuperscript{K2E/S5P/I7L}, mD1\textsuperscript{K2E/S5P/H6D}, and mD1\textsuperscript{K2E/S5P}). Conversely, the introduction of mouse-specific Lys\textsuperscript{2} and Ser\textsuperscript{5} substitutions into hDectin-1 (hDectin-1\textsuperscript{K2K/PSS}) resulted in the reduction of its reporter activity against laminarin (supplemental Fig. S4). Taken together, these results suggest that two amino acids derived from mDectin-1, Lys\textsuperscript{2} and Ser\textsuperscript{5}, are critical for “desensitizing” Dectin-1 to low-valency beta-glucan. The importance of these 2 amino acids are also supported by Dectin-1 derived from another species, rat. Although

**Figure 2. Fucan activates 2B4 NFAT-GFP reporter cells expressing hDectin-1 but not mDectin-1.** A and B, 2B4 NFAT-GFP reporter cells transduced without (Syk) or with hDectin-1 or mDectin-1 were stimulated with the indicated concentration of fucan (A) or curdlan (B) for 18 h and expression of GFP analyzed by flow cytometry. All data are presented as the mean ± S.D., and representative results from three independent experiments with similar results are shown.

**Figure 3. Westase treatment eliminates the hDectin-1 activity to fucan as well as laminarin.** A and B, 2B4 NFAT-GFP reporter cells were left unstimulated or stimulated with curdlan, fucan (A), or laminarin (B) at the indicated concentrations. Ligands were left untreated (Buffer) or treated with Westase (Westase). GFP expression was analyzed by flow cytometry. All data are presented as the mean ± S.D., and representative results from two independent experiments with similar results are shown.
rat Dectin-1 (rDectin-1) shares high homology with mDectin-1, it uniquely lacks the N-terminal 9-amino acid sequence that contains Lys2 and Ser5 in mouse (Fig. 8, A and B, and supplemental Fig. S7). In the reporter cell assay, rDectin-1 was found to respond well to laminarin (Fig. 8, C and D), supporting our idea that inactivation of Lys2 and Ser5 may sensitize Dectin-1 to low-valency β-glucan.

Low-valency Dectin-1 ligands activate human DCs but not murine DCs

To confirm the observed hDectin-1-specific phenomenon using primary cells, we finally compared the reactivity of human and murine myeloid cells to low-valency Dectin-1 ligand in a non-biased manner by stimulating human monocyte-derived dendritic cells (hMoDCs) and mouse bone marrow-derived dendritic cells (mBMDCs) with laminarin.
**Intracellular non-ITAM sequence sensitizes human Dectin-1**

**Discussion**

In this study, we report that hDectin-1 responds to low-valency β-glucan and its cytoplasmic region is critical for this function.

Within the cytoplasmic N terminus, we show that Glu² of hDectin-1 is one of the critical residues controlling the sensitivity of hDectin-1 to low-valency β-glucan, as a substitution of a mouse-specific residue in this position (hDectin-1E²K) impaired this activity. It appears that it is important that the residue at position 2 be a “non-Lys” residue, as substitutions to other residues did not reduce the activity (hDectin-1E²A or hDectin-1E²D) (supplemental Fig. S6). In line with these observations, Lys residues of mDectin-1 are reported to undergo ubiquitination, which results in the degradation and desensitization of mDectin-1 upon ligand binding (19). Thus, the inactivation of the Lys² residue might be one of the reasons that sensitize hDectin-1.

Meanwhile, the precise role of hDectin-1 Pro⁵, which is conserved in primates, is not yet clear. The reciprocal single substitution (mDectin-1S⁵P and hDectin-1P⁵S) had no impact (Fig. 7 and data not shown), whereas mDectin-1 carrying the double mutation (mDectin-1K²E/S⁵P) was active, suggesting that Pro⁵ renders hDectin-1 sensitive, albeit it is not sufficient in and of itself. One potential explanation is that mouse Ser⁵, which is conserved in most non-primates mammals (supplemental Fig. S7), may act to reduce the receptor signaling by promoting protein modification as Ser phosphorylation is linked to ubiquitination in several signaling molecules (20–22). Alternatively, Ser⁵ may interfere with the function of hemITAM through phosphorylation or other modifications. Indeed, phosphorylation of the Ser residue in the cytoplasmic region of FcRy and Igα is reported to inhibit tyrosine phosphorylation of their own ITAMs, which then inhibits downstream signaling (23, 24). More detailed analysis is needed to clarify whether Pro⁵ (human) cancels the negative function of Ser⁵ (mouse), or, alternatively, actively promotes downstream signaling.

From the analysis of the phylogenetic comparisons, one could speculate that “elimination” of Lys² and Ser⁵ by substitution (human) or truncation (rat) (Fig. 8A and supplemental Fig. S7) might be a common strategy to increase the sensitivity of Dectin-1 to low-valency β-glucan during evolution. In support of this idea, the 5’-UTR of the mRNA for rDectin-1 contains a sequence corresponding to the Ser⁵ found in mice (Fig. 8A), suggesting that Ser⁵ is preserved as a relic in rDectin-1, which has been inactivated by the introduction of a downstream start codon, as speculated in other immune receptors (25).

Despite the different sensitivities to low-valency β-glucan, the various Dectin-1 mutants used in this study retained substantial reactivities to curdlan. This suggests that Dectin-1, like other ITAM-coupled receptors such as the TCR and BCR, has the capacity to sense the quality of ligand. Recently, another CLR, mMincle and hMincle, recognize different ligands and induce distinct responses (26, 27). It is tempting to speculate that the CLR family members might have modulated their sensitivities and ligand spectrums during evolution to adapt to their environment, an idea that warrants further investigation.

**Figure 6.** The cytoplasmic region of hDectin-1 determines its sensitivity to laminarin. A, schematic structures and surface expression of WT and mutant Dectin-1 proteins. Red and blue boxes represent the regions derived from mDectin-1 and hDectin-1, respectively, and the numbers indicate the amino acid residues. Cells were left unstained (open area) or stained with anti-HA-PE antibody (gray area) and analyzed with flow cytometry. B and C, 2B4 reporter cells expressing Syk alone (−) or together with WT or chimeric Dectin-1 proteins (hDT or mDT) were stimulated with laminarin (B) or curdlan (C) at the indicated concentrations for 18 h. Induction of GFP was analyzed by flow cytometry. Data are presented as the mean ± S.D. All data are representative results from four independent experiments with similar results.

Laminarin potently activated hMoDCs to induce a set of inflammatory genes including IL1B, IL1A, and CLEC4E, which are reportedly induced by CLR-mediated signaling (17, 18). In contrast, the majority of mouse orthologues of genes upregulated in hMoDCs did not show substantial induction in mBMDCs (Fig. 9). Note that mBMDCs constitutively expressed Dectin-1 (see GSE98814 and GSE98825), and responded normally to other stimuli, such as LPS (supplemental Fig. S5). Collectively, these data support the idea that hDectin-1, but not mDectin-1, is an activating receptor for low-valency β-glucan.

**Discussion**

In this study, we report that hDectin-1 responds to low-valency β-glucan and its cytoplasmic region is critical for this function.

Within the cytoplasmic N terminus, we show that Glu² of hDectin-1 is one of the critical residues controlling the sensitivity of hDectin-1 to low-valency β-glucan, as a substitution of a mouse-specific residue in this position (hDectin-1E²K) impaired this activity. It appears that it is important that the residue at position 2 be a “non-Lys” residue, as substitutions to other residues did not reduce the activity (hDectin-1E²A or hDectin-1E²D) (supplemental Fig. S6). In line with these observations, Lys residues of mDectin-1 are reported to undergo ubiquitination, which results in the degradation and desensitization of mDectin-1 upon ligand binding (19). Thus, the inactivation of the Lys² residue might be one of the reasons that sensitize hDectin-1.

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Despite the different sensitivities to low-valency β-glucan, the various Dectin-1 mutants used in this study retained substantial reactivities to curdlan. This suggests that Dectin-1, like other ITAM-coupled receptors such as the TCR and BCR, has the capacity to sense the quality of ligand. Recently, another CLR, mMincle and hMincle, recognize different ligands and induce distinct responses (26, 27). It is tempting to speculate that the CLR family members might have modulated their sensitivities and ligand spectrums during evolution to adapt to their environment, an idea that warrants further investigation.
Thus, the role of Dectin-1 in fungal infection characterized by use of gene-deficient mice (10, 11) may not fully reflect the function of human orthologue. These findings demonstrate that the therapeutic approaches targeted to Dectin-1 requires re-evaluation of its functions in vivo by generating models expressing hDectin-1 in future studies.

**Experimental procedures**

**Reagents and antibodies**

Laminarin and curdlan were purchased from InvivoGen. Zymosan (Z4250) and LPS (L4516) were purchased from Sigma. Westase (9095) was purchased from TaKaRa. Fucan from *Cladosiphon novae-caledoniae* Kylin was kindly provided by Daiichi Sangyo and the supernatant was collected after centrifugation at 20,000 × g and used as a stimulant. Phycoerythrin (PE)-conjugated anti-HA Ab (clone 16B12) was purchased from Abcam.

**Cells**

2B4-NFAT-GFP reporter cells expressing various CLRs were co-transfected with FcRγ or DAP12 coupled receptors, reporter cells were co-transfected with FcRγ or DAP12, respectively. hemITAM-harbored receptors were co-expressed with Syk. Receptors that are not coupled to ITAM signaling were expressed as chimeric receptors by fusing to CD3ε. mBMDCs were prepared as previously described (29). Briefly, BM cells from a WT C57BL/6J mouse were suspended in RPMI1640 medium supplemented with 10% FBS, antibiotics, and β-mercaptoethanol at a density of 5 × 10^5 cells/ml in the presence of culture supernatant of MGM-5 (provided by Dr. S.
Nagata) as a source of GM-CSF containing conditioned medium, and cultured for 7 days at 37 °C. hMoDCs were also generated as previously described (30). Briefly, peripheral blood mononuclear cells (PBMCs) from a healthy donor were isolated by Lymphocyte Separation Solution (d = 1.077) (Nacalai Tesque) for gradient centrifugation. Human CD14+ monocytes were purified from PBMCs using anti-human CD14 MicroBeads (Miltenyi Biotech), and cultured in RPMI1640 supplemented with 10% FBS, non-essential amino acid, antibiotics, 10 ng/ml of human GM-CSF (PeproTech), and 10 ng/ml of human IL-4 (PeproTech) for 7 days at 37 °C. The collection and use of human PBMCs were approved by the institutional review boards of Research Institute for Microbial Diseases, Osaka University (29-4).

**In vitro stimulation**

The reporter cells were stimulated with curdlan, zymosan, fucan, or laminarin for 18 h at 37 °C. The GFP expression of reporter cells was evaluated by FACS Calibur flow cytometer (BD Biosciences).

**Westase treatment**

Curdlan and fucan were diluted to 1 mg/ml with buffer (Mcllvain’s disodium phosphate/citric acid buffer, pH 6.0)
containing 2 units/ml of Westase, β-1,3- and β-1,6-glucanase, or buffer alone and were incubated for 5 min at 100 °C to inactivate Westase following incubation for 12 h at 37 °C.

**Construction of chimeric Dectin-1 receptors**

For mouse/human Dectin-1 chimeras, constructs were generated by overlapping extension PCR. Primers used were listed on supplemental Table S1. The resulting constructs were cloned into pMX-IRES-hCD8 or pMX-puro retroviral vector containing HA tag at the C terminus and delivered into 2B4-NFAT-GFP cells expressing Syk as previously described (28, 29).

**Microarray analysis**

1 × 10⁶ hMoDCs from a healthy volunteer or 1 × 10⁶ mBMDCs from a WT mouse were left untreated or stimulated with 500 μg/ml of laminarin for 8 h at 37 °C. Total RNA was isolated by TRIzol (Thermo Scientific). DNA microarray analysis was performed using Human Gene 1.0 ST array (Affimetrix) or Mouse Gene 1.0 ST array (Affimetrix). A Z-score was calculated for each gene between each sample. Genes with both a Z-score exceeding (or equal to) 2 and a sample/reference ratio exceeding (or equal to) 1.5 were defined as up-regulated. The array data were deposited in the Gene Expression Omnibus (accession number GSE98826).

**Dectin-1-Ig fusion protein**

Extracellular domains of hDectin-1 (amino acids 68–247) or mDectin-1 (amino acids 98–244) were fused to the N terminus of the human IgG1 Fc region (hIgG1) as described previously (31). Zymosan diluted in isopropyl alcohol was added to 96-well plates followed by evaporation. Laminarin dissolved in NaHCO₃ buffer was coated on the plates by incubation at 37 °C for 8 h. 100 μg/ml of hIgG1-Fc, hDectin-1-Ig, or mDectin-1-Ig diluted in buffer consisting of 20 mM Tris-HCl, 150 mM NaCl, 1 mM CaCl₂, and 2 mM MgCl₂ (pH 7.0) was incubated with plate-coated ligands. Anti-hIgG-HRP was used for the detection of bound protein.

**Real-time PCR**

1 × 10⁶ hMoDCs from a healthy volunteer or 1 × 10⁶ mBMDCs from a WT mouse were stimulated or left untreated for 8 h at 37 °C. Total RNA was prepared using Sepasol RNA I Super G (Nacalai Tesque) and used to generate cDNA templates with ReverTra Ace (TOYOBO). Quantitative PCR was performed by using THUNDERBIRD SYBR qPCR mix (TOYOBO) and ABI PRISM 7000 (Applied Biosystems). Human and mouse β-actin mRNA were used for normalization. All primers for specific target genes are listed in supplemental Table S1.

**Author contributions**—T. T., C. M., and S. Y. designed the research. T. T. and T. I. performed the experiments. K. S. and Y. N. provided materials. T. T., C. M., and S. Y. wrote the manuscript.

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