Identification of Distinct Ligands for the C-type Lectin Receptors Mincle and Dectin-2 in the Pathogenic Fungus Malassezia

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Identification of distinct ligands for the C-type lectin receptors Mincle and Dectin-2 in the pathogenic fungus *Malassezia*

Running title: Fungal ligands for Mincle and Dectin-2

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SUMMARY

Various C-type lectin receptors (CLRs), including Mincle and Dectin-2, function as pattern-recognition receptors and play a central role in immunity to fungal pathogens. However, the precise structures of the CLR ligands in various pathogenic fungi have yet to be defined. Here we report that *Malassezia*, an opportunistic skin fungal pathogen, is cooperatively recognized by Mincle and Dectin-2 through distinct ligands. Solvent-based fractionation revealed that Mincle and Dectin-2 recognize lipophilic- and hydrophilic-components of *Malassezia*, respectively. Mass spectrometry and NMR revealed glyceroglycolipid and unique mannosyl-fatty acids linked to mannitol as two Mincle ligands. An *O*-linked mannobiose-rich glycoprotein was identified as a *Malassezia* ligand for Dectin-2. Cytokine production in response to the Mincle-ligands and the Dectin-2-ligand was abrogated in Mincle$^{-/-}$ and Dectin-2$^{-/-}$ dendritic cells, respectively. These results demonstrate that Mincle and Dectin-2 recognize distinct ligand in *Malassezia* to induce host immune responses.

Highlights

- Mincle and Dectin-2 cooperatively recognize *Malassezia* through distinct ligands
- Mincle ligands in *Malassezia* are glucosyl-glycolipid and mannosyl-glycolipid
- Dectin-2 ligand in *Malassezia* is *O*-linked mannobiose-rich glycoprotein
- Cytokine response to the respective ligands was impaired in Mincle$^{-/-}$ and Dectin-2$^{-/-}$ DCs

INTRODUCTION

Innate host response is mediated by pattern recognition receptors (PRRs) for pathogen-associated molecular patterns (PAMPs), including Toll-like receptors (TLRs), retinoic acid-inducible gene-I-like receptors (RLRs), nucleotide-binding oligomerization domain-like receptors (NLRs) and C-type lectin receptors (CLRs) (Robinson et al., 2006; Takeuchi and Akira, 2010). Among these PRRs, CLRs play a crucial role in recognizing the complex structures, composed of carbohydrate residues, of various fungal pathogens (Hardison and Brown, 2012; Robinson et al., 2006). Recently, some CLRs have also been demonstrated to directly transduce the signals to produce inflammatory cytokines through signaling subunits such as FcRγ (Robinson et al., 2006), however, the ligands of most CLRs remain largely unclear.

Mincle (also called Clec4e or Clecsf9) is a CLR expressed in activated macrophages and dendritic cells (DCs) subjected to several types of stress (Matsumoto et al., 1999). We have previously shown that Mincle is an activating receptor coupled with Fc receptor γ (FcRγ) chain, an immunoreceptor tyrosine-based activation motif (ITAM)-containing adaptor (Yamasaki et al., 2008). We found that Mincle recognizes

mycobacteria, and identified trehalose-6'6-dimicolate (TDM, also called cord factor) as a specific ligand (Ishikawa et al., 2009). TDM is a glycolipid derived from the mycobacterial cell wall, and it has been shown to possess potent adjuvant activity (Hunter et al., 2006). Although the TDM expression is restricted to mycobacteria, corynebacteria and *Nocardia*, we have recently found that Mincle also recognizes *Malassezia* species (Yamasaki et al., 2009). However, the Mincle ligand in *Malassezia* has not yet been identified, thus suggesting the existence of a ligand other than TDM in this fungus.

Fungi of the *Malassezia* genus are found in the normal flora of human skin. These species are considered to be harmless commensal organisms under normal circumstances, however, they are also widely known as opportunistic pathogens (Gaitanis et al., 2012; Guillot and Bond, 1999). They have been reported to be associated with diverse dermatological pathologies, including pityriasis versicolor, seborrheic dermatitis, atopic dermatitis and folliculitis (Ashbee and Evans, 2002; Guillot and Bond, 1999). *Malassezia* species also cause lethal systemic infections in newborn infants receiving intravenous lipid emulsions (Marcon and Powell, 1992;

Redline and Dahms, 1981). Intriguingly, *Malassezia* species are unique among other fungi in that they require lipid for their growth (Schmidt, 1997). However, the mechanism underlying the recognition of *Malassezia* by host cells has not been fully elucidated.

Dectin-2 (also called Clec4n) is another FcRγ-coupled CLR that is constitutively expressed on DCs, tissue macrophages and inflammatory monocytes (Sato et al., 2006; Taylor et al., 2005). Dectin-2 is reported to recognize a variety of fungi, including *Candida albicans*, *Saccharomyces cerevisiae*, *Histoplasma capsulatum*, *Microsporum audouini* and *Trichophyton rubrum* (McGreal et al., 2006; Ritter et al., 2010; Sato et al., 2006). It is therefore possible that Dectin-2 also recognizes *Malassezia*. Dectin-2 is reported to bind to high mannose structures (McGreal et al., 2006; Sato et al., 2006), especially mannans in *C. albicans* (Saijo et al., 2010), however, the precise structure of the Dectin-2 ligand has not yet been fully defined.

In this study, we demonstrate that two CLRs, Mincle and Dectin-2, cooperatively induce the immune response to the same fungus, *Malassezia*, through the recognition of distinct ligands.

RESULTS

Dectin-2 recognizes *Malassezia* species

We first tried to compare the recognition property of Mincle and Dectin-2. More than 45 species of pathogenic fungi were analyzed by using NFAT-GFP reporter cells expressing FcRγ with Mincle or Dectin-2. As we have previously reported, Mincle selectively recognizes *Malassezia* species (Figure S1A) (Yamasaki et al., 2009). In sharp contrast, Dectin-2 broadly recognizes a variety of fungi, including *Trichophyton*, *Aspergillus*, *Cladosporium*, *Candida* and *Malassezia* species (Figure 1). Interestingly, only *Malassezia* species represented an overlapping target for both receptors. We confirmed that *Malassezia* could not activate reporter cells expressing FcRγ alone (data not shown).

Mincle and Dectin-2 recognize *Malassezia* by distinct mechanisms

In order to identify the ligand(s) for Mincle and Dectin-2 in *Malassezia*, we tried to extract active fraction with various aqueous-organic solvents (Figure 2A). These extracts and solvent-treated fungal cells were then tested to determine their ability to

stimulate NFAT reporter cells. We found that *M. pachydermatis* treated with chloroform:methanol (C:M) selectively lost their Mincle-stimulating activity (Figure 2B). Simultaneously, we analyzed the activity of extracted fractions in plate-coated form, and found that only the C:M phase after C:M extraction showed strong stimulatory activity (Figure 2C). These findings suggested that Mincle recognizes some lipophilic component(s) in *Malassezia*.

On the other hand, the activity for Dectin-2 was efficiently extracted into the water phase, indicating that the Dectin-2 recognizes hydrophilic component(s) in *Malassezia* (Figures 2D and 2E). These results suggest that *Malassezia* may possess distinct ligand components that are recognized by two different CLRs, Mincle and Dectin-2.

Identification of two glycolipids as Mincle ligands

We first tried to identify the Mincle ligand(s) in *Malassezia*. The C:M-soluble fraction was separated into 49 fractions by silica gel column chromatography. Extracts from these fractions showed strong ligand activity that peaked at fractions #44-45

(Figure 3A, top). A thin layer chromatography (TLC) analysis demonstrated that these fractions (#44-45) contain several spots that were considered to be candidates for Mincle ligands (Figure 3A, bottom). We further analyzed fraction #44 by means of high performance thin layer chromatography (HPTLC) and separated it into 20 subfractions to identify the active lipid components. Fraction #44 contained two peaks of ligand activity, 44-1 and 44-2, corresponding to the position of two purple-red spots detected by orcinol staining. These results showed that 44-1 and 44-2 contain glycolipids that are different from TDM (Figure 3B). We further purified 44-1 and 44-2 from fraction #44 by reversed-phase column chromatography and HPLC and their chemical structures were determined on the basis of the chemical and spectroscopic evidence using FAB-MS, ESI-TOFMS, ^{1}H NMR, ^{13}C NMR, and GC-MS.

We first analyzed 44-1 structure (Figure S2A). The negative FAB-MS spectrum of 44-1 showed a pseudo-molecular ion peaks $[M-H]^-$ at *m/z* 919. The fragment ion peaks due to fatty acid anions were observed at *m/z* 297 [$C_{18}H_{37}COO^-$] and 241 [$C_{14}H_{29}COO^-$] as shown in Figure 3C. The molecular formula of 44-1 was further determined to be $C_{49}H_{92}O_{15}$ by the ESI-TOFMS [*m/z* 943.6362, calcd. 943.6328

$(M+Na)^+$] (Figure S3A). The ^{1}H-NMR spectrum of 44-1 exhibited a strong broad signal due to the aliphatic methylenes at δ_H 1.22, a terminal methyl signal at δ_H 0.80 and several multiplets between δ_H 3.80 and 4.80 due to oxygenated methine and methylene protons. Two typical anomeric proton signals were also detected at δ_H 4.97 (1H, d, *J* = 7.9 Hz) and δ_H 4.78 (^{1}H, d, *J* = 7.6 Hz), suggesting two β-linked monosaccharides (Figure S3B). The ^{13}C-NMR spectrum exhibited two terminal methyl signals at δ_C 11.3 and 19.1, aliphatic methylenes at δ_C 25-35, fourteen oxygenated methylenes, methines at δ_C 62-78, two anomeric carbon signals at δ_C 104.4 and 104.8, and two estercarbonyl signals at δ_C 173.3 and 173.4. These results indicated that 44-1-2 was a glyceroglycolipid (Figure S3C).

The structure of the sugar and glycerol moiety was determined as follows. The ^{1}H-^{1}H COSY and TOCSY spectra revealed the two independent correlations from H-1 to H-6 of β-glucopyranoses, and H-1 to H-3 of a glycerol. The connectivity of two glucopyranoses and a glycerol was determined based on the HMBC correlations between the H-1" (δ_H 4.97) of β-Glc*p* and C-6' (δ_C 69.6) of β-Glc*p*, and between H-1' (δ_H 4.78) of Glc*p* and C-3 (δ_C 68.1) of glycerol. The terminal structure of ω3 branched

fatty acid was confirmed by the chemical shift value and HSQC correlations of terminal methyl signals (Figures S3D-S3G) (Pan et al., 2010). Composition of the neutral sugar and fatty acids was conducted by GC-MS analysis following methanolysis (Figures S3H-S3J).

Taken together, the less polar glycolipid (44-1) was a glyceroglycolipid having one glycerol, one gentiobiose (6-*O*-β-D-glucopyranosyl-D-glucopyranose), and *anteiso*-fatty acids (C_{14} and C_{18}), which are attached via ester bonds to the hydroxyl groups of the glycerol backbone (Figure 3D and Figure S3K). In addition to 44-1, small amount of related compounds that are similar to 44-1, except for the length of acyl chains, were identified. We confirmed that all these compounds possess comparable Mincle ligand activity (Figures S4A and S4B).

Interestingly, 44-1 has a structural similarity to the membrane anchor moiety of lipoteichoic acid (LTA), a bacterial component recognized by TLR2 (Schwandner et al., 1999). However, LTA did not act as a Mincle ligand (Figures S4C-S4E).

We next analyzed the structure of 44-2 (Figure S2B). The positive ESI-TOFMS spectrum of 44-2 showed a pseudo-molecular ion peaks at *m/z*

1700.0501$[M+Na]^+$ and gave molecular formula as $C_{84}H_{156}O_{32}Na$ (calcd. for 1700.0472) (Figure 3E). The ^{1}H-NMR spectrum of 44-2 exhibited a typical spectrum feature due to glycolipid (Figure S3L). Three anomeric proton signals at δ_H 4.96 (1H, brs), 5.00 (2H, brs), and 5.43 (1H, brs) were assignable to β-mannopyranose from the correlations of ^{1}H-^{1}H COSY, TOCSY, NOESY and HSQC spectra (Figures S3M-S3P). Because the molecular of 44-2 was so large to elucidate by only spectroscopic analysis, the chemical conversion methods were used for structure elucidation. GC-MS analysis following methanolysis revealed that 44-2 consists of D-mannose, mannitol and 10-hydroxystearic acid clearly (Figures S3Q-S3T). ^{1}H-NMR, TOCSY, HSQC and HMBC spectra of 44-2 at lower temperature clarified that the three acyl moieties were linked to the position 1, 3, 4 on mannitol (Figures S3U-S3X). The treatment of 44-2 with 0.5 M NaOMe gave two glycosyl fatty acids and L-mannitol (Figures S4F-S4U). The structure of two glycosyl fatty acids were determined to be 10-*O*-β-D-mannopyranosyl stearic acid methyl ester and 10-*O*-β-D-mannopyranosyl-(1->2)-β-D-mannopyranosyl stearic acid methyl ester using Mass and NMR analysis, respectively. Taken together, the analysis on native form

revealed that the polar glycolipid (44-2) has a mannitol backbone, which is attached to two 10-*O*-β-D-mannopyranosyl-10-hydroxy-stearic acids, and one 10-*O*-[β-D-mannopyranosyl-(1-2)-β-D-mannopyranosyl]-10-hydroxy-stearic acid *via* ester bonds (Figure 3F).

Thus, 44-2 is a Mincle ligand with unique structure that have not been previously reported in nature or synthesized in the laboratory. Interestingly, only weak activity was detected in glycoside components of 44-2 obtained by alkaline hydrolysis (Figures S4F-S4U), suggesting that intact form of 44-2 structure is required for the potent ligand activity.

Mincle is necessary and sufficient for the recognition of *Malassezia* glycolipid ligands

The ligand activity of these glycolipids was verified using Mincle-expressing reporter cells. 44-1 and 44-2 had a Mincle ligand activity as potent as TDM (Figure 4A). We further confirmed that Mincle directly binds to these glycolipids by using soluble Mincle-Ig protein (Figure 4B). These results indicate that *Malassezia* fungus possess

two Mincle ligands with unique structures.

To examine the contribution of endogenous Mincle as a receptor for these glycolipids, we tested the ability of 44-1 and 44-2 to activate dendritic cells. Wild-type bone marrow-derived dendritic cells (BMDCs) were able to secrete TNF in response to 44-1 and 44-2 (Figure 4C). This TNF production was almost completely suppressed in Mincle$^{-/-}$ DCs, indicating that Mincle is an essential receptor for 44-1 and 44-2 in DCs (Figure 4C). This finding also confirmed that the observed DC activation is not due to possible contaminating TLR ligands in these fractions, since TLR signaling is intact in Mincle$^{-/-}$ mice (Ishikawa et al., 2009). Dectin-2 was dispensable for the recognition of these glycolipids, which is consistent with the results of reporter cells. FcRγ, a signaling subunit of Mincle, was also essential for the response to the glycolipids, whereas zymosan induced a similar response in all these cells (Figure 4D). We therefore concluded that Mincle is an essential receptor for the cytokine production induced by two glycolipids derived from *Malassezia*.

Dectin-2 recognizes *Malassezia* through α-1,2-linked mannose

Dectin-2 contains a glutamic acid-proline-asparagine (EPN) motif, which is known to preferentially bind to mannose (Drickamer, 1992). To assess whether the recognition of *Malassezia* cells by Dectin-2 requires this motif, we substituted residues of the EPN motif of Dectin-2 to create a glutamine-proline-asparagic acid (QPD) motif, which is known as galactose-binding motif (Drickamer, 1992). *M. furfur* failed to activate reporter cells expressing Dectin-2 mutant (Dectin-2^{QPD}) (Figure 5A).

To determine the saccharide through which Dectin-2 recognizes *Malassezia*, we tried to block the recognition with various kinds of monosaccharides. An excessive amount of mannose was able to block the NFAT-GFP reporter activity induced by *Malassezia* cells, whereas glucose and galactose did not show any blocking activity (Figure 5B). These results suggest that Dectin-2 may recognize *Malassezia* through mannose related structure.

A hydrophilic component has Dectin-2 ligand activity

We next searched for the Dectin-2 ligand in *Malassezia*. The *Malassezia* water-soluble fraction (hereafter referred to as MWS) had Dectin-2 ligand activity

(Figure 5C). The MWS could activate cells expressing Dectin-2 in amounts as low as 50 ng (Figure 5C), whereas it had no effect on Mincle-expressing cells, even in amounts as high as 5 μg (data not shown). We have previously reported that the cell wall matrix glycoprotein of *Malassezia* contains cell wall β-glucan (Shibata et al., 2009). To enrich the Dectin-2 ligands, we digested β-glucan in MWS with westase (β-1,6-glucanase) (Shibata et al., 2009), because it has not been reported that Dectin-2 recognize β-glucan. We confirmed that the westase digestion of MWS did not impair ligand activity (data not shown).

The reaction product was fractionated by gel filtration chromatography to give four fractions, W1-W4, separated on the basis of their molecular mass (Figure 5D). W1 activated reporter cells expressing Dectin-2, whereas W2, W3 and W4 did not show strong Dectin-2 ligand activity (Figure 5E).

Identification of *O*-linked manno-protein as a Dectin-2 ligand

Dectin-2 recognizes several fungi that possess *N*-linked mannan on their surface (McGreal et al., 2006; Sato et al., 2006). However, the ^{1}H NMR analysis of W1

showed only two signals at the H-1 region (Figure 5F and Table 1), suggesting that W1 may possess structure distinct from *N*-linked mannan. The chemical shifts at 5.04 ppm of W1 indicated the presence of a non-reducing terminal α-1,2-linked mannose residue (Shibata et al., 2007).

We further analyzed the structure of W1 by methylation analysis. W1 has only a non-reducing terminal mannose residue and a 2-*O*-substituted mannose residue in the molar ratio of 1:1 (Table S1). These data suggest that W1 may be a manno-protein possessing predominantly *O*-linked manno-oligosaccharides, though such a cell wall matrix glycoprotein has not been identified in yeasts and fungi.

To test this idea, we treated W1 with 0.1 M NaOH to induce β-elimination, which selectively releases *O*-linked oligosaccharides connected to serine and/or threonine (Ser/Thr) residues. Bio-Gel P-2 column chromatography of the reaction product showed that about 90% of the carbohydrate was released and eluted only in the disaccharide fraction (Figure 5G). Sugar composition analysis revealed that the eluted disaccharide consists of only mannose (Figure S7A). This result is significantly different from that of the β-elimination of the mannans from *C. albicans*, the amount of

the released oligosaccharides from which comprise only about 3-5% of the total carbohydrate (data not shown).

The ^{1}H NMR and methylation analyses of the released biose fraction indicated that the *O*-linked oligosaccharide was an α-1,2-linked mannobiose, Manα1-2Man (Table 1, Figures S7B, S7C and Table S1). To study the molar ratio of Ser/Thr residues, we investigated the amino acid composition of the protein moiety of mannosyl W1, and found that W1 was abundant in serine (32.9%, Table S2).

On the basis of these results, we concluded that the structure of W1 was a mucin-like serine-rich glycoprotein and the *O*-linked oligosaccharide was predominantly α-1,2-linked mannobiose (Figure 5H). Thus, although Dectin-2 recognizes *Malassezia* as well as *C. albicans*, the ligand structure of *Malassezia* was quite different from that of *C. albicans*.

Dectin-2 is essential for the cytokine production induced by W1

Next, to assess whether Dectin-2 is an essential receptor for the *Malassezia* ligand in DCs, WT, Mincle$^{-/-}$, Dectin-2$^{-/-}$ and FcRγ$^{-/-}$ BMDCs were stimulated with

MWS and W1. MWS and W1 were capable to activate WT DCs to produce TNF. However, the TNF production was almost completely suppressed in Dectin-2$^{-/-}$ and FcR$\gamma^{-/-}$ cells, but not in Mincle$^{-/-}$ DCs (Figure 6A). These findings demonstrated that Dectin-2 is an essential receptor for the *Malassezia*-derived *O*-mannobiose-rich protein which can directly activate DCs to produce inflammatory cytokines.

Malassezia-derived ligands induces host immune responses

We then examined the effect of these *Malassezia*-derived ligands on immune responses. First, 44-2 and W1 were intraperitoneally injected in mice to assess innate immune responses against these ligands. 44-2 and W1 are capable of inducing neutrophil infiltration into peritoneal cavities (Figure 6B). Furthermore, whole *Malassezia* cells and 44-2 showed adjuvanticity toward acquired immune responses, such as IFNγ production, in response to recall antigen stimulation. Although Mincle ligand is capable of driving Th17 responses (Werninghaus et al., 2009), IL-17 was not detected in our limited condition (Figure S6).

Mincle and Dectin-2 contribute to cytokine production in response to whole *Malassezia* cells

Finally, we investigated the contribution of the two CLRs to the recognition of whole *Malassezia* cells. The production of TNF in response to *M. furfur* was decreased in the absence of Mincle or Dectin-2, thus suggesting that both Mincle and Dectin-2 can mediate DC activation in response to *M. furfur* (Figure 6C). In line with these observations, the TNF production was severely impaired in DCs lacking FcRγ, a common subunit of Mincle and Dectin-2. We found that *M. furfur* also induces IL-10 production, which was also dependent on Mincle and Dectin-2 (Figure 6D). These results suggest that Mincle and Dectin-2 cooperatively contribute to cytokine production in response to the *Malassezia* species.

DISCUSSION

Here we have shown that the identification of distinct ligands for Mincle and Dectin-2 in *Malassezia* fungus.

The general principle of the Mincle ligand structure has not yet been clearly defined. Mincle recognizes TDM and its analogue trehalose dibehenate (TDB), but not mycolate or trehalose alone (Ishikawa et al., 2009; Schoenen et al., 2010). Trehalose is a disaccharide formed by two glucose with an α,α-1,1 linkage. In this study, we found that Mincle ligand 44-1 has one gentiobiose, a disaccharide composed of two units of glucose with a β-1,6 linkage, and two fatty acids, which are attached *via* ester bonds to the hydroxyl groups of the glycerol backbone. In contrast, 44-2 has two mannosylated fatty acids, and one β-1,2-linked mannobiosylated fatty acid, which are attached to the mannitol backbone, although there was minor structural variability such as number of mannose or hydroxyl fatty acid residues. A bipolar glycolipid with disaccharide composed of glucose or mannose attached to fatty acids may represent a potential minimal ligand structure for Mincle. Furthermore, comparisons of the fatty acids moieties of 44-1, 44-2, TDM and TDB suggest that the length of the fatty acids may not

critically influence the Mincle ligand activity.

The reason why Mincle selectively recognizes *Malassezia* among the various fungi remains unclear. Given that *Malassezia* uniquely requires lipid for their growth (Schmidt, 1997), the uptake of exogenous lipid as nutrition may be required for the biosynthesis of long-chain fatty acid moiety of Mincle ligands in fungus. Currently, the roles of 44-1 and 44-2 in the physiology and pathogenesis of *Malassezia* still remain unclear, although it is possible that other glycolipids are synthesized in other *Malassezia* strains.

On the other hand, it has been reported that Dectin-2 recognizes the terminal mannose of *N*-linked glycan (McGreal et al., 2006). In the present study, we demonstrated that *O*-linked manno-protein could be a Dectin-2 ligand. α-1,2-mannosyl residues of W1 were necessary and sufficient for the recognition by Dectin-2 (Figures S7D and S7E). Although we detected similar *O*-linked manno-protein in *M. pachydermatis* and *M. sympodialis* (Figure S1B and S1C), other *Malassezia* strains may contain different forms of Dectin-2 ligands. Taken together, active Dectin-2 ligand could be defined as a high density of terminal α-1,2-mannose attached to glycans,

proteins, and presumably any kind of scaffold. In line with this hypothesis, *C. guillermondii* and *S. cerevisiae* were not recognized by Dectin-2 (Figure 1), most likely because the cell wall of these fungi contains α-1,2-mannose masked with β-1,2-mannose and α-1,3-mannose, respectively (Romero et al., 1999; Shibata et al., 1996).

Protein mannosylation is an important process in fungal physiology. It has been reported that protein *O*-mannosyl transferases (PMTs) and α-1,2-mannosyl transferases (MNTs) mediate *O*-mannosylation of proteins in several fungi (Deshpande et al., 2008). Deficiency of these enzymes in several fungi results in an attenuation in their virulence, adherence to host cells, biofilm formation, and cell interaction during mating (Munro et al., 2005; Timpel et al., 1998). Therefore, the *O*-mannosylated products would be one of the appropriate pathogen-associated molecular patterns (PAMPs) for the host to induce an immune response against the fungus. Characterization of W1 core protein by SDS-PAGE suggested that apparent molecular mass of protein moiety is approximately 10 kDa (Figure S5). The identification of mannosyl transferase in *Malassezia* may help to clarify the mechanism regulating the

biosynthesis of such PAMPs.

Several reports have suggested that *Malassezia* species are associated with atopic dermatitis (Ashbee and Evans, 2002; Scheynius et al., 2002). Many kinds of antigens in *Malassezia* have been demonstrated to react with patient IgE (Ashbee and Evans, 2002). However, the precise molecular mechanisms underlying the pathogenesis remain unclear. The reactivity of the *O*-mannosyl protein to patient IgE would be an intriguing issue to be addressed.

Malassezia is known to alter the antigens expressed throughout their growth cycle and culture conditions (Ashbee and Evans, 2002; Shibata et al., 2009). Indeed, It is therefore possible that the relative amount, localization and structure of Mincle/Dectin-2 ligands in *Malassezia* may also vary according to the life cycle, nutritional status, temperature or substrains. Taken together, the acquisition of two CLRs recognizing different ligands in the same fungus would enable the host to exert stable immune responses against variable pathogens.

A synthetic LTA anchor, which has structural similarity to 44-1, has been reported to induce TNF production from macrophages in a TLR-independent manner

(Morath et al., 2002). Mincle might be a responsible receptor for this response.

Although *Malassezia* is a pathogenic fungus, it is usually a harmless commensal found in healthy skin. It is important to examine whether the expression/function of Mincle/Dectin-2 in langerhans cells or dermal DCs is downregulated in healthy skin. Alternatively, some inhibitory receptors recognizing *Malassezia* may be expressed in such dermal cells to prevent unnecessary DC activation. IL-10, an anti-inflammatory cytokine induced by *Malassezia*, may also play a role in regulating host immune responses to *Malassezia*.

It has recently been revealed that several CLRs recognize the damage-associated molecular patterns (DAMPs) derived from damaged tissue (Aragane et al., 2003; Nauta et al., 2003; Ogden et al., 2001; Oka et al., 1998; Yamasaki et al., 2008; Yuita et al., 2005). On the other hand, terminal mannose residues of glycoproteins become exposed upon inflammation and stresses (Franz et al., 2006), although they are normally masked with complex branched sugars during protein maturation in vertebrates (Green et al., 2007). It is therefore possible that Dectin-2 may recognize damaged-self through terminal mannose residues of self protein. The identification of

endogenous ligand for Mincle, Dectin-2 and other CLRs may help to elucidate the immune responses to damaged-tissue through DAMPs-PRRs interaction.

EXPERIMENTAL PROCEDURES

Mice. Dectin-2-deficient mice and FcRγ-deficient mice on the C57BL/6 background were described previously (Park et al., 1998; Saijo et al., 2010). Mincle-deficient mice, described previously (Yamasaki et al., 2009), were backcrossed for at least nine generations with C57BL/6 mice. All mice were maintained in a filtered-air laminar-flow enclosure and given standard laboratory food and water *ad libitum*. All animal protocols were approved by the committee of Ethics on Animal Experiment, Faculty of Medical Sciences, Kyushu University.

Fungi. *M. pachydermatis* (IFM No. 48586) was grown on agar plates (Wako) or liquid medium with potato dextrose broth (Difco Laboratories) for 5 days at 32°C. *M. furfur* (IFM No. 52635) was grown in potato dextrose agar supplemented with 100 μl olive oil (Figure 1), or grown in potato dextrose liquid medium supplemented with 1% Tween-80 (Nacalai tesque) (Figures 6C and 6D) for 5 days at 32°C.

Reagents. TDM, D-glucose, D-mannose and D-galactose were purchased from Nacalai tesque. Zymosan (Z4250), LTA (L4015), OVA (A5503) and α-mannosidase (M7257) were purchased from Sigma-Aldrich. Westase (9095) and *Candida albicans* cell wall mannan (MG001) were obtained from Takara Bio. Other reagents used for chemical analyses were described in Supplemental Information.

***In vitro* stimulation.** To stimulate the cells, TDM was dissolved in C:M (2:1) at 1 mg/ml in isopropanol. Then, these extracts from fungi were added to 96-well plates at 20 μl/well, followed by evaporation of the solvent, as described previously (Ishikawa et al., 2009).

***In vivo* stimulation.** For innate immune responses, mice were intraperitoneally injected with 44-2 or W1 ligands in oil-in-water consisting of mineral oil (9%), Tween-80 (1%) and PBS (90%), or in PBS alone, respectively. At 20 h after injection, peritoneal cells were collected and stained with anti-CD11b and anti-Gr1 mAb and analyzed by flow cytometry. For acquired immune responses, mice were immunized with 4 x 10^7 *M.*

furfur (i.p.) or 200 μg ovalbumin (OVA) together with 200 μg *Malassezia*-derived ligands (s.c.). At 7 days after immunization, splenocytes or inguinal lymph node cells were collected and cultured at 3 x 10^5 cells/200 μl with *Malassezia* antigen or OVA for 72 h. Cytokine concentrations in the culture supernatants were determined by ELISA.

Cells. 2B4-NFAT-GFP reporter cells expressing Mincle, Dectin-2 and Dectin-2^{QPD} mutant (E168Q/N170D) were prepared as previously described (Yamasaki et al., 2008). For BMDC preparation, BM cells were suspended in RPMI 1640 medium supplemented with 10% (vol/vol) FCS and β-mercaptethanol, and were plated at a density of 5 ×10^6 cells/ml in the presence of culture supernatant of MGM-5 (provided by Dr. S. Nagata) as a sourse of GM-CSF, and were cultured for 6 days at 37°C. For BMDMs, L929-conditioned medium were used as a source of M-CSF, and adherent cells were used for the *in vitro* experiments. ELISA kit for TNF, IL-10, IL-4, IFNγ and IL-17 were purchased from BD Biosciences or R&D Systems.

Preparation of *Malassezia* lipophilic fraction. *M. pachydermatis* was treated with C:M (2:1; vol/vol), hexane, acetone, 1-butanol (BuOH), or distilled water. The insoluble fractions were collected. The soluble fractions were further partitioned by C:M:W (8:4:3; vol/vol) into a lower organic phase (C:M) and upper aqueous phase (M:W). The upper aqueous phase (M:W) was further partitioned by 1-butanol:water (1:1; vol/vol) into an upper butanol phase (BuOH) and a lower aqueous phase (water). Each fraction was resuspended in a volume of 2-propanol relative to the original cell pellet weight, and tested as lipid extracts (Morita et al., 2005).

Preparation of *Malassezia* water-soluble fraction (MWS). *M. furfur* cells were washed with deionized water and dehydrated with acetone. The crude cell surface matrix glycoproteins were extracted with deionized water at 120°C for 2 h. After centrifugation, the soluble extract was dialyzed against running tap water for 2 days, then were evaporated and lyophilized and used as *Malassezia* water-soluble fraction (MWS).

Preparation of W1. MWS was dissolved in 25 ml of 100 mM McIlvain buffer (pH 6.0) and 50 units of westase were added and incubated at 37°C for 24 h. The enzyme was inactivated by heating at 100°C for 5 min, and the supernatant was separated by centrifugation at 3,000 rpm for 10 min, followed by evaporation. The westase reaction product was applied onto a column (4.0 x 40 cm) of Sephacryl S-100 and eluted with deionized water to yield four fractions, W1, W2, W3, and W4. The amount of W1 was approximately 0.5% of the dried cell mass.

Chemical analysis. Fast atom bombardment mass spectrometry (FAB-MS), electrospray ionization-time of flight mass spectrometry (ESI-TOFMS), gas chromatography-mass spectrometry (GC-MS) and Nuclear magnetic resonance spectroscopy (^{1}H NMR and ^{13}C NMR) were performed as described in Supplemental Information.

Release of *O*-linked oligosaccharides from W1 by β-elimination. W1 (20 mg) was dissolved in 0.5 M $NaBH_4$/0.1 M NaOH and incubated at 25°C for 18 h. The reaction

mixture was neutralized with acetic acid and repeatedly evaporated with methanol to remove boric acid. The reaction mixture was dissolved in 1 ml of water and was applied onto a column (2.5 x 100 cm) of Bio-Gel P-2 and eluted with water. The released oligosaccharide was analyzed by ^{1}H NMR and the methylation analyses.

Carbohydrate composition analysis. For the analysis of the carbohydrate composition, samples were hydrolyzed with 4 M trifluoroacetic acid (TFA) at 100°C for 3 h. The resulting monosaccharide mixtures were reduced by treatment with $NaBH_4$ and acetylated by acetic anhydride/pyridine (1:1, v/v). The reagents were evaporated and analyzed by GC/MS.

Monosaccharide linkage analysis. The methylation analysis was carried out according to the method reported by Ciucanu and Kerek (Ciucanu and Kerek, 1984). The manno-protein or oligosaccharide was dissolved in a NaOH/dimethylsulfoxide suspension prepared using powdered NaOH. After stirring for 30 min, methyl iodide was added, and the suspension was stirred for another 30 min. The methylated product

was extracted into chloroform and washed with water. The permethylated carbohydrates were then hydrolyzed in 2 M trifluoroacetic acid at 110°C for 2 h. The partially methylated monosaccharides were reduced with 1% $NaBD_4$ at room temperature for 18 h. Following borate removal by drying from methanol, the partially methylated alditols were acetylated by adding acetic anhydride/pyridine (1:1, v/v) and incubating them at 50°C for 3 h. The reagents were evaporated and analyzed by GC/MS.

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FIGURE LEGENDS

Figure 1. Dectin-2 recognizes *Malassezia* species.

Screening of pathogenic fungi for Dectin-2 ligand activities. The reporter cell lines expressing Dectin-2 + FcRγ were co-cultured with the indicated pathogenic fungi for 18 h. The NFAT-GFP induction was analyzed by flow cytometry (See also **Figure S1**). Representative results from two independent experiments with similar results are shown.

Figure 2. Isolation of Mincle and Dectin-2 ligands in *Malassezia.*

(A) A schematic diagram of the solvent-based fractionation of *M. pachydermatis*. Solvent-treated fungal cells (ppt, gray boxes) and soluble extracts (sup, open boxes) were subjected to reporter assays. C:M, chroloform:methanol.

(B-E) Solvent-treated fungal cells (B and D) and plated-coated soluble extracts (C and E) of live *M. pachydermatis* were co-cultured with reporter cells expressing Mincle + FcRγ (B-C) or Dectin-2 + FcRγ (D-E). The NFAT-GFP induction was analyzed by flow cytometry (See also **Figure S2**).

All data (B-E) are the means ± SD for triplicate assays, and representative results from three independent experiments with similar results are shown.

Figure 3. Identification of Mincle ligands in *Malassezia*.

(A) Silica gel column purification. C:M extract of *M. pachydermatis* was subjected to silica gel column chromatography (Silica gel 60, Merck) and eluted with $CHCl_3$/MeOH/H_2O (9/1/0.1 to 1/1/0, v/v/v) to give 49 fractions. Each fraction was coated onto a plate to stimulate reporter cells expressing Mincle and FcRγ (top). Each fraction was also separated by TLC followed by orcinol staining (bottom). The arrowhead indicates Fraction #44.

(B) TLC separation. Fraction #44 (Fr. 44) was analyzed by HPTLC and divided into 20 subfractions. Each subfraction was coated onto a plate to stimulate reporter cells expressing Mincle and FcRγ. The black arrowheads show the origin and solvent fronts. The red arrowheads indicate spots corresponding to 44-1 and 44-2 by orcinol staining. Purified TDM was used as a control.

(C) Mass spectrum of 44-1. The negative FAB-MS spectrum of 44-1 showed a pseudo-molecular ion peaks $[M-H]^-$ at *m*/*z*: 919. The fragment ion peaks due to fatty acid anions were observed at *m*/*z*: 297 [$C_{19}H_{37}COO^-$] and 241 [$C_{14}H_{29}COO^-$].

(D) The chemical structure of Mincle ligand 44-1. (See also **Figure S3**)

(E) Mass spectrum of 44-2. The molecular formula of 44-2 was determined to be $C_{84}H_{156}NaO_{32}$ by the ESI-TOFMS [*m*/*z* 1700.0501, calcd. 1700.0472 $(M+Na)^+$].

(F) The chemical structure of Mincle ligand 44-2. (See also **Figure S3**)

Figure 4. Mincle is necessary and sufficient for the recognition of two glycolipids from *Malassezia*.

(A) 44-1 and 44-2 derived from *M. pachydermatis* activated Mincle-expressing cells. NFAT-GFP reporter cells expressing Mincle + FcRγ were co-cultured for 18 h with plates coated with indicated amount of 44-1, 44-2 or TDM (0.27 μg/well) as a control (See also **Figure S4**).

(B) Binding of Mincle-Ig fusion protein to 44-1 and 44-2. Serially diluted Ig control (Ig) and Mincle-Ig fusion proteins were allowed to react with 44-1 and 44-2 coated on

ELISA plates (1 μg/well). W1 and TDM were used as negative and positive control, respectively. The bound Fc fusion proteins were detected using HRP-conjugated anti-hIgG.

(C-D) Essential role of Mincle-FcRγ axis in cytokine production induced by 44-1 and 44-2. BMDCs from WT, FcRγ$^{-/-}$, Dectin-2$^{-/-}$ and Mincle$^{-/-}$ mice were stimulated with plates coated 44-1 or 44-2 (C) or zymosan (10 μg/ml) as a control (D). The culture supernatants were collected at 48 h and their concentrations of TNF were determined by ELISA.

The data (B-D) are the means ± SD for triplicate assays, and representative results from two independent experiments with similar results are shown.

Figure 5. Identification of the Dectin-2 ligand in *Malassezia*.

(A) Role of mannose-binding motif of Dectin-2 in *Malassezia* recognition. NFAT-GFP reporter cells expressing FcRγ together with Dectin-2^{WT} or Dectin-2^{QPD} were co-cultured for 18 h with *M. furfur*.

(B) Blocking of *Malassezia* recognition by monosaccharides. NFAT-GFP reporter cells expressing Dectin-2 + FcRγ were co-cultured with 3×10^6 of *M. furfur* in the presence of glucose, mannose or galactose.

(C) Activation of Dectin-2-expressing cells by hydrophilic fraction of *M. furfur*. NFAT-GFP reporter cells expressing Dectin-2 + FcRγ were co-cultured for 18 h with plates coated MWS (0.05 – 5 μg/well) or *Candida albicans* cell wall mannan (*C. a.* mannan; 0.1 μg/well) as a control.

(D) Size fractionation by gel filtration chromatography. Separation of MWS was performed with a column (4.0 x 40 cm) of Sephacryl S-100, and the carbohydrate content in each fraction was assayed by the phenol/sulfuric acid method. Four fractions, W1-W4, were collected on the basis of their molecular mass and subjected to the following assay.

(E) Detection of ligand activity in W1 fraction. NFAT-GFP reporter cells expressing Dectin-2 or Mincle were co-cultured for 18 h with plates coated W1, W2, W3 and W4 (0.01 – 1 μg/well) or TDM (0.27 μg/ml/well) as a control.

(F) The ^{1}H NMR analysis of *M. furfur* W1. W1 was dissolved in D_2O. The ^{1}H NMR

spectra were recorded by a JNM-LA600 spectrometer (JEOL) at 45°C. (See also **Table S2**)

(G) Gel filtration chromatography. The elution profiles of β-elimination products of *M. furfur* W1 were shown. Elution was performed with a column (2.5 x 100 cm) of Bio-Gel P-2, and the carbohydrate content in each fraction was assayed by the phenol/sulfuric acid method. The arrows indicate the fraction corresponding to monosaccharide (mono), disaccharide (di), trisaccharide (tri) and polysaccharide (poly). (See also **Figure S5**)

(H) Schematic representation of a possible structure of W1. X is any amino acid.

The data (A, B, C and E) are the means ± SD for triplicate assays, and representative results from three independent experiments with similar results are shown.

Figure 6. Mincle and Dectin-2 mediate *Malassezia*-induced immune responses.

(A) BMDCs from WT, FcRγ$^{-/-}$, Dectin-2$^{-/-}$ and Mincle$^{-/-}$ mice were stimulated with plates coated W1 or MWS or zymosan (10 μg/ml) as a control. Culture supernatants were collected at 48 h, and their concentrations of TNF were determined by ELISA.

(B) Mice were intraperitoneally injected with 200 μg 44-2 in oil-in-water emulsion consisting of mineral oil (9%), Tween-80 (1%) and PBS (90%). Mice were also injected i.p. with 200 μg W1 in PBS. At 20 h after injection, peritoneal cells were stained with CD11b and Gr1 and analyzed by flow cytometry. Each symbol represents an individual mouse. (See also **Figure S6**) *, $P < 0.05$.

(C-D) BMDCs from WT, FcR$\gamma^{-/-}$, Dectin-$2^{-/-}$ and Mincle$^{-/-}$ mice were stimulated with *M. furfur* or zymosan (10 μg/ml) as a control. After 48 h culture, the concentrations of TNF (C) and IL-10 (D) were determined by ELISA.

The data (A) are the means ± SD for triplicate assays, and representative results from three independent experiments with similar results are shown. The data (C-D) are the means ± SD for triplicate assays, and representative results from two independent experiments with similar results are shown.

Figure 1

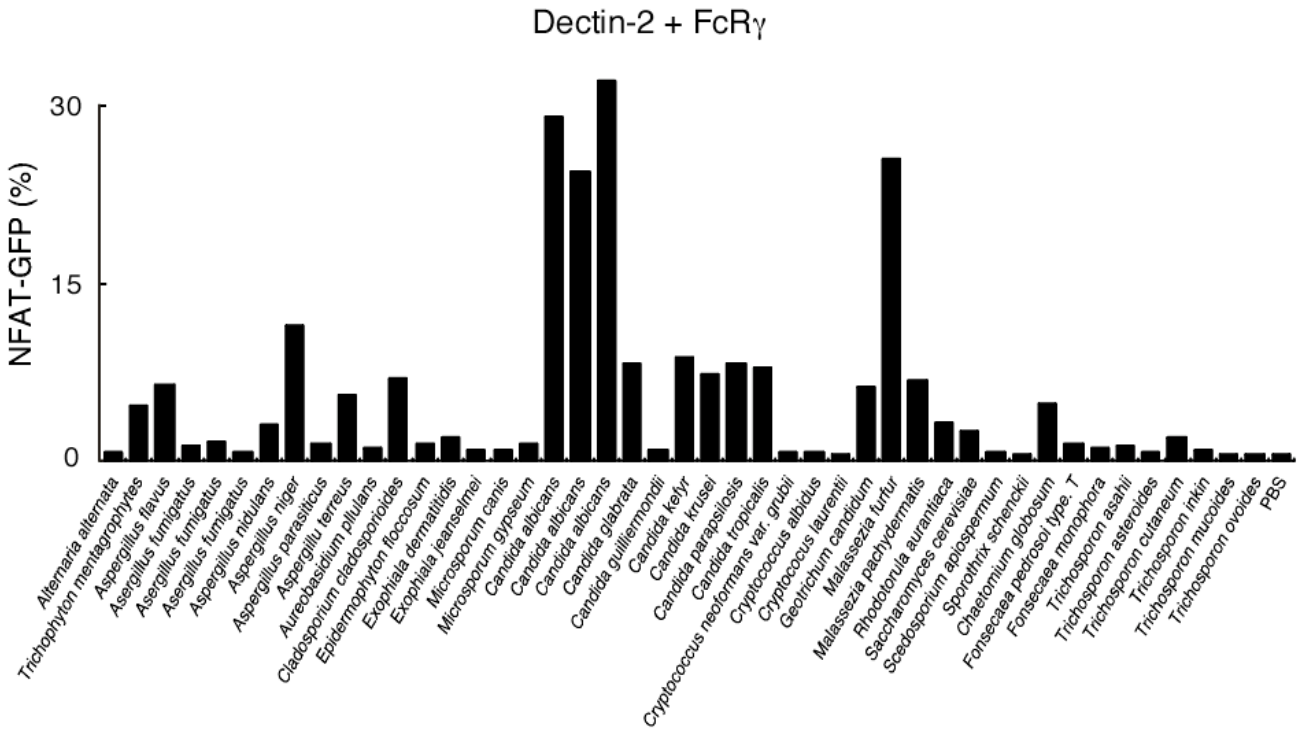
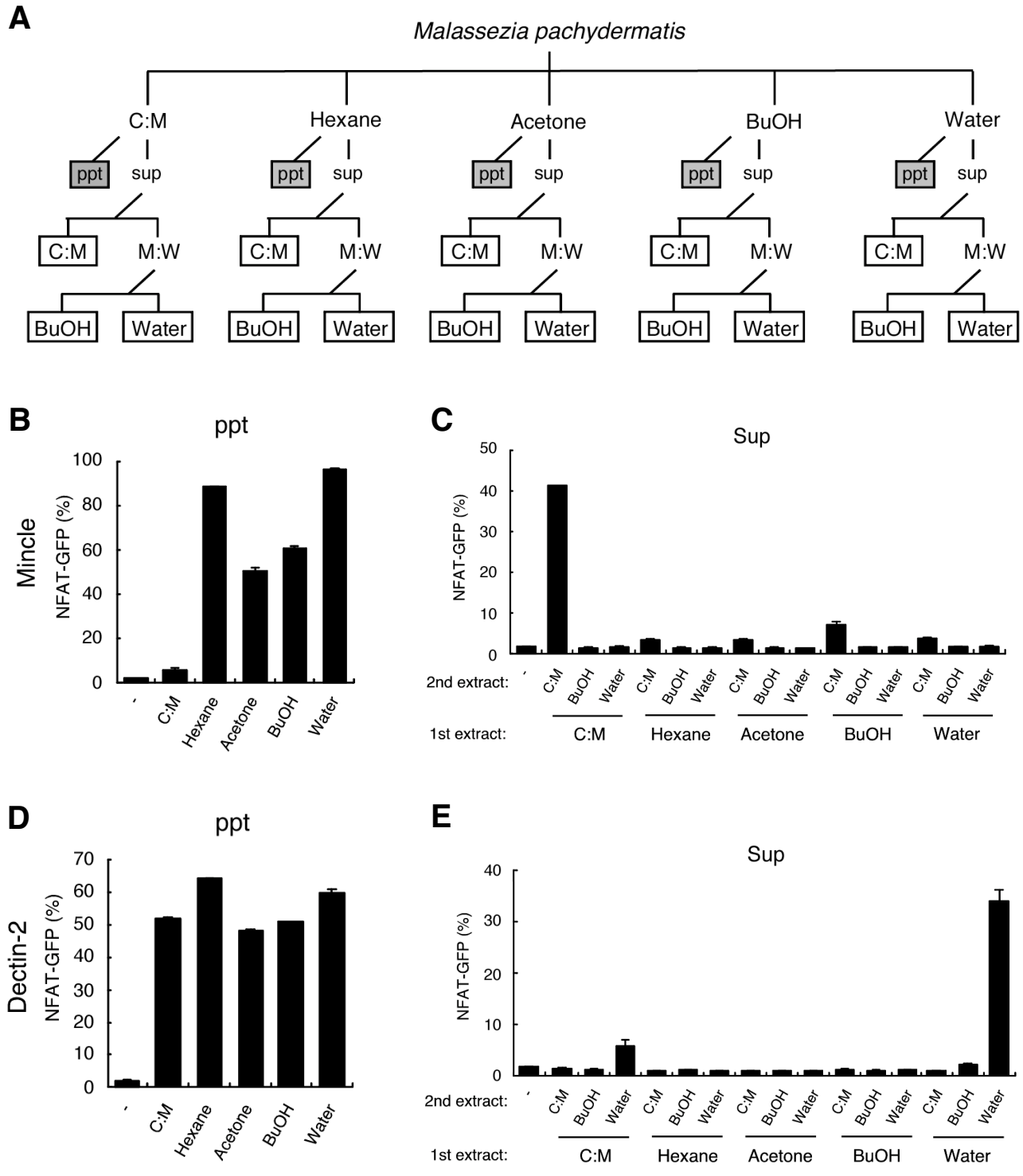

Dectin-2 + FcRγ
NFAT-GFP (%)
30
15
0
Alternaria alternata
Trichophyton mentagrophytes
Aspergillus flavus
Asergillus fumigatus
Asergillus fumigatus
Asergillus fumigatus
Aspergillus nidulans
Aspergillus niger
Aspergillus parasiticus
Aspergillu terreus
Aureobasidium pllulans
Cladosporium cladosporioides
Epidermophyton floccosum
Exophiala dermatitidis
Exophiala jeanselmei
Microsporum canis
Microsporum gypseum
Candida albicans
Candida albicans
Candida albicans
Candida glabrata
Candida guilliermondii
Candida kefyr
Candida krusei
Candida parapsilosis
Candida tropicalis
Cryptococcus neoformans var. grubii
Cryptococcus albidus
Cryptococcus laurentii
Geotrichum candidum
Malassezia furfur
Malassezia pachydermatis
Rhodotorula aurantiaca
Saccharomyces cerevisiae
Scedosporium apiospermum
Sporothrix schenckii
Chaetomium globosum
Fonsecaea pedrosoi type. T
Fonsecaea monophora
Trichosporon asahii
Trichosporon asteroides
Trichosporon cutaneum
Trichosporon inkin
Trichosporon mucoides
Trichosporon ovoides
PBS

Figure 2


A
Malassezia pachydermatis
C:M
Hexane
Acetone
BuOH
Water
ppt
sup
C:M
M:W
BuOH
Water
B
ppt
Mincle
NFAT-GFP (%)
100
80
60
40
20
0
-
C:M
Hexane
Acetone
BuOH
Water
C
Sup
NFAT-GFP (%)
50
40
30
20
10
0
2nd extract:
-
C:M
BuOH
Water
1st extract:
C:M
Hexane
Acetone
BuOH
Water
D
ppt
Dectin-2
NFAT-GFP (%)
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60
50
40
30
20
10
0
E
Sup
NFAT-GFP (%)
40
30
20
10
0

Figure 3

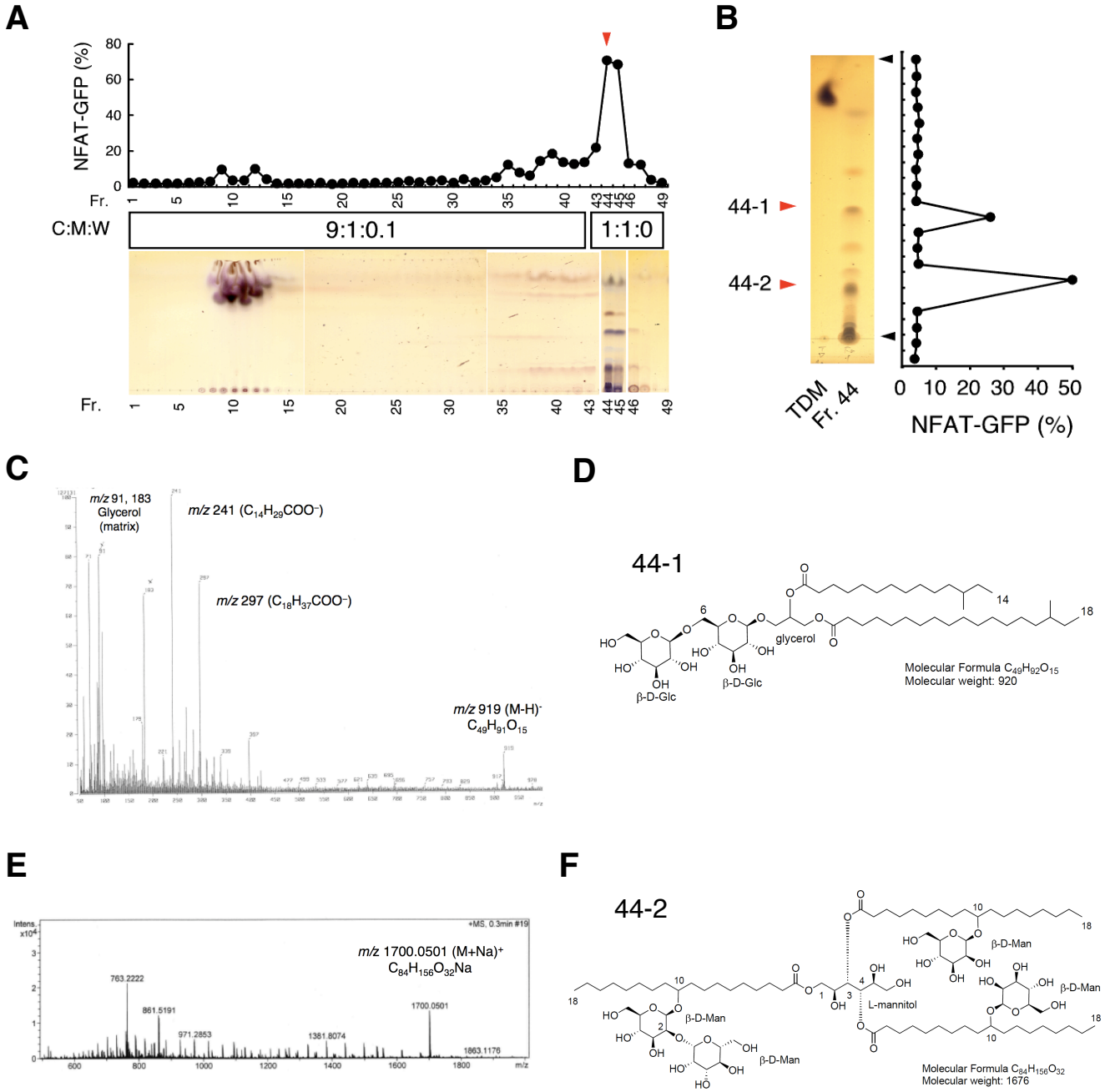

A
NFAT-GFP (%)
80
60
40
20
0
Fr.
1 5 10 15 20 25 30 35 40 43 44 45 46 49
C:M:W
9:1:0.1
1:1:0
Fr.
1 5 10 15 20 25 30 35 40 43 44 45 46 49
B
44-1
44-2
TDM Fr. 44
0 10 20 30 40 50
NFAT-GFP (%)
C
m/z 91, 183
Glycerol
(matrix)
m/z 241 (C14H29COO-)
m/z 297 (C18H37COO-)
m/z 919 (M-H)-
C49H91O15
D
44-1
glycerol
β-D-Glc
β-D-Glc
Molecular Formula C49H92O15
Molecular weight: 920
E
+MS, 0.3min #19
m/z 1700.0501 (M+Na)+
C84H156O32Na
763.2222
861.5191
971.2853
1381.8074
1700.0501
1863.1176
600 800 1000 1200 1400 1600 1800 m/z
F
44-2
β-D-Man
β-D-Man
β-D-Man
L-mannitol
Molecular Formula C84H156O32
Molecular weight: 1676

Figure 4

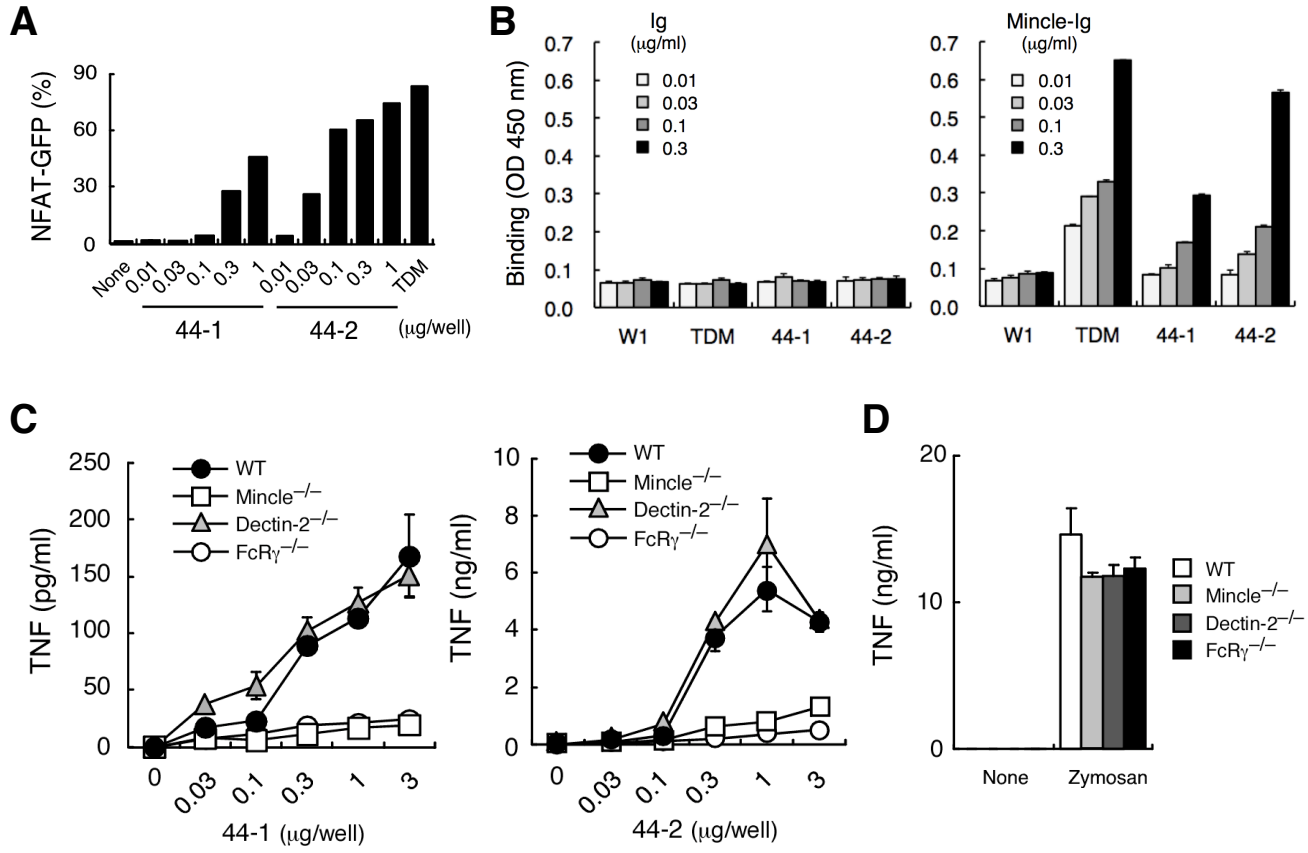

A
NFAT-GFP (%)
90
60
30
0
None
0.01
0.03
0.1
0.3
1
0.01
0.03
0.1
0.3
1
TDM
44-1
44-2
(μg/well)
B
Ig
(μg/ml)
0.01
0.03
0.1
0.3
Binding (OD 450 nm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
W1
TDM
44-1
44-2
Mincle-Ig
(μg/ml)
0.01
0.03
0.1
0.3
W1
TDM
44-1
44-2
C
TNF (pg/ml)
250
200
150
100
50
0
WT
Mincle−/−
Dectin-2−/−
FcRγ−/−
0
0.03
0.1
0.3
1
3
44-1 (μg/well)
TNF (ng/ml)
10
8
6
4
2
0
WT
Mincle−/−
Dectin-2−/−
FcRγ−/−
0
0.03
0.1
0.3
1
3
44-2 (μg/well)
D
TNF (ng/ml)
20
10
0
None
Zymosan
WT
Mincle−/−
Dectin-2−/−
FcRγ−/−

Figure 5

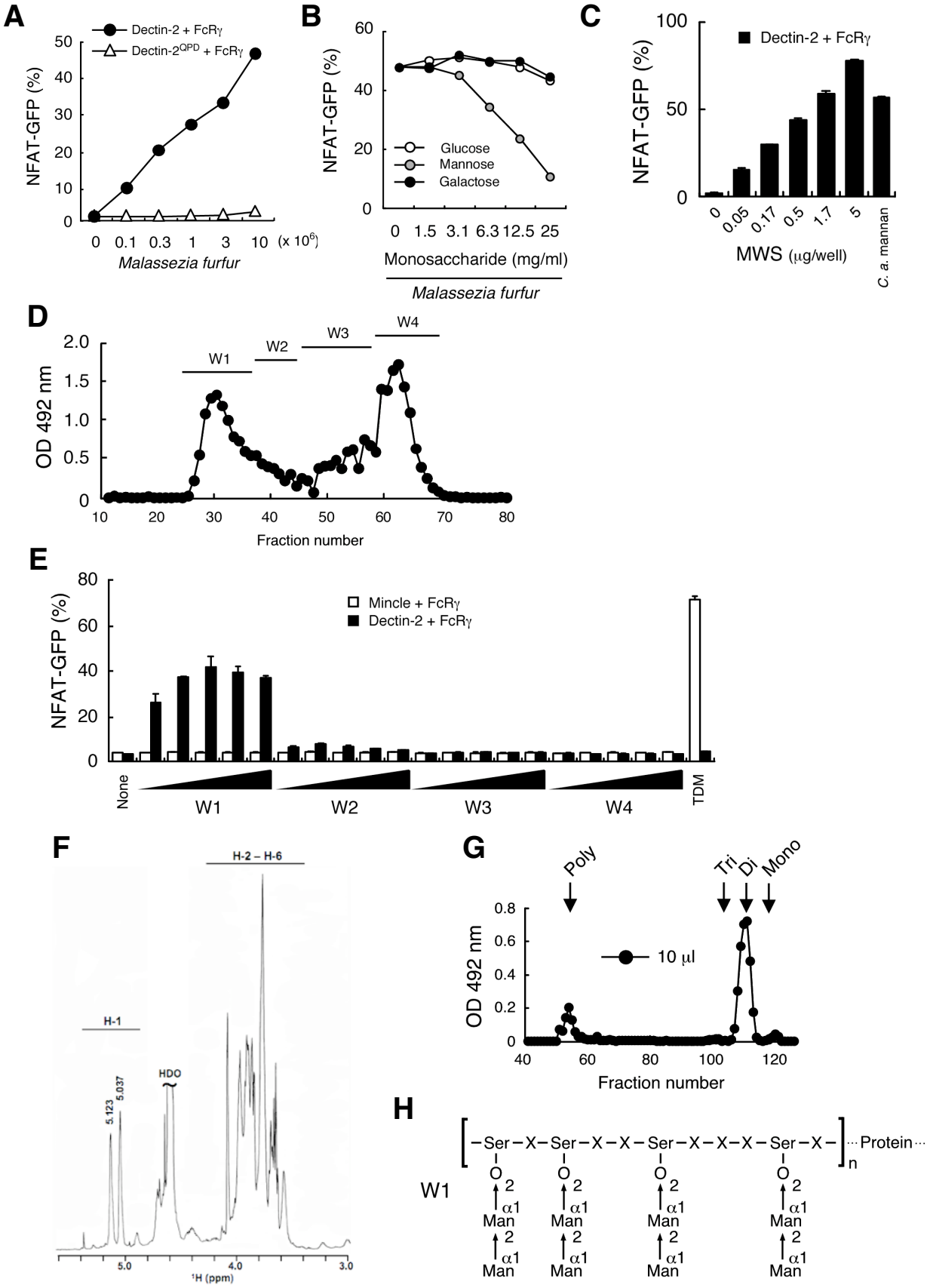

A
Dectin-2 + FcRγ
Dectin-2QPD + FcRγ
NFAT-GFP (%)
0 0.1 0.3 1 3 10 (x 10^6)
Malassezia furfur
B
NFAT-GFP (%)
Glucose
Mannose
Galactose
0 1.5 3.1 6.3 12.5 25
Monosaccharide (mg/ml)
Malassezia furfur
C
Dectin-2 + FcRγ
NFAT-GFP (%)
0 0.05 0.17 0.5 1.7 5
MWS (μg/well)
C. a. mannan
D
W1
W2
W3
W4
OD 492 nm
Fraction number
E
Mincle + FcRγ
Dectin-2 + FcRγ
NFAT-GFP (%)
None
W1
W2
W3
W4
TDM
F
H-2 – H-6
H-1
HDO
5.123
5.037
5.0
4.0
3.0
¹H (ppm)
G
Poly
Tri
Di
Mono
10 μl
OD 492 nm
Fraction number
H
W1
Ser X Ser X X Ser X X X Ser X
Protein
n
O 2 α1 Man
2 α1 Man

Figure 6

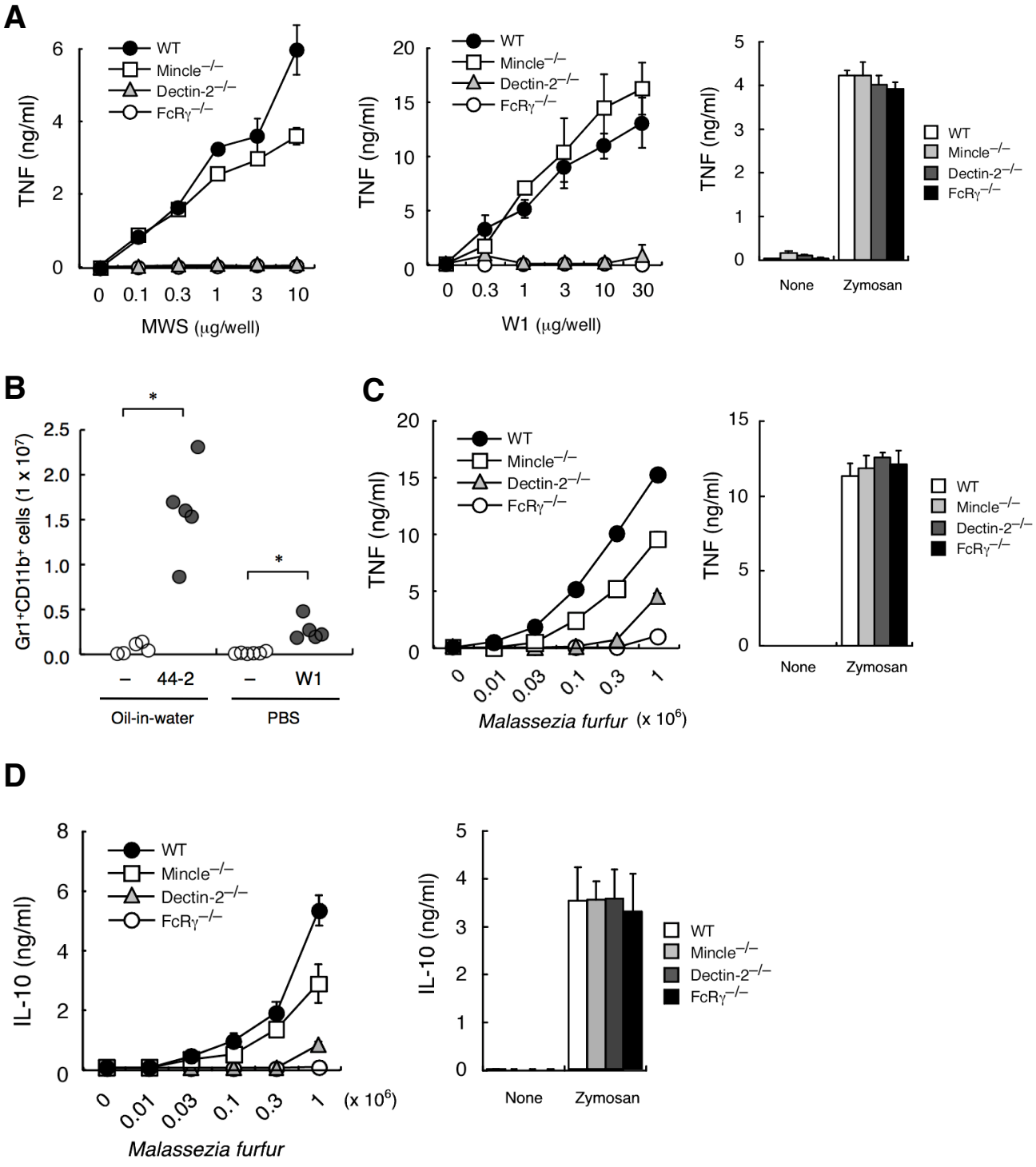

A
TNF (ng/ml)
WT
Mincle−/−
Dectin-2−/−
FcRγ−/−
MWS (μg/well)
W1 (μg/well)
None
Zymosan
B
Gr1+CD11b+ cells (1 x 10^7)
44-2
W1
Oil-in-water
PBS
C
Malassezia furfur (x 10^6)
D
IL-10 (ng/ml)
Malassezia furfur (x 10^6)

Table 1. ^{1}H and ^{13}C chemical shifts (δ, ppm)[a] for W1[b] and mannobiose released from W1 by β-elimination.

Mannoprotein or Oligosaccharide	Residue	H-1 ($J_{H1, H2}$)	H-2 ($J_{H2, H3}$)	H-3 ($J_{H3, H4}$)	H-4	H-5	H-6/H-6'
mannoprotein							
W1	→2Manα-O-Ser/Thr	5.123	3.960	3.783	3.636	-[d]	-
	Manα1→	5.037	4.073	3.843	3.652	-	-
β-elimination							
Man_2-ol	→2Man-ol[e]	3.926	3.783	4.021	3.690	3.768	3.877/3.679
	Manα1→	5.001	3.989	3.871	3.668	3.799	3.908/-
		(1.8)[c]	(3.3)	(9.6)			

	Residue	C-1	C-2	C-3	C-4	C-5	C6
Man_2-ol	→2Man-ol	62.07	80.27	68.49	70.40	71.91	63.98
	Manα1→	102.00	71.32	71.20	67.68	74.06	61.81

[a] Sample was dissolved in D_2O. The ^{1}H NMR spectra were recorded by a JNM-LA600 spectrometer (JEOL) at 45°C. The proton and carbon chemical shifts were referenced relative to the internal acetone at δ 2.225 and 31.07, respectively.

[b] W1 was isolated from *M. furfur*.

[c] J = Hz.

[d] Not determined.

[e] Man-ol; mannitol

Supplemental Information

Identification of distinct ligands for Mincle and Dectin-2 in pathogenic fungus *Malassezia*

Tetsuaki Ishikawa, Fumie Itoh, Sayumi Yoshida, Shinobu Saijo, Tetsuhiro Matsuzawa, Tohru Gonoi, Takashi Saito, Yoshio Okawa, Nobuyuki Shibata, Tomofumi Miyamoto and Sho Yamasaki

Figure S1

A

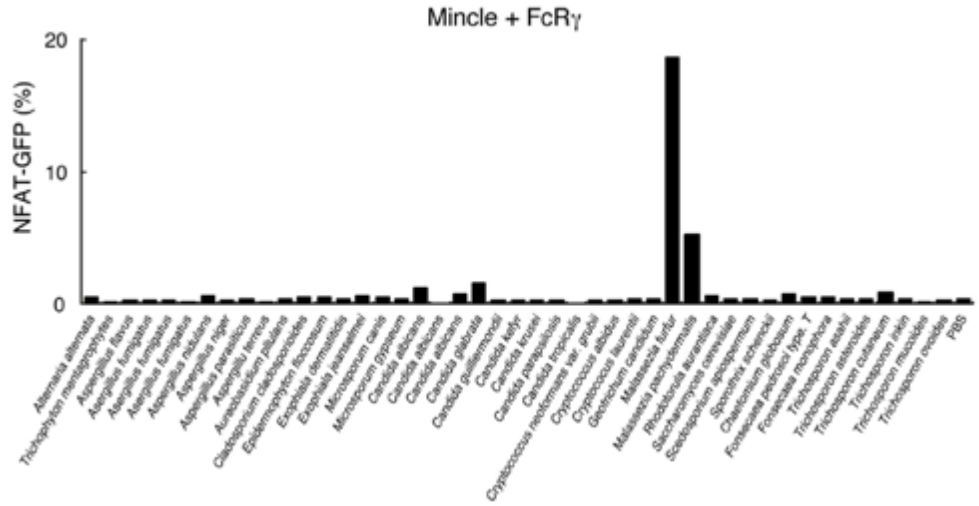
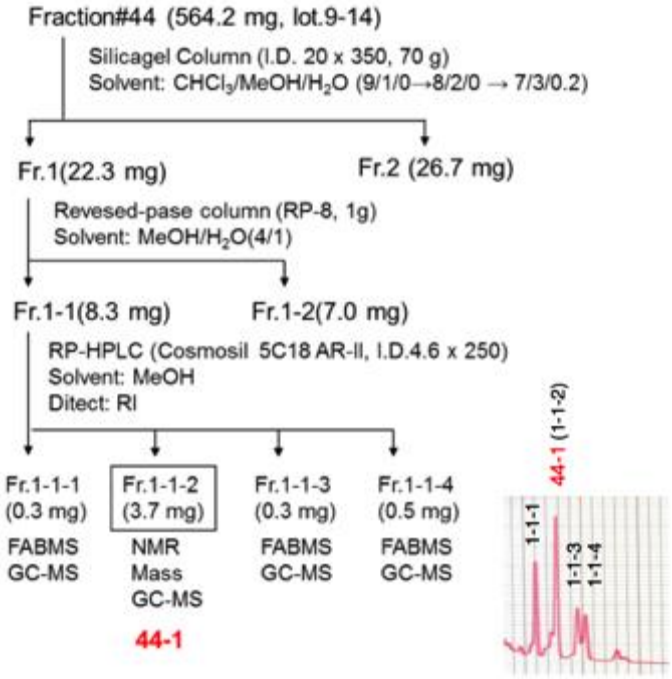


Figure S1. Recognition of *Malassezia* species by Mincle. (related to Figure 1)
(A) Screening of pathogenic fungi for Mincle ligand activities. The reporter cell line expressing Mincle + FcRγ was co-cultured with the indicated pathogenic fungi for 18 h. The NFAT-GFP induction was analyzed by flow cytometry. Representative results from two independent experiments with similar results are shown.

Figure S2

A



B

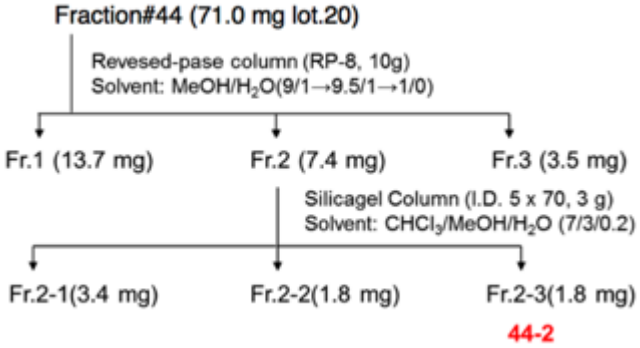


Figure S2. Isolation of 44-1 and 44-2. (related to Figure 2)

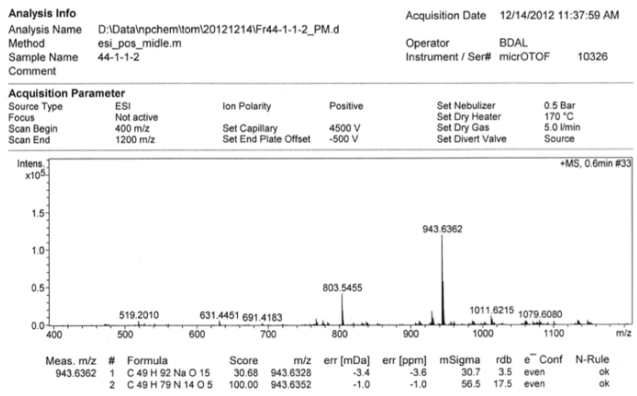
(A) Purification of 44-1. Fraction#44 (lot.9-14) was concentrated *in vacuo* to give a residue (564.2 mg), which was subjected to silica gel column chromatography (Silica gel 60, Merck, Darmstadt, Germany) eluted with $CHCl_3$/MeOH/H_2O (9/1/0 to 8/2/0 to 7/3/0.2, v/v/v) to give two fractions, Fraction 1 and 2 (11.7 mg). Fraction 1 (22.3 mg)

was further chromatographed on a reversed phase column chromatography (LiChroprep RP-8, Merck, Darmstadt, Germany), eluted with MeOH/H_2O (4/1, v/v) to give two fractions, Fraction 1-1 and 1-2. Fraction 1-1 (8.3 mg) was further subjected to reversed phase HPLC (Cosmosil 5C18 AR-II, Nacalai Tesque, Tokyo, Japan), eluted with MeOH to give five fractions, Fraction 1-1-1 (0.3 mg), 1-1-2 (3.7 mg), 1-1-3 (0.3 mg), and 1-1-4 (0.5 mg). We named Fraction 1-1-2 as 44-1 (shown in red).

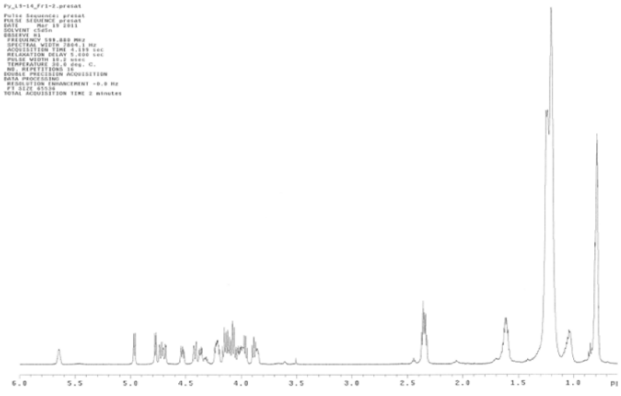
(B) Purification of 44-2. Fraction#44 (lot.20) was concentrated *in vacuo* to give a residue (71.0 mg), which was subjected to reversed-phase column chromatography (RP-8) eluted with MeOH/H_2O (9/1/ to 9.5/1 to 1/0, v/v) to give three fractions. Fraction 2 (7.4 mg) was further chromatographed on a silica gel column chromatography eluted with $CHCl_3$/MeOH/H_2O (7/3/0.2, v/v/v) to give three fractions. We named Fraction 2-3 as 44-2 (shown in red).

Figure S3

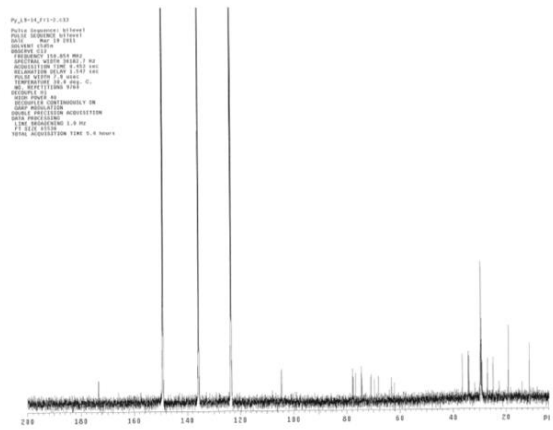
A



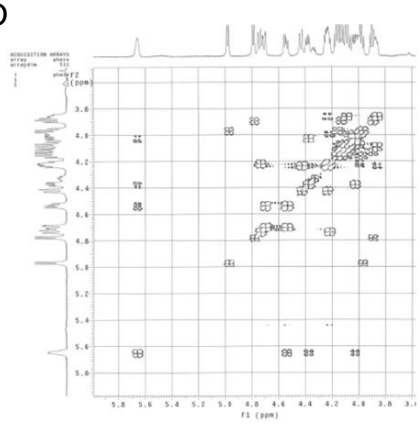
B



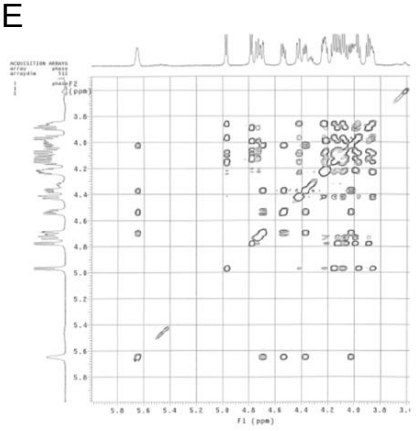
C



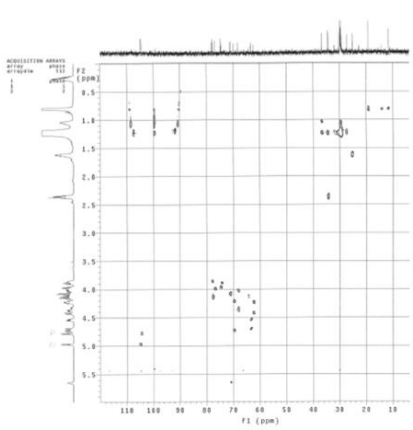
D



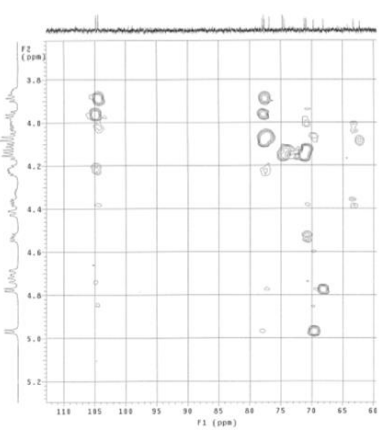
E



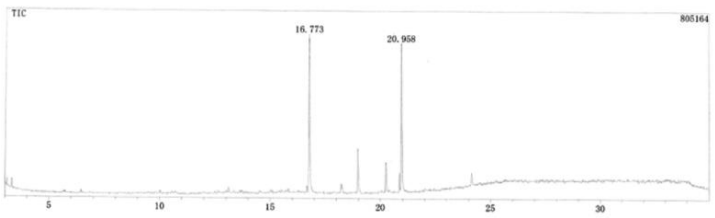
F



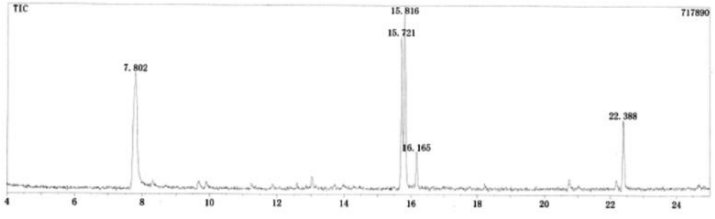
G



H



J



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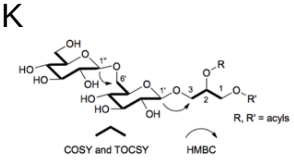

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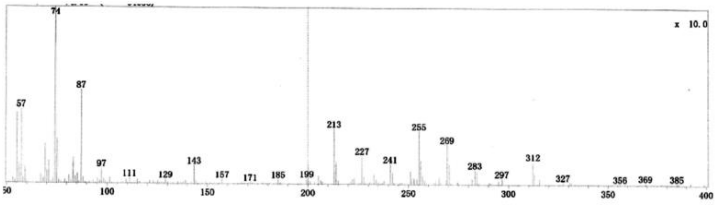

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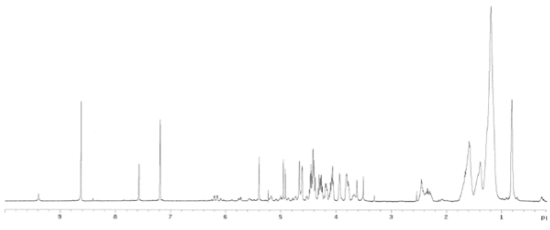

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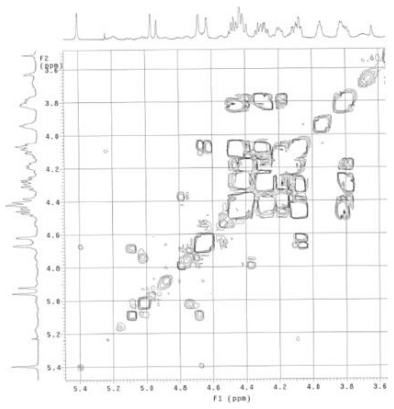

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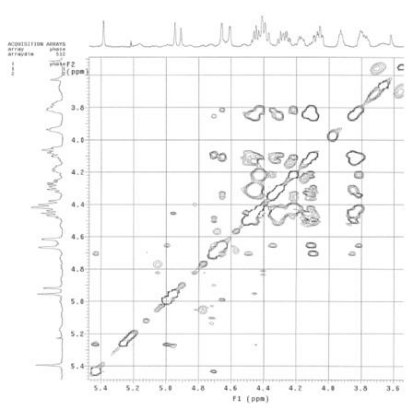

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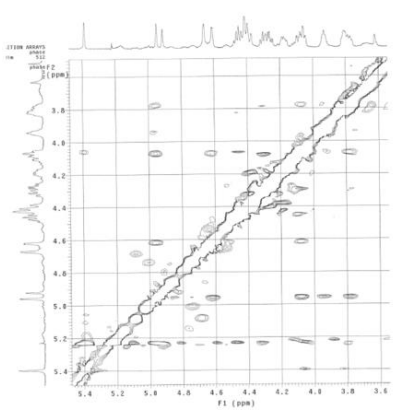

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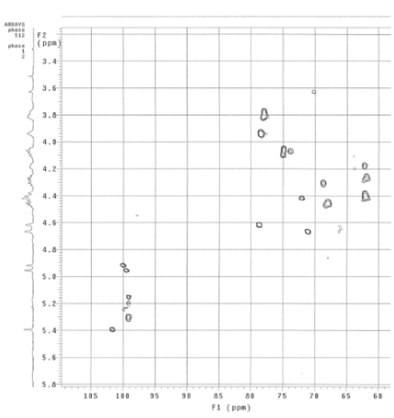
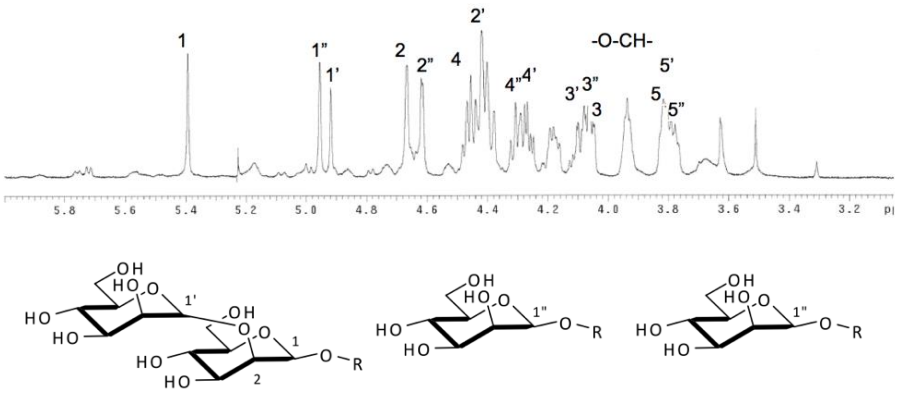
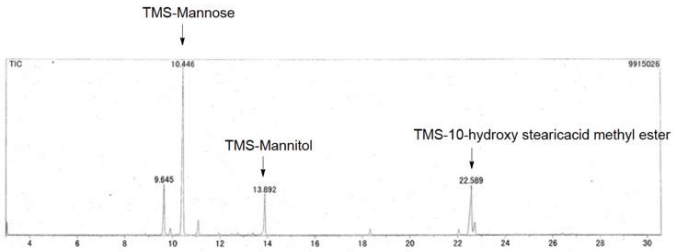
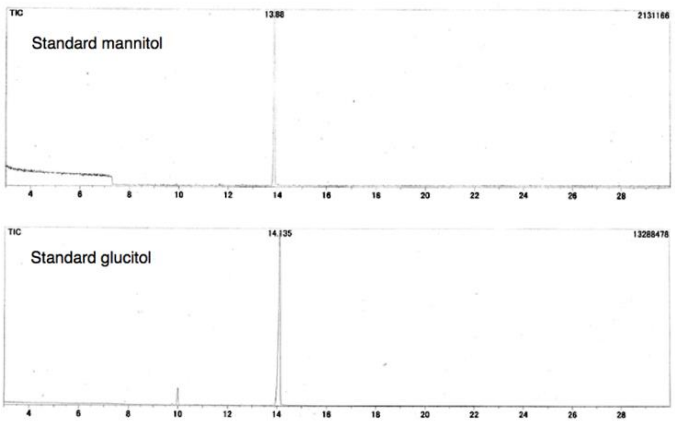




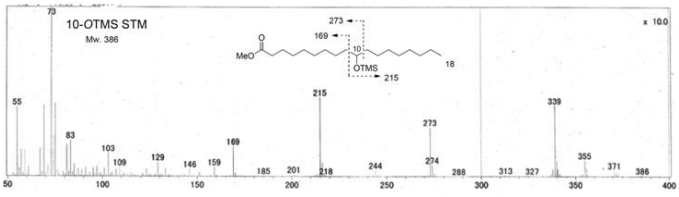
Q



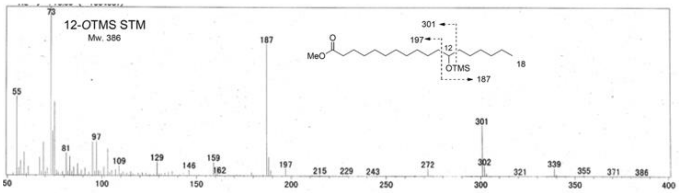
R



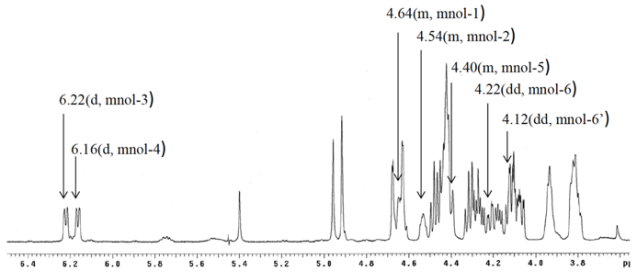
S



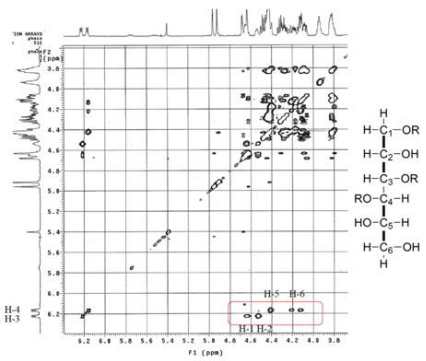
T



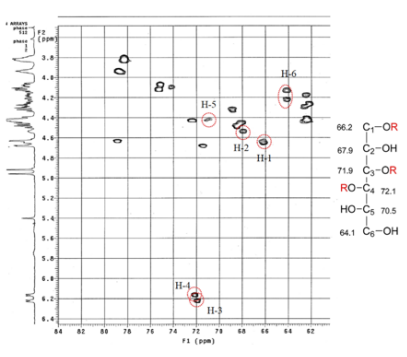
U



V



W



X

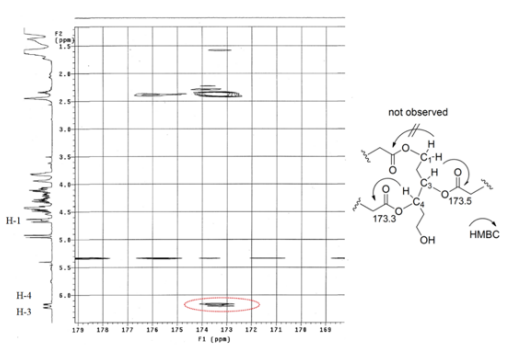


Figure S3. Structure determination of 44-1 and 44-2. (related to Figure 3)

(A) ESI-TOFMS spectrum of 44-1.

The molecular formula of 44-1 was determined to be $C_{49}H_{92}O_{15}$ by ESI-TOFMS [*m*/*z* 943.6362, calcd 943.6328 $(M+Na)^+$].

Apparatus: microTOF II (Bruker Daltonics, MA, USA)

(B) ^{1}H NMR spectrum of 44-1.

^{1}H-NMR(C_5D_5N/D_2O (20/1), 600MHz, δ_H): 0.80 (12H, m, methyls), 1.2 (m, methylenes), 1.6 (4H, m, methylenes), 2.35 (4H, m, methylenes), 3.86 (1H, m, glc-5'), 3.89 (1H, t, 8.1, glc-2), 3.96 (1H, t, 8.1, glc-2'), 4.00 (1H, m, glc-5), 4.02 (1H, dd, 9.3, 14.5, gly-1), 4.90 (2H, m, glc-4 & glc-4'), 4.13 (1H, m, glc-3), 4.16 (1H, t, 9.1, glc-3'), 4.22 (2H, m, glc-6 & glc-6'), 4.37 (1H, dd, 5.5, 11.0, gly-1), 4.42 (1H, dd, 2.1, 12.0, glc-6'), 4.53(1H, dd, 7.2, 12.2 gly-3), 4.69 (1H, dd, 3.1, 12.2, gly-3), 4.73 (1H, brd, 10.2, glc-6), 4.78 (1H, d, 7.6, glc-1'), 4.97 (1H, d, 7.9, glc-1), 5.64 (1H, m, gly-2)

Apparatus: Varian Inova 600 spectrometer (Varian, MA).

(C) ^{13}C NMR spectrum of 44-1.

^{13}C-NMR (C_5D_5N/D_2O (20/1), 150 MHz, δ_C): 11.3 (CH_3), 19.1 (CH_3), 25-35 (methylenes), 62.2 (CH_2, glc-6'), 63.2 (CH_2, gly-3), 68.1 (CH_2, gly-1), 69.6 (CH_2, glc-6), 70.8 (CH, gly-2), 70.8 (CH, glc-4'), 71.2 (CH, glc-4), 74.3 (CH, glc-2), 74.6 (CH, glc-2'), 76.8 (CH, glc-5), 77.6 (CH, glc-3), 77.7 (CH, glc-3'), 77.9 (CH, glc-5'), 104.4 (CH, glc-1), 104.8 (CH, glc-1'), 173.3 (COO), 173.4 (COO).

(D-G) 2D-NMR spectrum of 44-1.

The ^{1}H-^{1}H COSY (D) and TOCSY (E) spectra revealed the two independent correlations from H-1 to H-6 of β-glucopyranoses, and H-1 to H-3 of a glycerol. The connectivity of two glucopyranoses and a glycerol was determined based on the HMBC correlations (F) between the H-1" (δ_H 4.97) of β-Glc*p* and C-6' (δ_C 69.6) of β-Glc*p*, and between H-1' (δ_H 4.78) of Glc*p* and C-3 (δ_C 68.1) of glycerol. The terminal structure of ω3 branched fatty acid was confirmed by the chemical shift value and HSQC correlations (G) of terminal methyl signals (ref).

(H-J) GC-MS spectrum of 44-1.

GC-MS analysis of fatty acid methylesters (FAMEs) and trimethylsilyl (TMS) ether of methyl glycoside: 44-1 (fraction 1-1-2: *ca.* 0.1mg) was heated with 10% HCl/MeOH (0.2 ml) in a sealed tube at 80°C for 2 h. The reaction mixture was diluted with MeOH

(1.0 ml) and extracted with *n*-hexane, and the *n*-hexane extract was concentrated *in vacuo* to give a mixture of FAMEs. The FAMEs was dissolved in acetone and subjected to GC-MS (H) [Shimadzu QP-5050A with TC-1701, GL Science Inc. Tokyo, Japan, column temperature 180-320°C, rate of temperature increase: 4°C/min]. The remaining MeOH layer was neutralized with Ag_2CO_3, and filtrated. The filtrate was dried *in vacuo* then dissolved in pyridine (0.05 ml) and 0.05 ml of 1-(trimethylsilyl) imidazole was added. The reaction mixture was heated at 60°C for 20 min, and the TMS ether of glycoside was analyzed by GC-MS. FAMEs: methyl 12-methyltetradecanoate t_R = 16.77 min, *m*/*z* = 256 (M^+), 227, 213, 199, 87, 74; (I) methyl 16-methyloctadecanoate t_R = 20.96 min, *m*/*z* = 312(M^+), 283, 269, 255, 87, 74; (J) *O*Me-TMS-glucose t_R = 15.72, 15.82, 16.17 min (standard of glucose t_R = 15.76, 15.85, 16.21 min).

(K) The structure of 44-1 as diglycosyldiacylglycerol.

COSY, correlation spectroscopy; HMBC, hetero-nuclear multi-bond connectivity. R = $C_{14}H_{29}COOH$, R' = $C_{18}H_{37}COOH$.

(L) ^1H NMR spectrum of 44-2 at 313K.

^1H-NMR (C_5D_5N/D_2O (20/1), 600MHz, 313K, δ_H): 0.80 (12H, m, methyls), 1.2-1.8 (m, methylenes), 2.2-2.5 (m, methylenes), 3.85 (3H, m, man-5), 3.98 (1H, H-10 of FA), 4.11 (3H, m, man-3), 4.32 (2H, m, man-4), 4.45 (2H, m, man-2), 4.50 (1H, t, man-4), 4.66 (1H, brd, man-2), 4.70 (1H,brs, man-2), 4.96 (1H, s, man-1), 5.00 (2H, s, man-1), 5.43 (1H, s, man-1).

(M-P) 2D-NMR spectrum of 44-2 at 313K.

The ^1H-^1H COSY (M), TOCSY (N), NOESY (O), and HSQC (P) spectra revealed the three independent correlations from H-1 to H-5 of β-mannopyranoses.

(Q-T) GC-MS analysis of 44-2.

GC-MS analysis of TMS ether of methyl mannose, mannitol, glucitol, and 10-hydroxy and 12-hydroxylstearic acid methyl esters: 44-2 (fraction 2-3: *ca.* 0.1 mg) was heated with 10% HCl/MeOH (0.1 ml) in a sealed tube at 80°C for 2h. The reaction mixture was diluted with MeOH (0.5 ml) and neutralized with Ag_2CO_3, and filtrated. The filtrate was dried *in vacuo* then dissolved in pyridine (0.05 ml) and 0.05 ml of 1-(trimethylsilyl) imidazole was added. The reaction mixture was heated at 60 °C for 20 min, and the TMS ethers were analyzed by GC-MS. (Q) FAMEs: *O*Me-TMS-mannose t_R = 9.65, 10.45, (standard of mannose t_R = 9.64, 10.44 min), TMS-mannitol t_R = 13.89, (R)

Standard of TMS-mannitol t_R = 13.88 min, Standard of TMS-glucitol t_R = 14.14 min. (S) 10-*O*TMS-hydroxymethystearate t_R = 22.59 min, *m*/*z* = 386 (M^+), 339, 273, 215, 179; (T) 12-*O*TMS-hydroxymethystearate t_R = 22.88 min, *m*/*z* = 386 (M^+), 339, 301, 197, 187.

Absolute configuration of mannose was performed as previously described (Hara, et al., 1987). 44.2 (*ca*. 0.1 mg) was heated with 2M HCl (0.1 ml) in a sealed tube at 90°C for 7h. The reaction mixture was extracted with n-hexane, and the aqueous layer was lyophilized. The lyophilized product was dissolved in pyridine (0.05 ml) and added L-cysteine methyl ester hydrochloride (ca. 0.5 mg) and reacted at 70°C for 1h, then 1-(trimethylsilyl) imidazole was added. TMS ethers of methyl 2-(polyhydroxyalkyl)-thiazolidine-4(*R*)-carboxylates were analyzed by GC-MS.

Mannose of 44-2 t_R = 24.95 min, standard D-mannose t_R = 24.95 min, standard L-mannose t_R = 24.81 min.

Standard sapmples: D-mannose, mannitol, glucitol (Kishida Chemicals, Osaka, Japan), D-glucose, 12-hydroxystearic acid (Wako Chemicals, Osaka, Japan), L-mannose (Sigma-Aldrich, St. Louis, USA).

[Shimadzu QP-5050A with Inert Cap 5MS/Sil I.D. 0.25 x 30 m, GL Science Inc. Tokyo, Japan, column temperature 150-300°C, rate of temperature increase: 4°C /min].

Reference: Hara, S., Okabe, H., and Mihashi, K. (1987). Gas-liquid chromatographic separation of aldose enantiomers as trimethylsilyl ethers of methyl 2-(polyhydroxyalkyl)-thiazolidine-4(R)-carboxylates. Chem. Pharm. Bull., *35*, 501-506.

(U-X) Acyl position on L-mannitol.

(U) ^{1}H-NMR(C_5D_5N/D_2O (20/1), 600MHz, 300K, δ_H): 4.12 (1H, dd, *J*=3.0, 11.4, mnol-6), 4.22 (1H, dd, 3.6, 11.4, mnol-6'), 4.40 (1H, m, mnol-5), 4.54 (1H, m, mnol-2), 4.64 (2H, m, mnol-1), 6.16 (1H, brd, *J* = 7.9, mnol-4), 6.22 (1H, brd, *J* = 7.9, anol-3).

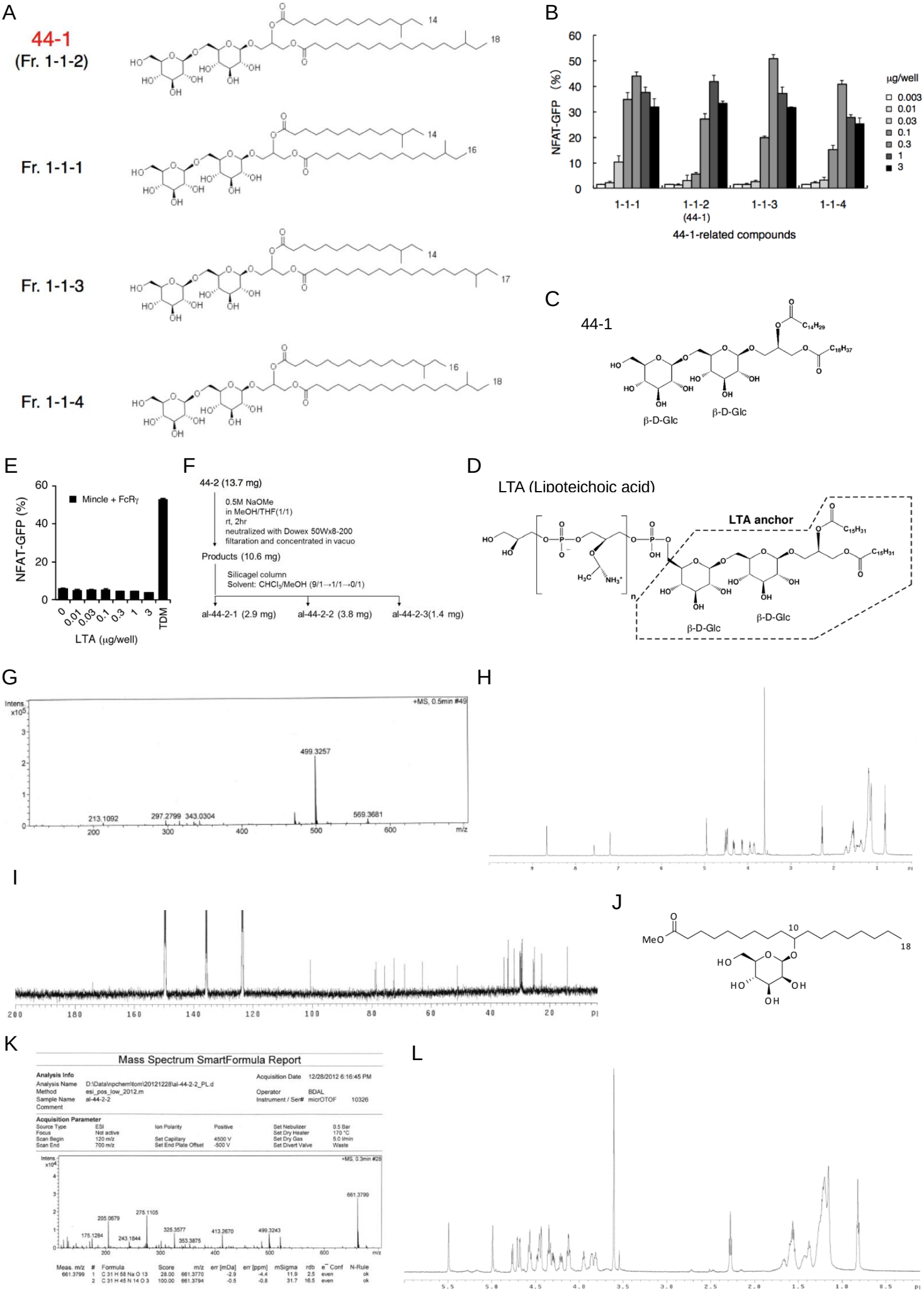
(V) The TOCSY spectrum revealed the correlations from H-3 to H_2-1, and H-4 to H_2-6 of mannitol. Although the correlation between H-3 and H-4 has not been observed, it thought to be the dihedral angle of H-3-C3-C-4-H-4 was nearly 90 degrees and the $^3J_{H\text{-}H}$ was very small.

(W) HSQC spectrum gave the ^{13}C chemical shifts assignment of L-mannitol, 66.2 (C1), 64.1 (C6), 67.9 (C2), 70.5 (C5), 71.9 (C3), 72.1 (C4).

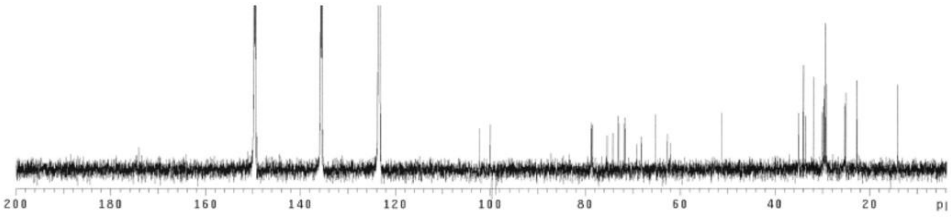
(X) HMBC spectrum revealed the correlations from H-3 and H-4 of mannitol to ester

carbonyl carbons of fatty acids. Although, the correlation between H_2-1 of mannitol to acyl carbon, the remaining fatty acid was linked to oxymethylene (C1 or C6) of mannitol, because the esterification shift was confirmed of the one oxymethylene proton and carbon (δ_H 4.64 and δ_C 66.2). As a matter of convenience, this oxymethylene was assumed to be C1 of mannitol.

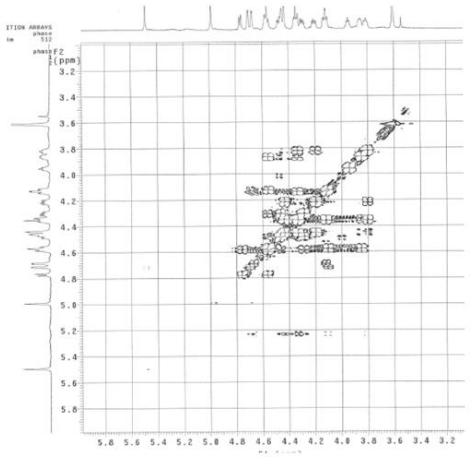
Figure S4


A
44-1
(Fr. 1-1-2)
Fr. 1-1-1
Fr. 1-1-3
Fr. 1-1-4
B
NFAT-GFP (%)
μg/well
0.003
0.01
0.03
0.1
0.3
1
3
1-1-1
1-1-2
(44-1)
1-1-3
1-1-4
44-1-related compounds
C
44-1
β-D-Glc
β-D-Glc
D
LTA (Lipoteichoic acid)
LTA anchor
β-D-Glc
β-D-Glc
E
NFAT-GFP (%)
Mincle + FcRγ
LTA (μg/well)
TDM
F
44-2 (13.7 mg)
0.5M NaOMe
in MeOH/THF(1/1)
rt, 2hr
neutralized with Dowex 50Wx8-200
filtaration and concentrated in vacuo
Products (10.6 mg)
Silicagel column
Solvent: CHCl3/MeOH (9/1→1/1→0/1)
al-44-2-1 (2.9 mg)
al-44-2-2 (3.8 mg)
al-44-2-3(1.4 mg)
G
+MS, 0.5min #49
499.3257
213.1092
297.2799
343.0304
569.3681
H
I
J
K
Mass Spectrum SmartFormula Report
+MS, 0.3min #28
661.3799
205.0679
275.1105
325.3577
413.2670
499.3243
175.1284
243.1844
353.3875
L

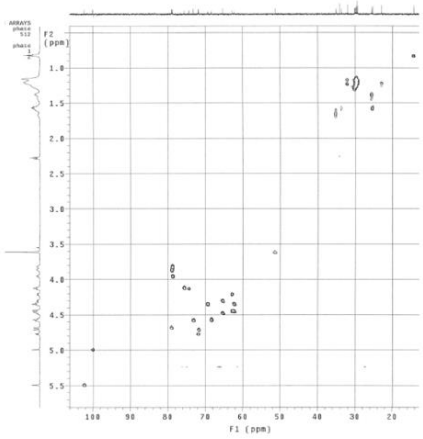
M



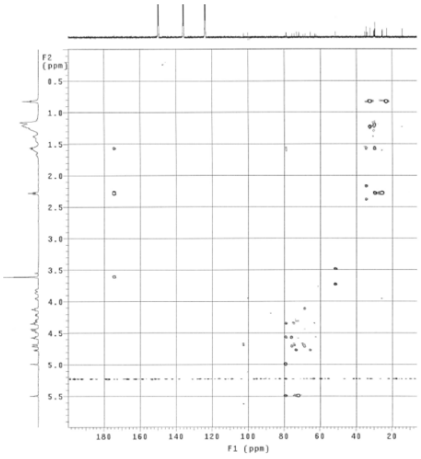
N

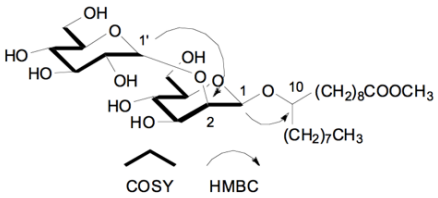


O

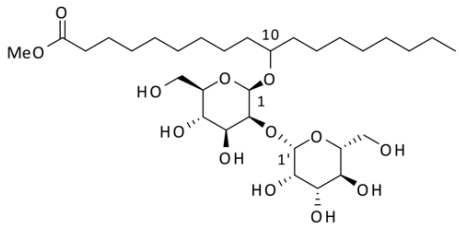


P

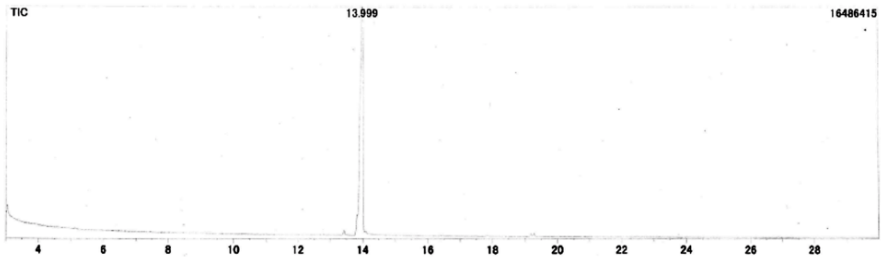



OH
O
1'
HO
HO
OH
OH
O
O
1
O
10
(CH2)8COOCH3
HO
HO
2
(CH2)7CH3
COSY
HMBC

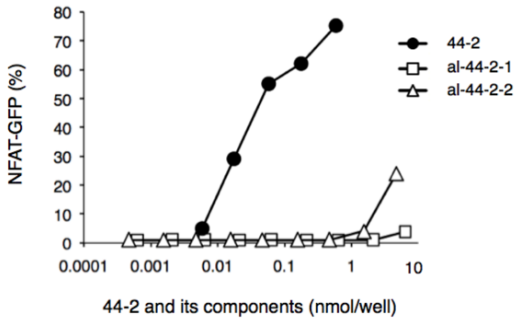
Q


MeO
O
10
HO
O
1
HO
OH
1'
OH
HO
OH
OH

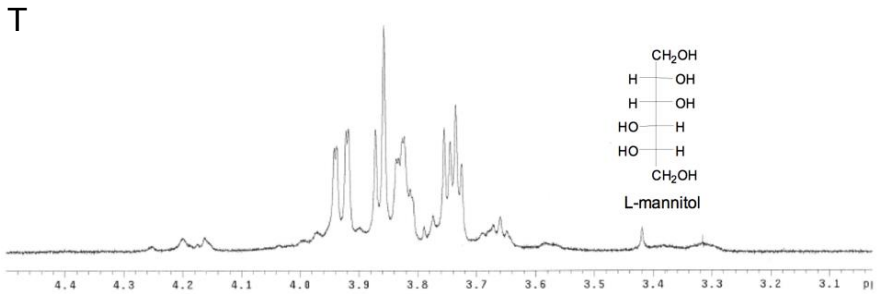
S


TIC
13.999
16486415

R


NFAT-GFP (%)
44-2
al-44-2-1
al-44-2-2
0.0001
0.001
0.01
0.1
1
10
44-2 and its components (nmol/well)

T


CH2OH
H
OH
H
OH
HO
H
HO
H
CH2OH
L-mannitol

U

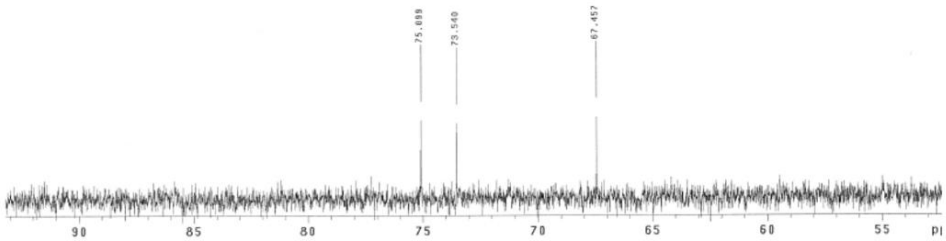

75.698
73.540
67.457

Figure S4. Structures and activities of 44-1- and 44-2-related compounds. (related to Figure 4)

(A) The structures of 44-1-related compounds.

The structures of fraction 1-1-1, 1-1-3, and 1-1-4 were determined using FAB-MS and GC-MS analysis.

Fraction 1-1-1: FAB-MS (negative, glycerol) *m/z*: 891[M-H]$^-$, 269 (R-COO$^-$), 241 (R'-COO$^-$); FAMEs: methyl 12-methyltetradecanoate t_R = 16.77 min, methyl 14-methylhexadecanoate t_R = 18.96 min, *m/z* = 284(M^+), 269, 255, 241, 87, 74.

Fraction 1-1-3: amorphous powder, negative ion FAB-MS (glycerol) *m/z*: 933[M-H]$^-$, 311 (R-COO$^-$), 241 (R'-COO$^-$); FAMEs: methyl 12-methyltetradecanoate t_R = 16.77 min, methyl 17-methylnonadecanoate t_R = 22.13 min, *m/z* = 326 (M^+), 295, 283, 269, 87, 74.

Fraction 1-1-4: amorphous powder, negative ion FAB-MS (glycerol) *m/z*: 947[M-H]$^-$, 297 (R-COO$^-$), 269 (R'-COO$^-$); FAMEs: methyl 14-methylhexadecanoate t_R = 18.97 min, methyl 16-methyloctadecanoate t_R = 20.96 min.

(B) Mincle ligand activity of 44-1-related compounds.

Mincle-expressing reporter cells were stimulated with plate-coated 44-1 and related compounds for 18 h. The NFAT-GFP expression was analyzed by flow cytometry. The data (B) are means ± SD for triplicated assays, and representative results from two independent experiments with similar results are shown.

(C) The chemical structure of 44-1.

(D) The chemical structures of the prototypical LTA and LTA anchor.

(E) NFAT-GFP reporter cells expressing Mincle + FcRγ were co-cultured for 18 h with plates coated LTA (0.01 - 3 μg/well) or TDM (0.27 μg/well) as a control. The data (E) are the means ± SD for triplicate assays, and representative results from three independent experiments with similar results are shown.

(F) Alkaline hydrolysis and purification of 44-2 components.

44-2 (fraction 1: 13.7 mg) was treated with 0.5M NaOMe/MeOH/THF (0.4 ml) at room temperature for 2h. The reaction mixture was diluted with MeOH (0.5 ml) and neutralized with Dowex and filtrated. The filtrate was subjected to silica gel column chromatography eluted with $CHCl_3$/MeOH (9/1-1/1-0/1) to give three compounds, al-44-2-1 (2.9 mg), al-44-2-2 (3.8 mg) and al-44-2-3 (1.4 mg).

(G-J) Structure elucidation of al-44-2-1.

(G) The molecular formula of al-44-2-1 was determined to be $C_{25}H_{48}O_8$ by ESI-TOFMS

[*m/z* 499.3257, calcd 499.3241 $(M+Na)^+$]. (H) ^{1}H-NMR(C_5D_5N/D_2O (20/1), 600MHz, δ_H): 0.80 (3H, t, -CH_3), 1.1-1.7 (m, methylenes), 2.28 (t, -CH_2-CO), 3.60 (3H, s, OMe), 3.85 (1H, m, man-5), 3.95 (1H, t, H10), 4.13 (1H, dd, man-3), 4.32 (1H, dd, man-6), 4.48 (1H, d, man-2), 4.50 (1H, m, man-6), 4.51 (t, man-4), 4.96 (1H, s, man-1). (I) ^{13}C-NMR(C_5D_5N/D_2O (20/1), 150MHz, δ_C) 14.1 (q, C18), 22-36 (t, methylenes), 51.2 (q, OMe), 62.9 (t, man-6), 68.9 (d, man-4), 72.6 (d, man-2), 75.6 (d, man-3), 78.5 (d, C10), 78.9 (d, man-5), 174.0 (s, C2). (J) Structure of al-44-2-1.

(K-Q) Structure elucidation of al-44-2-2.

(K) The molecular formula of al-44-2-2 was determined to be $C_{31}H_{58}O_{13}$ by ESI-TOFMS [*m/z* 661.3799, calcd 661.3770 $(M+Na)^+$]. (L) ^{1}H-NMR(C_5D_5N/D_2O (20/1), 600MHz, δ_H): 0.82 (3H, t, -CH_3), 1.1-1.7 (m, methylenes), 2.28 (t, -CH_2-CO), 3.61 (3H, s, OMe), 3.30 (1H, m, man-5), 3.35 (1H, m, man-5'), 3.95 (1H, t, H10), 4.11 (2H, m, man-3 and 3'), 4.20 (1H, dd, man-6), 4.35 (2H, m, man-4 and man-6'), 4.45 (2H, m, man-6 and man-6'), 4.56 (1H, t, man-4'), 4.69 (1H, d, man-2), 4.70 (1H, d, man-2'), 4.99 (1H, s, man-1), 5.50 (1H, s, man-1'). (M) ^{13}C-NMR(C_5D_5N/D_2O (20/1), 150MHz, δ_C) 14.1 (q, C18), 22-35 (t, methylenes), 51.2 (q, OMe), 62.0 (t, man-6'), 63.0 (t, man-6), 68.2 (d, man-4'), 69.2 (d, man-4), 71.6 (d, man-2'), 74.2 (d, man-3), 75.4 (d, man-3'), 78.6 (d, C10), 78.6 (d, man-2), 78.8 (d, man-5), 78.8 (d, man-5'), 100.0 (d, man-1), 102.2 (d, man-1'), 174.0 (s, C2). ^{1}H-^{1}H COSY spectrum revealed the correlations of two β-D-mannopyranoses (N), HSQC spectrum gave the ^{1}H and ^{13}C chemical shifts assignment of mannopyranoses (O), and HMBC spectrum showed the correlations of H-1' of man-1' to C-2 of man-1, and H-1 of man-1 to C-10 of fatty acid (P). (Q) Structure of al-44-2-2.

(R) Mincle ligand activity of hydrolyzed product of 44-2.

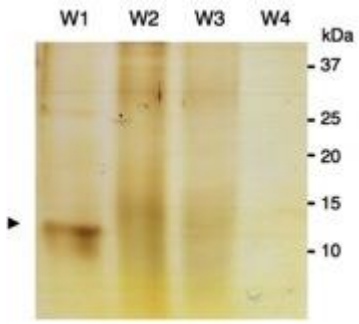
Mincle-expressing reporter cells were stimulated with al-44-2-1, al-44-2-2 and intact 44-2 for 18 h. The NFAT-GFP expression was analyzed by flow cytometry.

(S-U) Structure elucidation of al-44-2-3.

(S) GC-MS chromatogram of TMS-ether of al-44-2-3, t_R = 13.99 min. (T) ^{1}H-NMR(D_2O, 600MHz, δ_H): 3.74 (2H, H-1 and H-6), 3.82 (2H, H-2 and H-5), 3.86 (2H, H-3 and H-4), 3.93 (2H, H-1' and H-6'). (U) ^{13}C-NMR (D_2O, 150MHz, δ_C) 67.5 (C1 and C6), 73.5 (C3 and C4), 75.1 (C2 and C5). $[\alpha]_D^{26}$ –16.0 (H_2O, c = 0.03), standard D-mannitol: $[\alpha]_D^{26}$ +15.4 (H_2O, c = 0.07).

Figure S5

A



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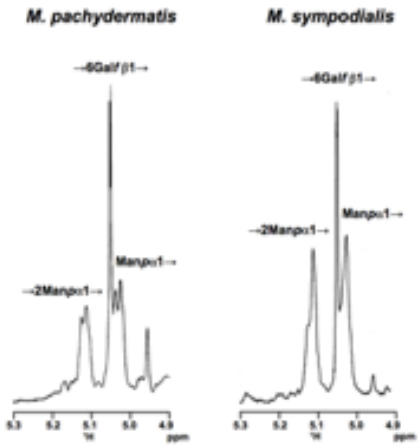

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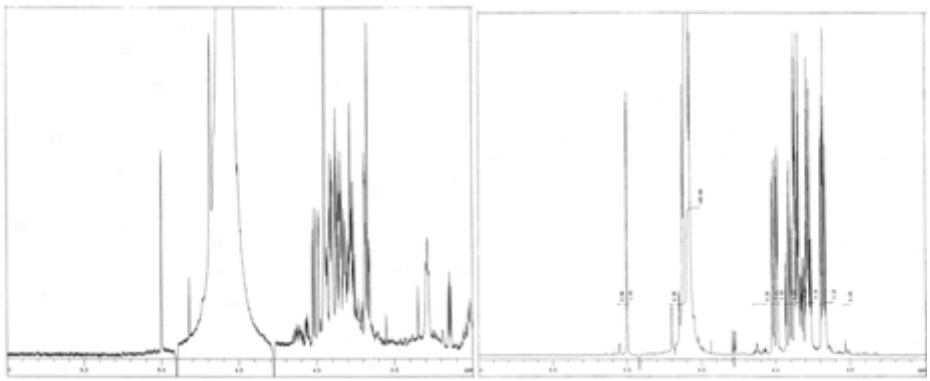
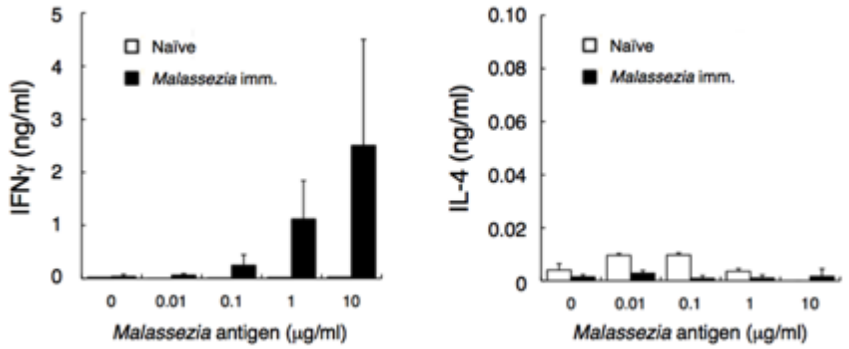


Figure S5. Characterization of W1. (related to Figure 5)

(A) SDS-PAGE analysis of W1 core protein. W1 after β-elimination (*O*-deglycosylation) was separated by 15% SDS-PAGE gel and visualized by silver staining. The same amount of inactive fraction W2, W3 and W4 were also analyzed as control. The numbers on the right indicate the sizes (kDa) of the molecular mass marker. (B) The ^{1}H NMR analysis of intact W1 fraction from *M. pachydermatis* and *M. sympodialis*. (C) The ^{1}H NMR analysis of the oligosaccharides released from W1 fractions by β-elimination. *O*-linked α-1,2-mannobiose was detected in both strains. The ^{1}H NMR spectra were recorded by a JNM-LA600 spectrometer (JEOL) at 45°C.

Figure S6

A



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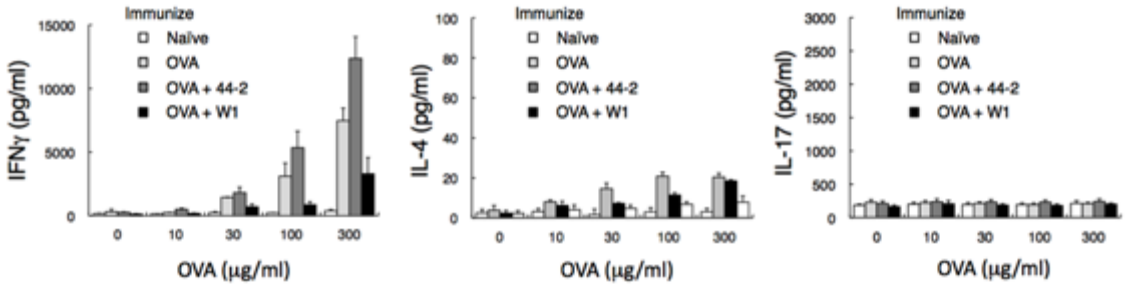


Figure S6. Effect of *Malassezia* components on acquired immunity. (related to Figure 6)

(A) Effect of *Malassezia* on T cell recall responses.

Mice were intraperitoneally injected with 4 x 10^7 *M. furfur*. Splenocytes were collected at 7 days after injection and stimulated with antigen extract from *Malassezia*-derived antigen for 72 h. Concentration of IFNγ and IL-4 were determined by ELISA. Concentration of IL-17 was below detection level. Each group included at least three mice. The data (A) are the means ± SD for triplicate assays.

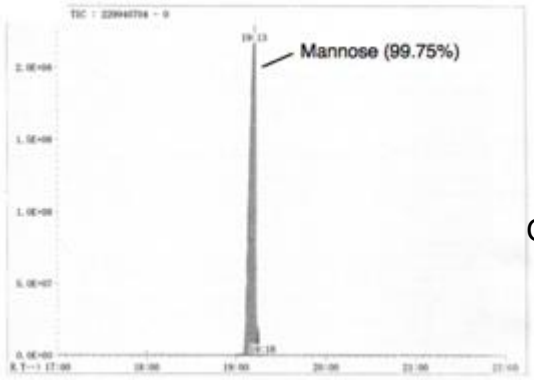
(B) Effect of 44-2 and W1 on antigen-specific T cell responses.

Mice were immunized with OVA in oil-in-water emulsion of 44-2 and W1-PBS solution at the base of the tail. Inguinal LNs were collected at 7 days after immunization and stimulated with OVA for 72 h. Concentrations of IFNγ, IL-4 and IL-17 were determined by ELISA. Each group included at least four mice. The data (B) are the

means ± SD for triplicate assays, and representative results from two independent experiments with similar results are shown.

Figure S7

A



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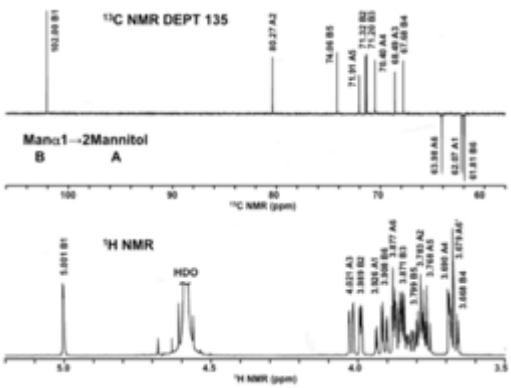
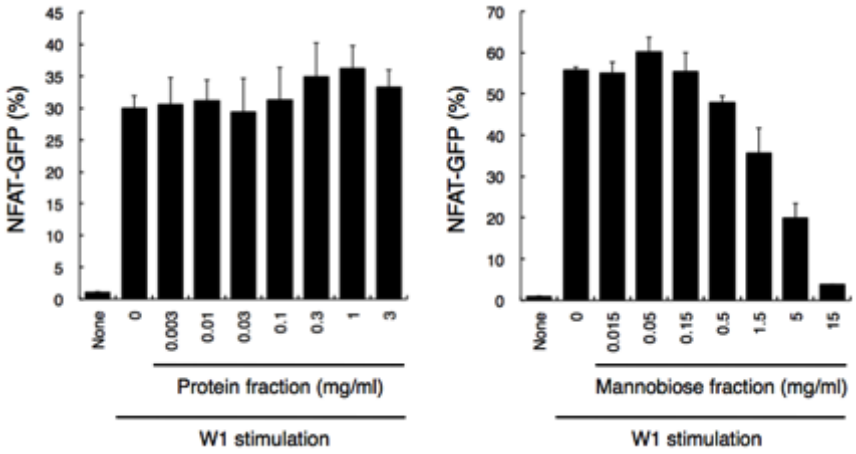


C

D



E

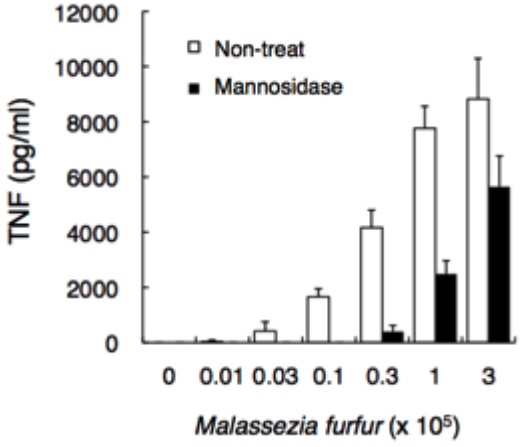


Figure S7. Carbohydrate analysis and activities of W1. (related to Table 1)

*(*A) *O*-linked disaccaride fraction purified from W1 was hydrolyzed with 4 M trifluoroacetic acid (TFA) at 100°C for 3 h. The resulting monosaccharide mixtures were reduced by treatment with $NaBH_4$ and acetylated by acetic anhydride/pyridine (1:1, v/v). The reagents were evaporated and analyzed by GC/MS.

(B) ^{13}C NMR DEPT 135 spectrum of the disaccharide. Negative signals in the spectrum imply that the carbon atom must have two protons and therefore correspond to the C-6 of mannose residue and C-1 and C-6 of mannitol residue.

(C) ^{1}H NMR spectrum of the disaccharide.

The proton and carbon chemical shifts were referenced relative to the internal acetone at δ 2.225 and 31.07, respectively.

(D) Blocking activity of W1 components. Protein fraction and mannobiose fraction were separated by gel filtration after β-elimination of W1. NFAT-GFP reporter cells expressing Dectin-2 and FcRγ were stimulated with plate-coated W1 (0.003 – 3 μg/well) in the presence of the protein fraction (left) or mannobiose fraction (right) for 18 h. NFAT-GFP expression was determined by flow cytometry.

(E) Stimulatory activity of *Malassezia* after mannobiose depletion. BMDCs from WT mice were stimulated with non-treated (non-treat) or α-mannosidase-treated (mannosidase) *M. furfur*. After 48 h culture, the concentrations of TNF were determined by ELISA. The data (D and E) are the means ± SD for triplicate assays.

Table S1. GC-MS analysis[a] of *O*-methylalditol acetates derived from methylation analysis of W1 and mannobiose released from W1 by β-elimination. (related to Table 1)

O-methylalditol acetate	RRT[b]	Linkage	Molar ratio	
			W1	Man_2-ol
1,3,4,5,6-Penta-*O*-Me-mannitol	0.88	–> 2)-Man-ol[c]	-	0.80
2,3,4,6-Tetra-*O*-Me-mannitol	1.00	Man*p*-(1 –>	1.00	1.00
3,4,6-Tri-*O*-Me-mannitol	1.08	–> 2)-Man*p*-(1 –>	0.74	-
2,3,4-Tri-*O*-Me-mannitol	1.11	–> 6)-Man*p*-(1 –>	-	-
3,4-Di-*O*-Me-mannitol	1.20	–> 2,6)-Man*p*-(1 –>	-	-

[a] The partially methylated alditol acetates were analyzed by GC/MS using a capillary column (30 m x 0.25 mm; DB-5) with helium as the carrier gas and a temperature program (210-260°C at 5°C /min). The GC/MS analyses were carried out on a JMS-K9 (JEOL, Tokyo, Japan).

[b] Retention time relative to that of 2,3,4,6-tetra-*O*-methyl-1,5-di-*O*-acetyl-mannitol.

[c] Man-ol; mannitol

Table S2. Amino acid composition of mannosyl-W1. (related to Figure 5)

Amino acid	mol%
Alanine	11.13
Arginine	1.68
Aspartic acid	8.58
Cysteine	0.00
Glutamic acid	5.88
Glycine	9.31
Histidine	0.74
Isoleucine	4.15
Leucine	5.28
Lysine	1.06
Methionine	0.13
Phenylalanine	2.77
Proline	4.32
Serine	32.90
Threonine	5.77
Tyrosine	1.27
Valine	5.09
Total	100.00