Dectin-2 Recognises Mannosylated O-antigens of Human Opportunistic Pathogens and Augments Lipopolysaccharide Activation of Myeloid Cells

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Running Title: *Dectin-2 augments TLR4 signalling*

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ABSTRACT

Lipopolysaccharide (LPS) consists of a relatively conserved region of lipid A and core-oligosaccharide, and a highly variable region of O-antigen polysaccharide. While lipid A is known to bind to the toll-like receptor 4 (TLR4)-myeloid differentiation factor 2 (MD2) complex, the role of the O-antigen remains unclear. Here we report a novel molecular interaction between dendritic cell-associated C-type lectin-2 (Dectin-2) and mannosylated O-antigen found in a human opportunistic pathogen *Hafnia alvei* PCM 1223, which has repeating unit of [-Man-α1,3-Man-α1,2-Man-α1,2-Man-α1,2-Man-α1,3-]. *H. alvei* LPS induced higher levels of TNFα and IL-10 from mouse bone marrow-derived dendritic cells (BM-DCs), when compared to *Salmonella enterica* O66 LPS which has a repeat of [-Gal-α1,6-Gal-α1,4-[Glc-β1,3]GalNAc-α1,3-GalNAc-β1,3-]. In a cell-based reporter assay, Dectin-2 was shown to recognise *H. alvei* LPS. This binding was inhibited by mannosidase treatment of *H. alvei* LPS and by mutations in the carbohydrate-binding domain of Dectin-2, demonstrating that *H. alvei* LPS is a novel glycan ligand of Dectin-2. The enhanced cytokine production by *H. alvei* LPS was Dectin-2 dependent, as Dectin-2 knockout BM-DCs failed to do so. This receptor crosstalk between Dectin-2 and TLR4 involved events including spleen tyrosine kinase (Syk) activation and receptor juxtaposition. Furthermore, another mannosylated LPS from *Escherichia coli* O9a, also bound to Dectin-2 and augmented TLR4 activation of BM-DCs. Taken together, these data indicate that mannosylated O-antigens from several gram-negative bacteria augment TLR4 responses through interaction with Dectin-2.

Lipopolysaccharide (LPS)[2] consists of the relatively conserved region of lipid A and core-oligosaccharide, and the highly variable region of O-antigen polysaccharide (1). The conserved lipid A is recognised by the toll-like receptor 4 (TLR4)-myeloid differentiation factor 2 (MD2) receptor complex expressed on innate

immune cells such as dendritic cells (DCs) and macrophages (2). Lipid A binding induces TLR4 dimerization, and activates further downstream signalling, leading to inflammation-associated gene expression, such as cytokines and chemokines (3). In addition, previous studies have suggested a regulatory role of the variable O-antigen in LPS activation. For instance, in the LPS-induced sepsis mouse model, disease severity varies depending on the nature of the O-antigen glycan structure (4). An *in vitro* mechanistic study suggests that the O-antigen affects the kinetics of cytokine production from macrophages (5). Further, a recent report suggests a contribution of O-antigen to the pain occurring during the LPS-induced shock (6).

Glycan-binding proteins (lectins) expressed on the cell surface of innate immune cells have been reported to recognise O-antigens and the binding may influence TLR4 signalling (7). For example, the macrophage mannose receptor binds to LPS from various *Klebsiella pneumoniae* strains (8); the dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) binds to LPS isolated from *Helicobacter pylori* (9); and the Sialic acid binding Ig-like lectin-7 (Siglec-7) binds to lipooligosaccharide of *Campylobacter jejuni* (10).

Dendritic cell-associated C-type lectin-2 (Dectin-2) is a single transmembrane lectin expressed on various myeloid cells in mouse and man, including DCs, monocytes, and macrophages (11-14). Dectin-2 recognises α-linked mannose structure as a glycan ligand and elicits various cellular responses including cytokine production (15,16), cell surface marker induction (17), ligand endocytosis (18), and antigen presentation to CD8T cells (19). Dectin-2 signalling pathway involves the adaptor molecule Fc receptor common gamma-chain (FcRγ) that harbours the immunoreceptor tyrosine-based activation motif (ITAM) in the cytoplasmic domain (16-18). Upon Dectin-2 binding to the glycan ligands, the ITAM motif gets phosphorylated and induces spleen tyrosine kinase (Syk) activation (15,16). While glycan ligands of Dectin-2 have been identified in various microbes including *Candida albicans*, *Malassezia pachydermatis*, and mycobacteria (16,17,20,21), the nature of Dectin-2 ligands from gram-negative bacteria remains unclear.

According to the microbial polysaccharide database (http://csdb.glycoscience.ru/bacterial/), α-linked mannose containing O-antigens are found in various gram-negative bacterial species such as *Citrobacter braakii*, *Citrobacter werkmanii* O21 (22,23), *Escherichia coli* O8, O9, O68, and K12 (24-27), *Klebsiella pneumoniae* O3 and O5 (24,25), *Hafnia alvei* PCM 1223 (28), and *Serratia marcescens* O28 (29). Some of these bacteria can cause nosocomial infections in lung and urinary tract (30-34). Of note, 11% of *K. pneumoniae* clinical isolates were shown to be serotype O3 and O5 (35). Therefore it is of great importance to determine whether Dectin-2 recognises the mannosylated O-antigens.

In this study, we investigated the contribution of the α-linked mannosylated O-antigen in the LPS activation of myeloid cells. We compared DC response and Dectin-2 binding to the mannosylated LPS (Man-LPS) from *H. alvei* PCM 1223 and *E. coli* O9a with the LPS from *Salmonella enterica* O66 or *K. pneumonia* O1, which has the galactosylated O-antigen (Gal-LPS) (**Fig. 1A**). We observed binding between Man-LPS and Dectin-2, which led to augmentation of TLR4 response in mouse DCs and human monocytes. These results demonstrate a novel role of mannosylated O-antigen in activation of TLR4 in myeloid cells.

RESULTS

Man-LPS produced a higher level of TNFα and IL-10 from bone marrow-derived DCs (BM-DCs) than Gal-LPS.

To address the contribution of O-antigen in the LPS activation of innate immune cells, we tested two structurally defined LPS. The Man-LPS from *H. alvei* PCM 1223 is built of [-Man-α1,3-Man-α1,2-Man-α1,2-Man-α1,2-Man-α1,3-] repeating units (28), whereas Gal-LPS from *S. enterica* O66 contains [-Gal-α1,6-Gal-α1,4-[Glc-β1,3]-GalNAc-α1,3-GalNAc-β1,3-] repeating units (**Fig. 1A**) (36). The core-oligosaccharide and lipid A of these two LPS are relatively conserved (37-40). TLR4 activation by these two types of LPS was first measured using the TLR4-MD2 expressing HEK293 reporter cells with Man-LPS being 4-fold more potent as compared to Gal-LPS (**Fig. 1B**). Based on this result, we standardised TLR4 activation by using a 4-fold higher concentration of Gal-LPS

compared to Man-LPS in the rest of the study. Under these conditions, Man-LPS induced 2-fold more TNFα and IL-10 from mouse BM-DCs than Gal-LPS (**Fig. 1C**). Man and Gal-LPS induced the co-stimulatory molecule CD80 and mouse MHC class II molecule I-A^b to a similar extent (data not shown).

Man-LPS is a novel glycan ligand of Dectin-2.

Since O-antigen of Man-LPS consists of α-linked mannose, a glycan ligand of Dectin-2, we tested Dectin-2 binding to the purified LPS in a cell-based reporter assay, in which the lectin-glycan interaction is monitored as β-galactosidase expression (41). The Dectin-2 BWZ cells were cultured in a 96-well plate coated with Man and Gal-LPS. In this assay, Dectin-2 bound to Man-LPS, but not to Gal-LPS (**Fig. 2A**). No binding was observed between mock-BWZ cells and LPS, indicating specific binding of Dectin-2 to Man-LPS (**Fig. 2A**). In addition, plant derived galactan, β-linked mannan (**Fig. 2B**), and LPS from *K. pneumoniae* O1 (**Fig. 2C**), which has a homopolymeric Gal O-antigen (42), failed to bind to Dectin-2, confirming Dectin-2 binding to Man-LPS is sugar composition and linkage specific, rather than non-specific binding to homopolymeric carbohydrate polymer. Furthermore, the lipid A isolated from Man-LPS failed to bind to Dectin-2 (**Fig. 2D**), and treatment of Man-LPS with α-mannosidase inhibited the binding (**Fig. 2E**), suggesting that binding was mediated by the mannosylated O-antigen of Man-LPS. To assess whether the binding is through the carbohydrate-recognition domain of Dectin-2, we compared the binding of wild-type (WT) Dectin-2 and the QPD-mutant that no longer recognises mannose (17,43). As shown in **Fig. 2F**, the binding was significantly reduced by the mutations. These results demonstrate that Dectin-2 recognises the α-linked mannosylated O-antigen of *H. alvei* LPS. Since mannosylated O--antigen is found in other gram-negative bacteria including *E. coli* O9a (44), we tested whether Dectin-2 recognises mannosylated O-antigen from *E. coli* O9a. We found that

E. coli O9a LPS bound to Dectin-2, whereas the rough mutant LPS, which lacks the O-antigen (45), failed (**Fig. 2G**). We also tested the binding of Dectin-2 to *H. alvei* in the reporter assay. Dectin-2 bound to paraformaldehyde (PFA)-fixed *H. alvei*, whereas the QPD mutant did not (**Fig. 2H**), suggesting the role of Dectin-2 as a recognition receptor for gram-negative bacteria with α-linked mannosylated O-antigens.

Man-LPS activation involves a synergy between Dectin-2 and TLR4.

To assess the involvement of Dectin-2 in Man-LPS activation of immune cells, we generated BM-DCs from Dectin-2 KO mice (**Fig. 3A**). In contrast to WT BM-DCs, Dectin-2 KO BM-DCs were unable to enhance TNFα and IL-10 production in response to Man-LPS (**Fig. 3B**), indicating that Dectin-2 augments TLR4 activation by Man-LPS. This was reproducible when we used another Gal-LPS from *K. pneumoniae* O1 (data not shown). Of note, in the TLR4 KO BM-DCs, neither Man nor Gal-LPS induced the cytokine production (**Fig. 3B**), suggesting that the mannosylated O-antigen is not sufficient to activate Dectin-2 in the absence of TLR4. Similarly, Dectin-2-dependent enhancement in TNFα production by BM-DCs was seen in response to the WT *E. coli* O9a LPS, but not to the rough LPS (**Fig. 3C**). IL-10 response showed similar tendency, but was not statistically significant between WT and rough LPS. (**Fig. 3C**). The TLR4 reactivity of WT and rough LPS was indistinguishable (data not shown). We also assessed the contribution of Dectin-2 to the DC response toward *H. alvei*. IL-10 production in response to *H. alvei* was Dectin-2 dependent, suggesting a regulatory role of Dectin-2 in the recognition of *H. alvei* (**Fig. 3D**). Although TNFα production in response to Man-LPS was enhanced by Dectin-2 (**Fig. 3B**), the TNFα response to *H. alvei* was similar between WT and Dectin-2 KO BM-DCs (**Fig. 3D**), suggesting alternative molecular mechanisms leading to TNFα production such as TLR2 that recognises bacterial cell wall components (46).

The receptor synergy is Syk dependent and requires receptor juxtaposition.

To investigate the intracellular signalling events involved in this process, we assessed the impact of Man-LPS on Syk, a key molecule in the Dectin-2 pathway (15,16). Syk was found to be phosphorylated upon stimulation of BM-DCs by Man-LPS but not Gal-LPS (**Fig. 4A**). Furthermore, treatment of BM-DCs with the Syk inhibitor R406 abrogated the augmented cytokine production in response to Man-LPS (**Fig. 4B**), demonstrating Syk-dependent synergy between Dectin-2 and TLR4. Next, we assessed

the impact of the Syk activation by Man-LPS on the activation of NF-κB and MAPK pathways, hallmark of TLR4 activation (3). The phosphorylation of p38 and degradation of IκB was indistinguishable between Man and Gal-LPS, respectively, suggesting that other signalling pathways are modified by Syk activation through Dectin-2 (**Fig. 4C)**.

Since Man-LPS has the binding epitopes for both Dectin-2 and TLR4, we hypothesised that receptor juxtaposition by Man-LPS is the mechanism underpinning the synergy. To test this hypothesis, BM-DCs were stimulated with Gal-LPS as a TLR4 ligand in the presence of yeast α-linked mannan, a known Dectin-2 ligand. As shown in **Fig. 5**, addition of yeast mannan was not sufficient to enhance cytokine production, compared to the Gal-LPS, demonstrating that receptor juxtaposition is required to achieve synergy.

Human monocytes recapitulate the enhanced cytokine production in response to Man-LPS.

Man-LPS activation was also tested on human myeloid cells expressing Dectin-2. We found that human peripheral blood monocytes expressed Dectin-2 at a high level, and blood DCs and monocyte-derived DCs (Mo-DCs) expressed at a negligible level (**Fig. 6A**), which is consistent with previous reports analysing human Dectin-2 mRNA expression among human immune cells (11,12). Human monocytes produced higher levels of TNFα and IL-10 in response to Man-LPS compared to Gal-LPS (**Fig. 6B**). The anti-Dectin-2 antibody (Ab), however, failed to block the enhanced cytokine production (data not shown), implying the potential involvement of other lectins.

DISCUSSION

In line with our findings, recent reports show interactions between mammalian lectins and O-antigens. Several strains of *H. alvei* are targeted by Ficolin-3, a complement-associated soluble lectin (47,48). Langerin, a C-type lectin specific to α-linked mannose, is suggested to recognise the internal Man-α1,2-Man repeat found in O-antigens from *Escherichia coli* O106 and *Shigella boydii* B10 in the pathogen glycan array (49), implying that C-type lectins are capable of interacting with the internal glycan epitopes. At this moment, it is unclear whether the binding of Dectin-2 to the mannosylated O-antigen is mediated by the terminal mannose residue at the non-reducing end, the internal α-linked mannose repeats, or both.

Our findings have identified novel carbohydrate ligands of Dectin-2, and provided a deeper understanding in host-microbe interactions mediated by Dectin-2. Previous studies demonstrate that Dectin-2 plays a key role in fungal and mycobacterial infection, and house dust allergy (14,16,17,50). In this report, we have revealed a group of gram-negative bacteria recognised by Dectin-2. LPS from *H. alvei* PCM 1223 and *E. coli* O9a bound to Dectin-2 and enhanced TLR4 responses of BM-DCs in a Dectin-2 dependent manner, suggesting a novel role of Dectin-2 in the interaction between host and gram-negative bacteria bearing α-linked mannosylated O-antigen. Of note, *H. alvei* LPS was more potent than *E. coli* O9a LPS in Dectin-2 engagement in BM-DCs (**Fig. 3B and C**), this may be due to the different O-chain length, as the sugar structure is identical between these two bacteria (44).

Our findings that Dectin-2 interacts with TLR4 upon Man-LPS stimulation strengthens the proposed model that lectins are capable of regulating TLR pathways in various ways. For instance, stimulation of DCs and macrophages with β-glucan in the presence of various TLR ligands afforded to enhance TNFα and IL-10 production (51-53). Co-stimulation of DCs with DC-SIGN and TLR ligands selectively enhances IL-10 production. (54,55). While these studies clearly demonstrate the crosstalk between C-type lectins and TLRs, the molecular mechanisms underpinning such receptor crosstalk remain elusive. One potential mechanism is receptor juxtaposition, as previously proposed for the ITAM-coupled lectin Siglec-H; positioning Siglec-H in a close proximity of TLR9 in the endosome would enhance the TLR9 activation (56). Here we showed that such synergy was observed between Dectin-2 and TLR4. Whether this is a common strategy for the modulation of TLR functions by membrane-bound lectins remains to be demonstrated.

We have identified Syk as a key molecule for crosstalk between Dectin-2 and TLR4. Syk has been proposed as a regulator of TLR4 signalling. Several studies have reported that in DCs and macrophages Syk gets

phosphorylated upon LPS stimulation (57), and Syk deficiency results in enhanced TNFα and reduced IL-10 (58). Of note, most of the studies employed LPS from *E. coli* O111:B4 (59). In our study, since Gal-LPS failed to induce phosphorylation of Syk, it is likely that O-antigen structure influences Syk activation. Thus in order to address the role of Syk in TLR4 signalling, it would be essential to test whether reported Syk activation by *E. coli* O111 LPS involves Dectin-2 or other lectins.

Although our findings have revealed a novel function of mannosylated O-antigen in LPS activation of innate immune cells (**Fig. 7**), the role of core-oligosaccharides in the TLR4 activation remains elusive. In this regard, SIGNR1 is reported to recognise the core-oligosaccharide of *E. coli* LPS and augment cytokine responses (60). Further studies of lectin recognition of both highly variable O-antigen and the conserved core-oligosaccharide are warranted to gain a better understanding of LPS recognition by innate immune cells.

EXPERIMENTAL PROCEDURES

Mice- C57BL/6J (WT), TLR4 KO (a gift from Dr. JS Frick , University of Tuebingen, Tuebingen, Germany), and Dectin-2 KO mice were maintained in the specific pathogen free animal facilities at the University of East Anglia (Norwich, UK), University of Tuebingen (Tuebingen, Germany), and Chiba University (Chiba, Japan), respectively. Animal use in this study was in the accordance with the UK home office guidelines, the Regierungspraesidium Tuebingen, and the ethics committee of Chiba University.

Reagents- All the chemical reagents were obtained from Sigma-Aldrich, unless otherwise stated. Abs used in the flow cytometry were obtained from Biolegend, unless otherwise indicated, and include those against mouse CD80 (GL-1), I-A^b (AF6-120.1), human Dectin-2 (R&D systems, 545943), CD3 (OKT3), CD14 (M5E2), CD16 (3G8), CD19 (HIB19), CD20 (2H7), and CD56 (HCD56), CD11c (3.9), and HLA-DR (L243). The Abs used for western blot were obtained from Cell signalling and include Syk (D3Z1E), phospho-Syk (C87C1), p38 (D13E1), phospho-p38 (D3F9), IkB (rabbit polyclonal Ab), β-actin (13E5). LPS from *H. alvei* PCM 1223, *S. enterica* O66, *K. pneumoniae* O1, *E. coli* O9a and the rough mutant were isolated as described previously (28,42,45,61,62). The lipid A from the Man-LPS was isolated by hydrolysis as previously described (28). Galactan and β-linked mannans are from Megazyme. The Syk inhibitor R406 was purchased from InvivoGen. ELISA kits for mouse and human TNFα and IL-10 were from Biolegend and used according to the manufacturer's instructions.

Cells and bacteria- BWZ.36 cells harboring IL-2-driven β-galactosidase cassette (63) and the retrovirus-packaging cell line Plat-E were obtained from Dr. N. Shastri (University of California Berkeley Berkeley, CA, USA) and Dr. T. Kitamura (The University of Tokyo, Tokyo, Japan), respectively, and maintained as described before (64). *H. alvei* PCM 1223 was obtained from the Polish Collection of Microorganisms and cultured in Luria broth at 37 °C under shaking at 200 rpm (Innova 44 incubator, New Brunswick Scientific).

Flow cytometry- Cells were washed with Hank's balanced saline solution (Lonza) containing 0.1% BSA, 2 mM EDTA (FACS buffer), blocked with anti-mouse Fc block Ab (Biolegend) for 5 min at 25 °C, and stained with the Abs for 30 min at 4 °C. Stained cells were washed once with FACS buffer and analyzed by Fortessa (BD Biosciences). For dead cell exclusion, propidium iodide was added to the sample at a final concentration of 0.33 μg/ml before the analysis. Acquired data were analyzed with FlowJo (Tree Star).

TLR4 reporter assay- TLR4 reporter assay was performed using the HEK-Blue human TLR4 reporter cells that produce alkaline phosphatase in response to LPS, according to the manufacturer's instructions (InvivoGen). Briefly, the TLR4-HEK293 cells were cultured in the HEK-Blue detection medium containing the substrate for alkaline phosphatase in the presence of the LPS for 20 h. After incubation, the absorbance at 620 nm was measured.

Establishment of mouse Dectin-2 reporter cells- The Dectin-2 reporter cells were established as described previously (64). Briefly, the extracellular domain of mouse Dectin-2 (Gln42 through Leu209) was cloned into the retrovirus vector pMXs-IRES-EGFP-Ly49A-CD3ζ harbouring transmembrane region of the

mouse Ly49A and the cytoplasmic domain of the mouse CD3ζ (64). The pMXs-IRES-EGFP-Dectin-2-Ly49A-CD3ζ vector and the parental vector was used to establish Dectin-2 and Mock-BWZ cells, respectively for the retrovirus transduction using Plat-E cells (65). In order to establish reporter cells expressing carbohydrate-binding incompetent mutant of Dectin-2 (Dectin-2^{QPD}), two missense mutations (G502C and A508G) in the mannose recognition domain were introduced, which results in amino acid substitution of E168Q and N170D. The DNA fragment encoding extracellular domain of Dectin-2 with the two missense mutations was synthesised (Genscript) and cloned into the pMXs-IRES-EGFP-Ly49A-CD3ζ vector and used as described above.

Dectin-2 reporter assay- Reporter assay was performed as previously described (64). Briefly the 96 well flat-bottom ELISA plate (MaxiSorp, Thermo Scientific Nunc) was coated with 100 µl containing 40 ng of LPS or polysaccharides in 100 mM sodium bicarbonate buffer pH 9.5, for 16 h at 4 °C. Dectin-2 or Mock BWZ cells were then cultured in the well and β-galactosidase activity was monitored as previously described (64). For the mannosidase treatment, a 96-well plate was coated with 1.6 ng Man-LPS. After discarding the solution, wells were washed once with 200 µl PBS and blocked with 100 µl 4% bovine serum albumin in PBS for 1 h at 25 °C. Blocked wells were washed, and 50 unit of α1-2,3 mannosidase (New England BioLabs) was added to the wells and incubated at 37 °C for 14 h. The reporter cells were added to test the binding. For the reporter assay with bacteria, 1.0 x 10^6 of PFA-fixed *H. alvei* were immobilised on the 96-well plate as described above.

Generation of anti-Dectin-2 mAb- The anti-Dectin-2 mAb was generated as described previously (66). Briefly, two female Lewis rats (Japan SLC) were immunised with Dectin-2-BWZ cells emulsified with complete Freund's adjuvant (Difco Laboratories). Following with two injections of the cells emulsified with incomplete Freund's adjuvant (Difco Laboratories), the immunised rats were sacrificed and common iliac lymph nodes were harvested to generate hybridomas as described previously (66). The established hybridomas were screened by the reporter assay described previously (67). The animal experiments were performed in accordance with the institutional animal ethics committee at The University of Tokyo (Tokyo, Japan). The established hybridoma clone 2B4 produced anti-mouse Dectin-2 mAb, which isotype was determined as rat IgG2a, κ chain by flow cytometry (66). Monoclonal Ab 2B4 was purified from the culture supernatant and labelled with Alexa Flour-647 (Lifetechnologies) as descried previously (66).

Generation of BM-DCs- BM-DCs were generated by *in vitro* culture of mouse bone marrow cells as described previously (68). Briefly 3 x 10^6 mouse bone marrow cells were cultured in a 10-cm dish with 12 ml of the RPMI 1640 medium (Lonza) supplemented with 25 mM HEPES, 10% FBS (Thermo Scientific Gibco), 55 µM 2-mercaptoethanol, 100 U/ml penicillin and 100 µg/ml streptomycin (Lonza), 2 mM glutamine (Lonza), 1mM non-essential amino acids (Lonza), 1 mM Sodium Pyruvate (Lonza), and 20 ng/ml mouse granulocyte-macrophage colony-stimulating factor (GM-CSF, Peprotech). The culture was kept undisturbed for 6 days. On day 6, the cells were harvested and used as BM-DCs.

Stimulation of BM-DCs- BM-DCs (1 x 10^5 cells) were cultured in a 96-well plate using the RPMI medium described above without GM-CSF. Man and Gal-LPS were added to the culture at a final concentration of 1 or 4 µg/ml, respectively, and incubated for 7 h at 37 °C. The concentration was standardised for their reactivity toward TLR4 (**Fig. 1B**). For the cytokine analysis, culture supernatant was harvested and TNFα and IL-10 was measured by ELISA. When the Syk inhibitor R406 was used, it was added to the cell culture at a final concentration of 1 µM, and incubated 30 min before the addition of LPS. For CD80 and I-A^b expression analysis, stimulated BM-DCs were harvested and analysed by flow cytometry as described above.

Western blotting of intracellular proteins- One million BM-DCs were stimulated with 1 or 4 µg/ml of Man- and Gal-LPS, respectively, for the indicated time period. Cells were then processed as previously described with adaptation of lysis buffer volume to 75 µl (69). Two hundred thousand of cell-equivalent

lysate (20 µl) was subjected to SDS-PAGE using a 4-15% gradient TGX mini gel (Bio-Rad) for 90 min at 100 V. Proteins were transferred onto nitrocellulose membrane (GE Healthcare Life Sciences) at 100 V for 30 min. The membranes were blocked with 5% non-fat milk (Lonza) in PBS containing 0.05% Tween20 (PBS-T) for 1 h at 25 °C. The blocked membranes were washed 4 times by incubating in PBS-T for 5 min each. Membranes were then incubated with the primary antibodies in PBS containing 1% bovine serum albumin for 1 h at 25 °C at a dilution of 1:1000 for Syk, phospho-Syk, IκB, and β-actin and 1:5000 for p38 and phospho-p38. Membranes were washed as above and probed with anti-rabbit IgG conjugated with horseradish peroxidase (Cell Signalling) in 5% non-fat milk in PBS-T at a dilution of 1:3000 for 1 h at 25 °C. Membranes were washed as above, and incubated with the ECL detection reagent (GE Healthcare Lifesciences) and imaged using Fluorochem E (ProteinSimple).

Human blood monocytes- Human peripheral blood was obtained from the haemochromatosis patients undergoing a therapeutic venesection at the Norfolk and Norwich University Hospital (Norwich, UK). Human blood monocytes were isolated as described previously (70). The Dectin-2 expression on the monocytes was analysed by flow cytometry. The freshly isolated monocytes were stimulated with 1 ng/ml Man and 4 ng/ml Gal-LPS for 20 h at 37 °C. Human TNFα and IL-10 production was monitored by ELISA. Monocyte-derived DCs (Mo-DCs) were generated by *in vitro* culture of human blood monocytes as described previously (70). Blood collection in this study was approved by the ethics committee at the Faculty of Medicine and Health Sciences at University of East Anglia (Norwich, UK) (Ref: 2013/2014 - 14HT). Importantly, the monocytes in the patients with iron overload have shown to respond to LPS although the response was lower when compared to that of monocytes from healthy donors (71), justifying the use of monocytes from the patients to study human Dectin-2 function.

Statistical analysis-Student's t test and One-Way ANOVA followed by Tukey's test were used for statistical analysis on Prism software (GraphPad). $p < 0.05$ was considered as statistically significant.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS

AW and NK conceived and coordinated the study and wrote the paper. AW designed, performed and analysed all the experiments. DL performed and analysed the experiments in Figures 3 to 5. KMB arranged human blood sample collection. EK and YAK provided *H. alvei* and *S. enterica* LPS, respectively. CW provided LPS from *E. coli* O9a, the rough mutant, and K. pneumoniae O1. YI and SS provided Dectin-2 KO bone marrow cells. TN and NK established the Dectin-2-BWZ cells, and generated the anti-Dectin-2 mAb. NM and KY conceived and coordinated establishment of Dectin-2-BWZ cells and generation of the anti-Dectin-2 mAb. All authors analyzed the results and approved the final version of the manuscript.

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FOOTNOTES:

[2]The abbreviations used are: Ab, antibody; BM-DC, bone marrow-derived DCs; DCs, dendritic cells; DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin; Dectin-2, dendritic cell associated lectin-2; FcRγ, Fc receptor common gamma-chain; Gal, galactose; Gal-LPS, galactosylated-LPS; GalNac, *N*-acetylgalactosamine; Glc, glucose; ITAM, immunoreceptor tyrosine-based activation motif; LPS, lipopolysaccharide; Man, mannose; Man-LPS, mannosylated-LPS; MD2, myeloid differentiation factor 2; Mo-DCs, monocyte-derived DCs; PBMCs, peripheral blood mononuclear cells; PFA, paraformaldehyde; siglec, sialic acid binding Ig-like lectin; Syk, Spleen tyrosine kinase; TLR, toll-like receptor.

FIGURE LEGENDS:

Figure 1: Comparison of BM-DC response to Man and Gal-LPS. (A) Two LPS used in this study are shown. Man-LPS from *H. alvei* PCM 1223 has mannosylated repeating unit, whereas Gal-LPS from *S. enterica* O66 has galactosylated repeat. **(B)** HEK293 cells stably transfected with TLR4-MD2 were cultured in the presence of LPS. The TLR4 activation was monitored by measuring alkaline phosphatase activity using the substrate. **(C)** Mouse BM-DCs were stimulated with 1 μg/ml of Man-LPS or 4 μg/ml Gal-LPS for 7 h. The amount of TNFα and IL-10 in the culture supernatant was analysed by ELISA. Data are representative of three independent experiments with similar results.

Error bars indicate standard deviation (SD). Statistical analyses were performed by One-Way ANOVA followed by Tukey's test. ***, $p < 0.001$.

Figure 2: Dectin-2 recognises *H. alvei* O-antigen. (A, B, C, and D) Mouse Dectin-2 or mock-BWZ cells were cultured for 16 h in the 96 well plate coated with purified LPS, plant-derived polysaccharides, lipid A, or none. The β-galactosidase activity was measured using the substrate. The data are expressed as the absorbance at 570 nm subtracted with the reference absorbance at 630 nm. **(E)** The Man-LPS coated plate was incubated with the α1-2,3 mannosidase at 37 °C for 14 h. The wells were washed with PBS and the Dectin-2 reporter cells were added and analysed as in (A). **(F, G and H)** BWZ cells expressing WT Dectin-2 or the QPD mutant were cultured in the presence of Man-LPS, *E. coli* O9a LPS, and the rough mutant LPS, or 1.0 x 10^6 of PFA-fixed *H. alvei* PCM 1223. The binding was monitored as in (A). Data are representative of three independent experiments with similar results. Error bars indicate SD. Statistical analyses were performed by One-Way ANOVA followed by Tukey's test (A, B,C, D, G and H), or Student's *t*-test (E and F). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; n.s., not statistically significant.

Figure 3: The binding of Dectin-2 to the O-antigen augments BM-DC response. (A) Dectin-2 expression on mouse BM-DCs generated from WT and Dectin-2 KO mice is shown. Cells from *in vitro* culture of bone marrow cells were stained with anti-Dectin-2 (*black*) or isotype (*grey*) control Ab. The stained cells were analysed by flow cytometry. **(B and C)** BM-DCs from indicated background were incubated with the LPS and analysed for the cytokine production as in Fig. 1C. **(D)** BM-DCs were incubated with 1.0 x 10^6 of PFA-fixed *H. alvei* PCM 1223 for 7 h. Cytokine production was monitored by ELISA as in Fig 1C. Data are representative of three independent experiments with similar results. Error bars indicate SD. Statistical analyses were performed by One-Way ANOVA followed by Tukey's test *, $p < 0.05$; ***, $p < 0.001$; n.s., not statistically significant.

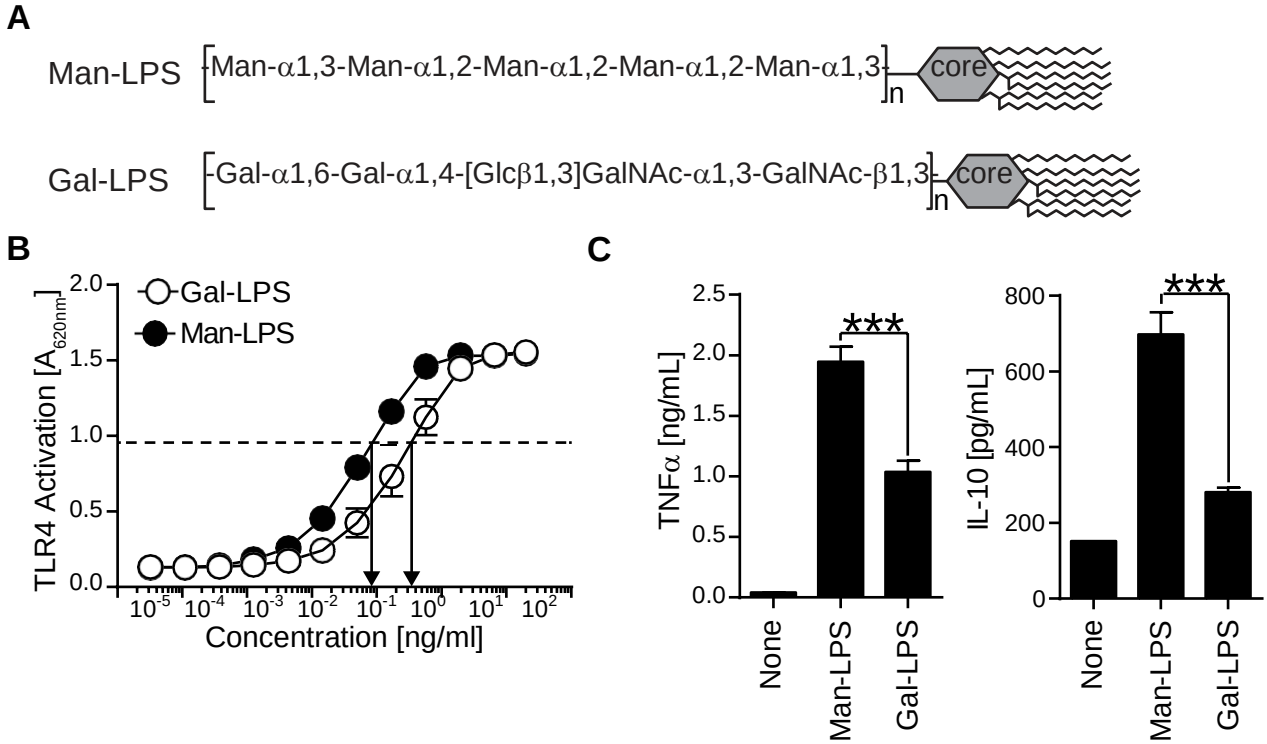
Figure 4: Syk-dependent BM-DC activation by Man-LPS. (A) BM-DCs were stimulated with 1 μg/ml of Man-LPS or 4 μg/ml Gal-LPS for the indicated time period. The stimulated cells were lysed and subjected to SDS-PAGE analysis. The proteins were transferred onto nitrocellulose membrane, and analysed for both phosphorylation and expression level of Syk. **(B)** BM-DCs were stimulated with the LPS in the presence or absence of the Syk inhibitor R406. The cytokine production was monitored by ELISA as in Fig. 1C. **(C)** Phosphorylation of p38 and degradation of IκB in response to LPS was analysed as in (A). Data are representative of three independent experiments with similar results. Error bars indicate SD. Statistical analyses were performed by One-Way ANOVA followed by Tukey's test. ***, $p < 0.001$; n.s., not statistically significant.

Figure 5: Co-stimulation of BM-DCs with Gal-LPS and yeast mannan failed to enhance cytokine production. BM-DCs were stimulated with the indicated stimuli and cytokine production was analysed as described in Fig. 1C. Data are representative of three independent experiments with similar results. Error bars indicate SD. Statistical analyses were performed by One-Way ANOVA followed by Tukey's test. **, $p < 0.01$; ***, $p < 0.001$; n.s., not statistically significant.

Figure 6: Human blood monocytes recapitulate the enhanced cytokine response to Man-LPS. **(A)** PBMCs or Mo-DCs were stained with anti-Dectin-2 (*black*) or isotype (*grey*) control Ab together with the cell surface markers for each lineage. Human blood monocytes (PI^-CD14^+), blood DCs ($PI^-CD3^-CD14^-CD16^-CD19^-CD20^-CD56^-CD11c^+HLA\text{-}DR^+$), and Mo-DCs ($PI^-CD11c^+$) were gated in the analysis. **(B)** Human blood monocytes were stimulated with the LPS as described in Fig. 1C. The cytokine production was monitored by ELISA. Data are representative of three independent experiments with similar results (A) and the results from three individuals (B). Error bars indicate SD. Statistical analyses were performed by One-Way ANOVA followed by Tukey's test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Figure 7: Role of mannosylated O-antigen in LPS activation of immune cells. Man-LPS is recognised by both Dectin-2 and TLR4, leading to activation of Syk. The Syk activation results in

enhanced TLR4 responses such as cytokine production. The signalling pathways affected by Syk are yet to be determined.


A
Man-LPS
Man-α1,3-Man-α1,2-Man-α1,2-Man-α1,2-Man-α1,3
core
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Gal-LPS
Gal-α1,6-Gal-α1,4-[Glcβ1,3]GalNAc-α1,3-GalNAc-β1,3
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Gal-LPS
Man-LPS
TLR4 Activation [A620nm]
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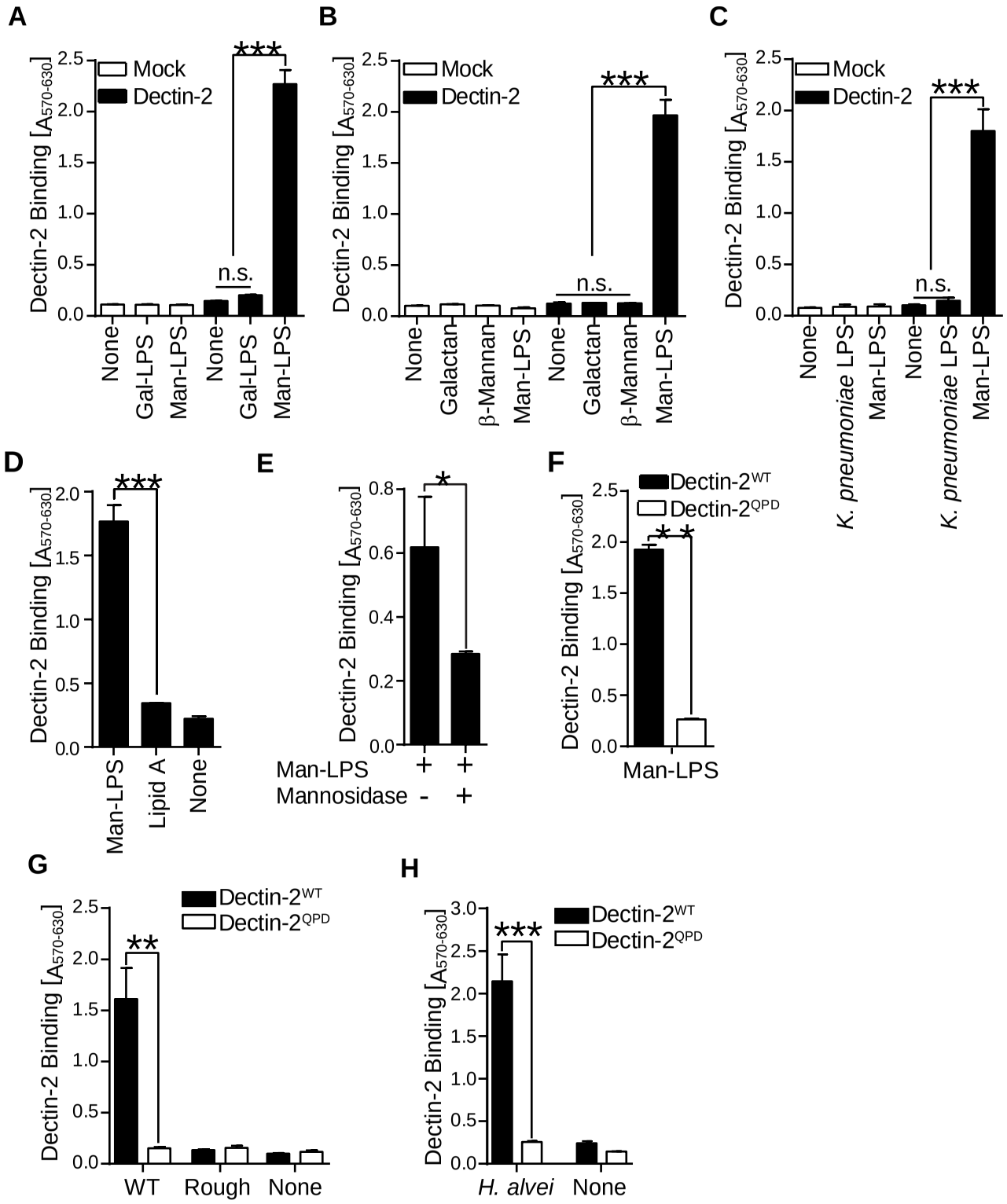
A
B
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Dectin-2 Binding [A570-630]
Mock
Dectin-2
n.s.
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None
Gal-LPS
Man-LPS
Galactan
β-Mannan
K. pneumoniae LPS
Lipid A
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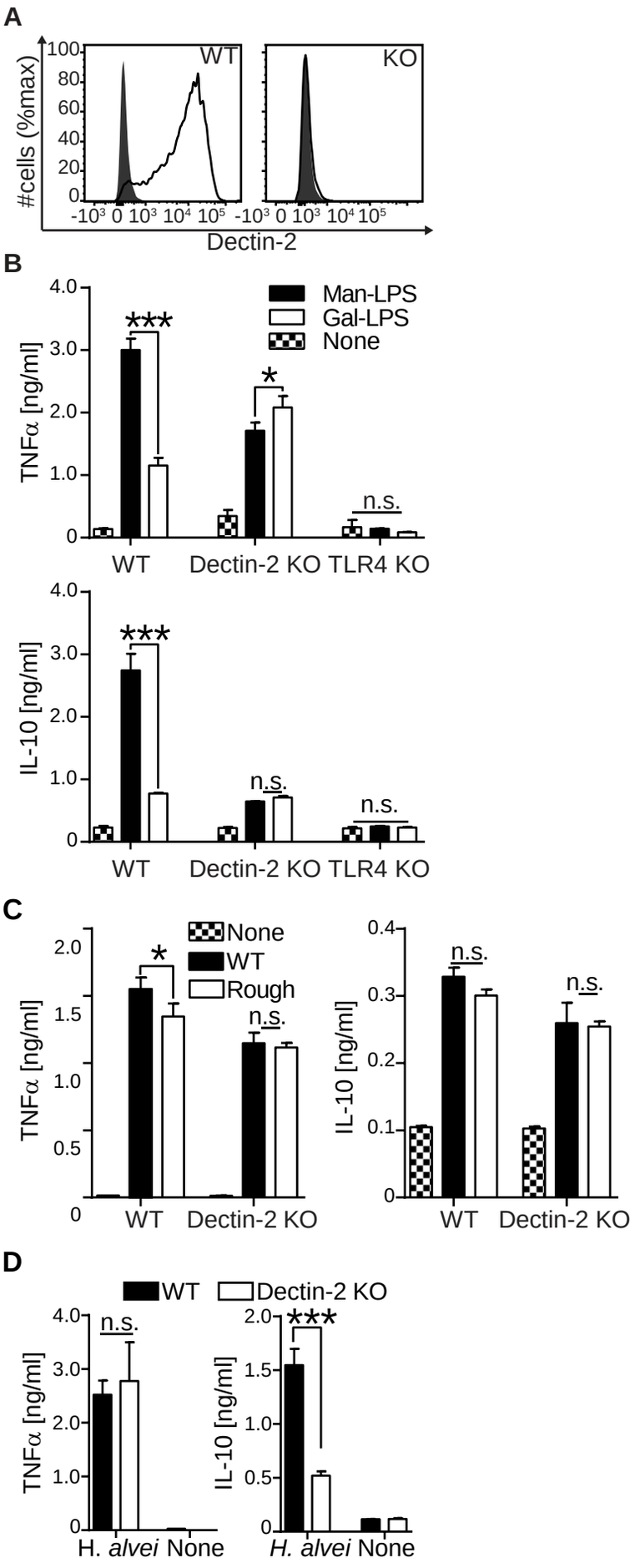
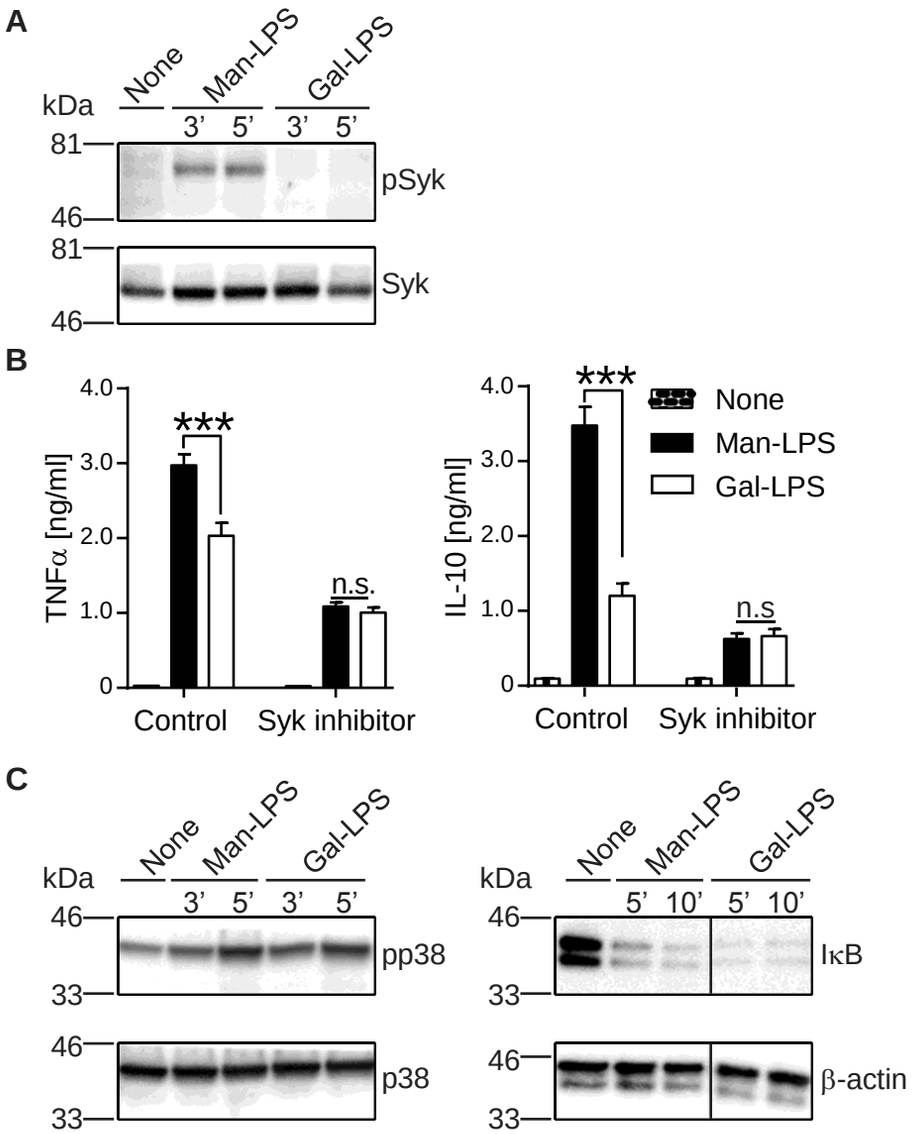
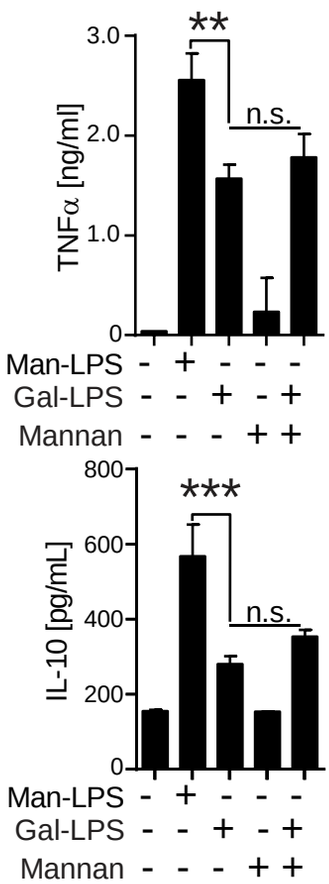
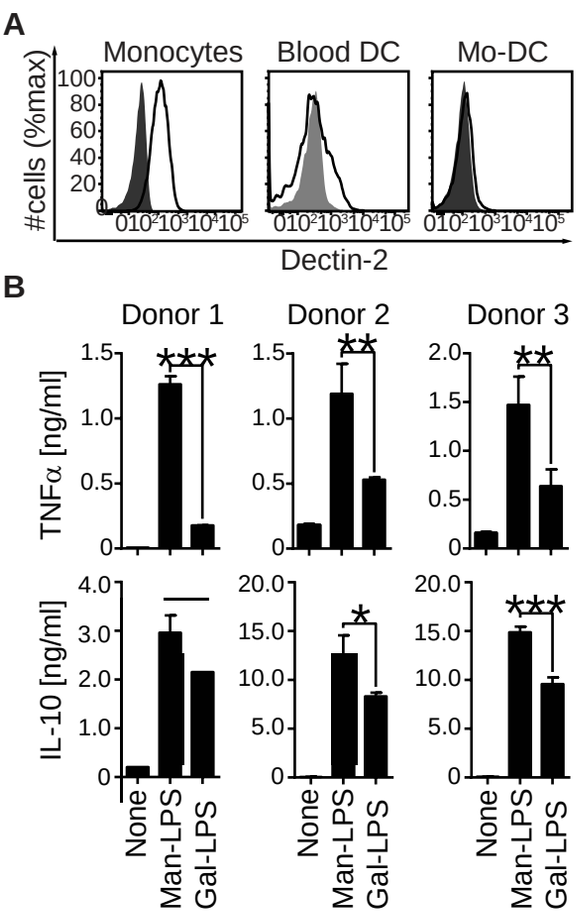
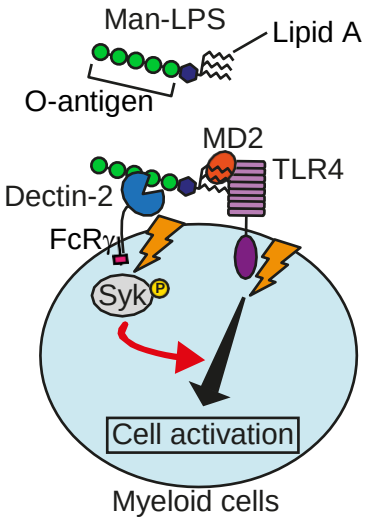
Man-LPS
+
Mannosidase
-
Dectin-2WT
Dectin-2QPD
WT
Rough
H. alvei


A
WT
KO
#cells (%max)
Dectin-2
B
Man-LPS
Gal-LPS
None
TNFα [ng/ml]
IL-10 [ng/ml]
n.s.
WT
Dectin-2 KO
TLR4 KO
C
None
WT
Rough
TNFα [ng/ml]
IL-10 [ng/ml]
n.s.
WT
Dectin-2 KO
D
WT
Dectin-2 KO
TNFα [ng/ml]
IL-10 [ng/ml]
n.s.
H. alvei
None


A
None
Man-LPS
Gal-LPS
kDa
3'
5'
3'
5'
81
46
pSyk
81
46
Syk
B
4.0
3.0
2.0
1.0
0
TNFα [ng/ml]
***
n.s.
Control
Syk inhibitor
IL-10 [ng/ml]
None
Man-LPS
Gal-LPS
n.s
C
pp38
p38
5'
10'
IκB
β-actin
46
33


**
n.s.
TNFα [ng/ml]
3.0
2.0
1.0
0
Man-LPS - + - - -
Gal-LPS - - + - +
Mannan - - - + +
***
n.s.
IL-10 [pg/mL]
800
600
400
200
0
Man-LPS - + - - -
Gal-LPS - - + - +
Mannan - - - + +


A
Monocytes
Blood DC
Mo-DC
#cells (%max)
100
80
60
40
20
0
0 10² 10³ 10⁴ 10⁵
Dectin-2
B
Donor 1
Donor 2
Donor 3
TNFα [ng/ml]
IL-10 [ng/ml]
1.5
1.0
0.5
0
2.0
4.0
3.0
2.0
1.0
20.0
15.0
10.0
5.0
***
**
*
None
Man-LPS
Gal-LPS


Man-LPS
Lipid A
O-antigen
MD2
TLR4
Dectin-2
FcRγ
Syk
P
Cell activation
Myeloid cells