

Activation of the innate immune receptor Dectin-1 upon formation of a ‘phagocytic synapse’

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Innate immune cells must be able to distinguish between direct binding to microbes and detection of components shed from the surface of microbes located at a distance. Dectin-1 (also known as CLEC7A) is a pattern-recognition receptor expressed by myeloid phagocytes (macrophages, dendritic cells and neutrophils) that detects β -glucans in fungal cell walls and triggers direct cellular antimicrobial activity, including phagocytosis and production of reactive oxygen species (ROS)^{1,2}. In contrast to inflammatory responses stimulated upon detection of soluble ligands by other pattern-recognition receptors, such as Toll-like receptors (TLRs), these responses are only useful when a cell comes into direct contact with a microbe and must not be spuriously activated by soluble stimuli. In this study we show that, despite its ability to bind both soluble and particulate β -glucan polymers, Dectin-1 signalling is only activated by particulate β -glucans, which cluster the receptor in synapse-like structures from which regulatory tyrosine phosphatases CD45 and CD148 (also known as PTPRC and PTPRJ, respectively) are excluded (Supplementary Fig. 1). The ‘phagocytic synapse’ now provides a model mechanism by which innate immune receptors can distinguish direct microbial contact from detection of microbes at a distance, thereby initiating direct cellular antimicrobial responses only when they are required.

Studies in mice and humans have demonstrated an important role for Dectin-1 in anti-fungal defence^{3–6}. Dectin-1 signals activate antimicrobial (phagocytosis, production of ROS) and inflammatory (cytokine and chemokine production) innate immune responses, and influence the development of adaptive immunity (reviewed in refs 1, 2). Although Dectin-1 has been demonstrated to collaborate with TLR signals to orchestrate immune responses to fungi^{7,8}, it activates a distinctly different signalling cascade. Dectin-1 signals via a motif in its cytoplasmic tail that resembles an immunoreceptor tyrosine-based activation motif (ITAM; reviewed in refs 1, 2). Like other ITAM-based receptors, including Fc receptors (FcR), T cell receptors (TCR), and B cell receptors (BCR), Dectin-1 signalling relies on activation of Src and Syk family kinases. However, in contrast to conventional ITAMs which comprise dual YXXL sequences, Dectin-1’s ‘hemITAM’ has only a single YXXL^{2,9}. Despite its unusual ITAM, Dectin-1 ligation by β -glucan-containing particles, such as zymosan and curdlan, triggers Src/Syk-dependent downstream signals in myeloid cells (macrophages, dendritic cells (DC) and neutrophils) to activate mitogen-activated protein (MAP) kinases, as well as NF- κ B and NFAT transcription factors (reviewed in refs 1, 2). In addition to inflammatory responses that are also triggered by TLRs, Dectin-1 induces distinct antimicrobial responses. Dectin-1 is a key phagocytic receptor for fungi and triggers a massive oxidative burst in response to fungal exposure^{5,6,10,11}.

TLRs sense soluble microbial stimuli and are activated by dimerization of intracellular signalling domains. The decades-old use of the small (6–8 kDa), soluble β -glucan laminarin (from *Laminaria digitata*) to ‘block’ β -glucan receptors on macrophages rather than activate them suggests that Dectin-1 may behave very differently¹². Indeed, in previous studies we have failed to detect Src, Syk and NFAT activation following treatment of macrophages with laminarin even though the material is a polymer of pure ligand^{11,13}. We proposed that a larger molecule may be required to provide a greater degree of receptor cross-linking to permit activation. We therefore compared the ability of whole glucan particles (WGP), a particulate *Saccharomyces cerevisiae* β -glucan preparation that lacks TLR-stimulating activity (Supplementary Fig. 2), with various molecular weight fractions of soluble *S. cerevisiae* β -1,3/1,6-glucans (see Fig. 1a).

WGP, like zymosan and curdlan, induced robust Dectin-1-dependent responses, including phagocytosis, induction of TNF- α , IL-6 and ROS by bone marrow-derived macrophages and DC (BMM and BMDC; Fig. 1b, c and Supplementary Figs 3–5, and data not shown). In contrast, none of the soluble β -glucans, not even the high molecular weight fraction, induced ROS, TNF- α or IL-6 production by either BMM or BMDC. Similar data were obtained using murine neutrophils and human monocytes and monocyte-derived macrophages (Supplementary Figs 4 and 6, and data not shown). Like zymosan, WGP induced Dectin-1 signalling (activation of Syk, p38 MAP kinase, NF- κ B and NFAT; Fig. 1d, e and Supplementary Figs 5d and 7–9). In contrast, none of the soluble β -glucans induced Dectin-1 signals. Thus simply increasing the size of the β -glucan polymer is not sufficient to activate Dectin-1 signalling.

It was demonstrated previously that a 150 kDa soluble *S. cerevisiae* β -glucan interacts with purified Dectin-1 with picomolar affinity¹⁴. We used a variety of approaches to verify that our *S. cerevisiae* β -glucans bind directly to cell surface Dectin-1. Fluorescently labelled soluble β -glucans bound to BMM and BMDC surfaces in a Dectin-1-dependent manner and all molecular weight fractions efficiently blocked (by at least 50%) binding of anti-Dectin-1 antibodies at the dose (50 μ g ml⁻¹) used in this study (Fig. 2a and Supplementary Figs 10 and 11, and data not shown). Furthermore, we observed significant soluble β -glucan binding to Dectin-1-expressing RAW264.7 macrophages, which, like primary macrophages/DC, respond robustly to particulate but not soluble β -glucans (Supplementary Figs 12 and 13). In contrast, parental RAW264.7 macrophages, which express very little Dectin-1 and mount only low responses to β -glucan particles, failed to bind soluble β -glucans (Supplementary Fig. 12a and data not shown). Furthermore, like laminarin, all the soluble β -glucans blocked Dectin-1-mediated particulate β -glucan responses in primary BMM and BMDC (Supplementary Figs 14 and 15). Thus, despite efficient binding to Dectin-1, soluble β -glucans are incapable of activating the receptor.

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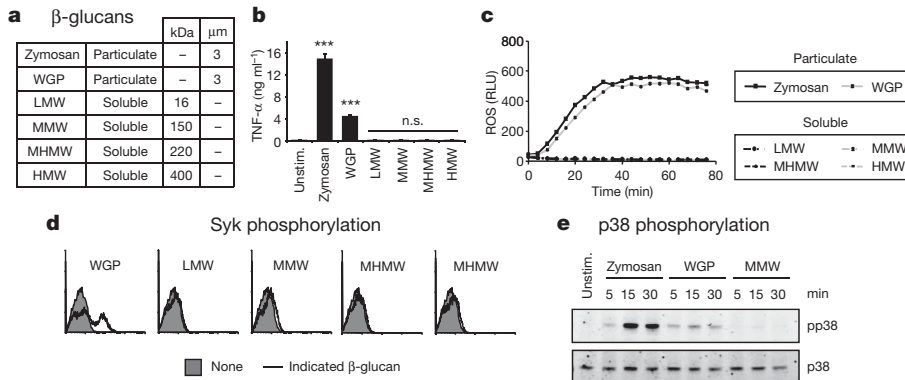


Figure 1 | Particulate, but not soluble, β -glucans induce Dectin-1 signalling. **a**, Size (molecular weight or diameter) of β -glucan preparations used in this study. **b–e**, Bone marrow-derived macrophages (BMM; **b–d**, IFN- γ -primed overnight) were stimulated with $50 \mu\text{g ml}^{-1}$ β -glucans. **b**, TNF- α production (24 h) was assessed by ELISA; data are means plus s.d. of triplicate culture (** $P < 0.001$; n.s., not significant). Unstim., unstimulated. **c**, ROS production

was assessed by luminol-enhanced chemiluminescence (ECL); data points are means of triplicate culture. RLU, relative light units. **d**, Syk activation (10 min) was assessed by intracellular flow cytometry. **e**, p38 MAP kinase activation at the indicated times was assessed by immunoblotting. All data are representative of at least three independent experiments.

These data indicate that the mode of presentation of the β -glucan may be critical for Dectin-1 signalling. We therefore examined whether immobilization of soluble β -glucans is sufficient to trigger Dectin-1 signalling. Like the β -glucan particles, soluble β -glucans immobilized on tissue culture plates or polystyrene latex beads ($0.5 \mu\text{m}$ diameter or larger) stimulated robust Dectin-1-dependent responses (Fig. 2b–e and Supplementary Figs 16–18).

The above data collectively demonstrate that to activate Dectin-1, β -glucans must be presented in an immobilized form, for example, on the surface of a phagocytosable particle such as a yeast cell. This scenario is reminiscent of the requirement for antigen presentation to the TCR by an antigen-presenting cell (APC). TCR signalling is regulated by CD45, a membrane protein with a large extracellular domain and intrinsic tyrosine phosphatase activity^{15,16}. CD45 is initially required for removal of an inhibitory phosphate to permit activation of Src family kinases, but subsequently must be isolated from the TCR complex due to its negative regulation of ITAM signalling. We therefore investigated whether Dectin-1 signalling is similarly regulated by CD45

and/or CD148, a CD45-related membrane tyrosine phosphatase with overlapping function that regulates ITAM signalling by the TCR, BCR and Fc γ R^{17,18}.

CD45-deficient BMM exhibited normal zymosan phagocytosis and only partially compromised WGP-induced ROS and TNF- α production, whereas CD148-deficient BMM showed no defect (Fig. 3a–c). In contrast, these Dectin-1 responses were severely compromised in BMM deficient in both CD45 and CD148 (Fig. 3a–c). TNF- α induction by various other stimuli was unaffected (Supplementary Fig. 19a) and Dectin-1 surface expression was normal (Supplementary Fig. 19b). WGP failed to induce Syk activation in BMM deficient in both phosphatases (Fig. 3d), which as previously reported¹⁸ had elevated basal levels of phosphorylation of the Src family kinase Lyn at its inhibitory tyrosine (Y507; Fig. 3e). Thus CD45 and CD148 have overlapping function in the regulation of Dectin-1 signalling in macrophages.

TCR activation is characterized by formation of an ‘immunological synapse’ between a T cell and an APC. Surface molecules on the two interacting cells are reorganized at the cell–cell interface to permit TCR

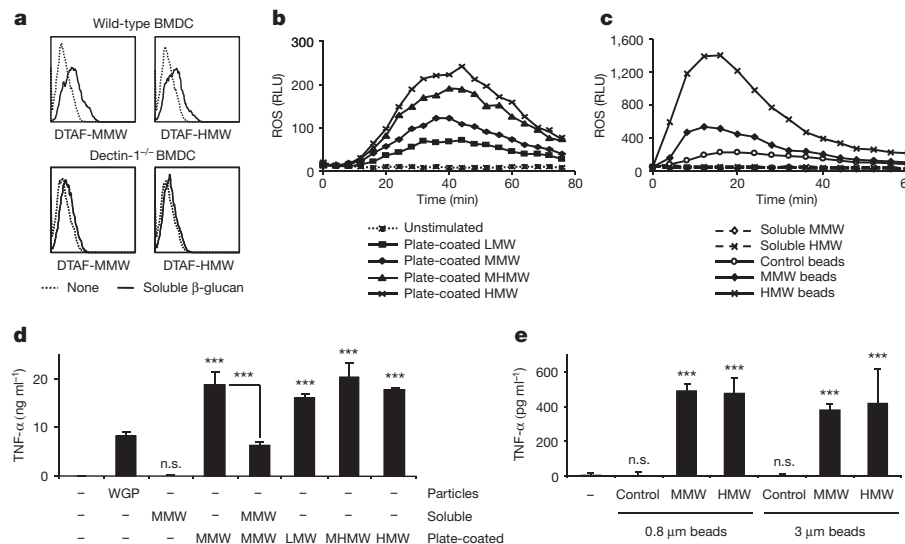


Figure 2 | Immobilized β -glucans induce Dectin-1 signalling. **a**, Soluble β -glucan binding ($50 \mu\text{g ml}^{-1}$, 10 min) to wild type and Dectin-1^{-/-} BMDC was assessed by flow cytometry. **b, c**, IFN- γ -primed BMM were stimulated with soluble β -glucans ($50 \mu\text{g ml}^{-1}$) or β -glucans immobilized on either tissue culture plates (**b**) or $0.8 \mu\text{m}$ polystyrene latex beads (**c**). ROS production was measured by luminol-ECL; data points are means of triplicate culture.

d, e, TNF- α production (24 h) by BMDC exposed to soluble/particulate ($50 \mu\text{g ml}^{-1}$), plate-immobilized or bead-coated soluble β -glucans was assessed by ELISA; data are expressed as means plus s.d. of triplicate culture (** $P < 0.001$; n.s., not significant). All data are representative of at least three independent experiments.

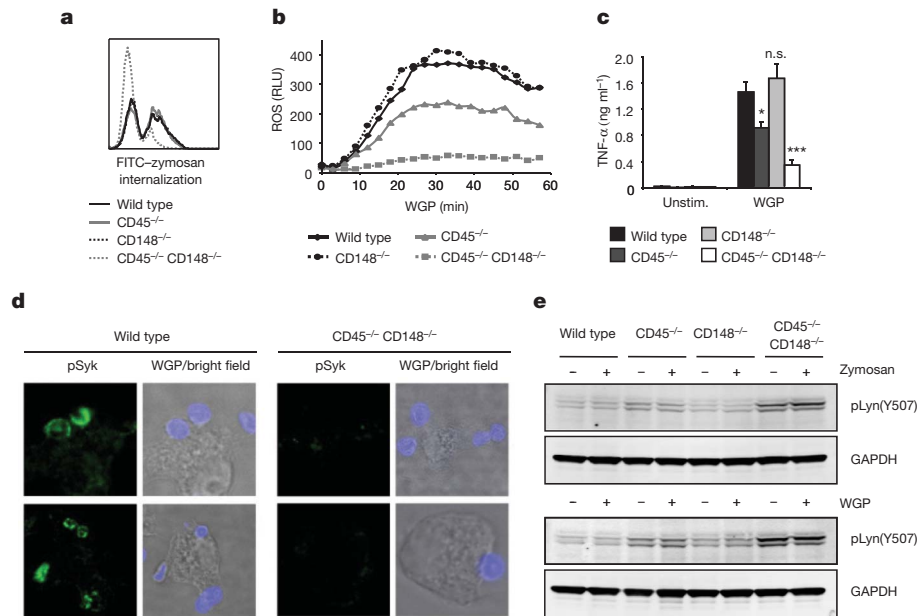


Figure 3 | CD45 and CD148 regulate Dectin-1 signalling. Responses of wild-type and CD45/CD148-deficient BMM (b, IFN- γ -primed) were examined. **a**, Internalization of FITC-labelled zymosan particles ($20 \mu\text{g ml}^{-1}$, 10 min) was assessed by flow cytometry. **b**, ROS production was measured by luminol-ECL; data points are means of triplicate culture. **c**, TNF- α production ($50 \mu\text{g ml}^{-1}$ WGP, 24 h) was assessed by ELISA; data are expressed as means plus s.d. of

triplicate culture ($*P < 0.01$; $***P < 0.001$; n.s., not significant). **d**, Syk activation (pSyk; green) by AlexaFluor647-labelled WGP ($20 \mu\text{g ml}^{-1}$, 1 min; blue) was assessed by confocal microscopy. **e**, Inactive Lyn (pY507) levels following zymosan/WGP stimulation ($50 \mu\text{g ml}^{-1}$, 10 min) were assessed by immunoblotting. All data are representative of three independent experiments.

signalling¹⁵. Following the initial CD45-promoted activation of Src family kinases, CD45 must be isolated from the TCR complex in order to remove its inhibitory phosphatase activity and permit propagation of the ITAM signal. We proposed that following binding of β -glucan-containing particles, CD45 and CD148 would similarly be sequestered from Dectin-1 to permit activation of Dectin-1 signalling.

We therefore examined macrophage surface molecule rearrangement following β -glucan particle binding. As we and others have observed previously^{8,19}, Dectin-1 clustered at β -glucan particle contact sites on the surface of Dectin-1-expressing RAW264.7 macrophages and phagocytic cups formed within 1 min of binding (Fig. 4a and Supplementary Fig. 20a). Recruitment of active Src and Syk kinases,

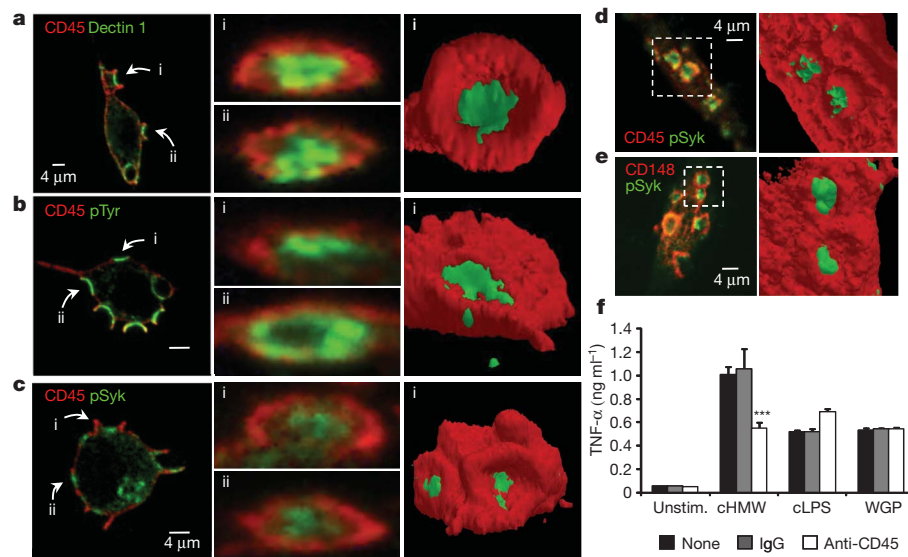


Figure 4 | CD45 and CD148 phosphatases are excluded from the β -glucan particle contact site. **a–c**, Confocal microscopy of SBPc-tagged Dectin-1-expressing RAW264.7 macrophages stimulated with zymosan ($20 \mu\text{g ml}^{-1}$, 1 min) and stained for CD45 (red) and SBPc tag (Dectin-1; green), phosphotyrosine (pTyr; green) or active Syk (pSyk; green). z-stacks were analysed to visualize the indicated particle contact sites (left panels) in cross-section (centre panels). Three-dimensional isosurface models of the indicated contact sites were generated using ImageJ and ImageSurfer (right panels). Scale bar = $4 \mu\text{m}$. **d**, Resident peritoneal macrophages stimulated with WGP ($20 \mu\text{g ml}^{-1}$, 1 min)

were stained (left panel) for CD45 (red) and active Syk (pSyk; green). **e**, Dectin-1-expressing RAW264.7 macrophages stimulated with WGP ($20 \mu\text{g ml}^{-1}$, 1 min) were stained (left panel) for CD148 (red) and active Syk (pSyk; green). Isosurface models (**d**, **e**, right panels) are of the indicated particle contact sites. **f**, BMDCs were added to tissue culture plates pre-coated with HMW soluble β -glucan (cHMW) or LPS (cLPS) and/or anti-CD45 or control IgG; some BMDCs were stimulated with WGP ($50 \mu\text{g ml}^{-1}$). TNF- α production (24 h) was assessed by ELISA; data are means plus s.d. of triplicate culture ($***P < 0.001$). All data are representative of at least three independent experiments.

as well as other tyrosine-phosphorylated proteins, to the contact sites of β -glucan particles with macrophages (Dectin-1-expressing RAW264.7 and primary murine macrophages) was also seen at this early time point (Fig. 4b–e and Supplementary Figs 20b–c and 21–23).

In contrast, CD45 was uniformly distributed on the surface of unstimulated macrophages, but upon contact with β -glucan particles was specifically absent from regions of membrane enriched for Dectin-1, active Src family and Syk kinases, and phospho-tyrosine staining (Fig. 4a–d and Supplementary Figs 20–24). Time course experiments showed that CD45 exclusion from the region of Dectin-1 clustering occurs before initiation of phagocytic cup formation (Supplementary Fig. 25 and Supplementary Movie 1), and is not dependent on actin dynamics (Supplementary Fig. 26). CD148 was similarly excluded from regions of Dectin-1 clustering and signalling induced by β -glucan particle binding (Fig. 4e). Three-dimensional images clearly show a 'bull's-eye' pattern of staining with a central Dectin-1 signalling region, from which CD45 and CD148 are excluded (Fig. 4a–e, Supplementary Fig. 27 and Supplementary Movie 2). We also observed clustering of Dectin-1 and exclusion of CD45 upon contact of Dectin-1-expressing RAW264.7 macrophages with live fungi (*S. cerevisiae*, *Candida albicans* and *Aspergillus fumigatus*) and β -glucan-coated plates (Supplementary Figs 28–32). In contrast, upon binding of soluble β -glucans to Dectin-1, CD45 remained colocalized with Dectin-1 at the cell surface (Supplementary Fig. 33).

CD45 and CD148 exclusion from the contact site of Dectin-1 with the β -glucan particle in the forming phagosome (or the contact surface during frustrated phagocytosis) is consistent with the hypothesis that following the initial activation of Src family kinases, the phosphatases must be isolated from the receptor to permit productive signalling. This model predicts that co-coating anti-CD45 antibodies on plates with β -glucans to prolong CD45 colocalization with β -glucan-bound Dectin-1 would suppress Dectin-1 signalling. Indeed, co-coated anti-CD45 reduced immobilized β -glucan-induced TNF- α production by about 45%, but did not affect TNF- α induction by WGP (which contact cells at points not bound to anti-CD45) or immobilized lipopolysaccharide (LPS; Fig. 4f).

Collectively, our data suggest a mechanism by which Dectin-1, unlike other innate pattern-recognition receptors such as TLRs, discriminates between soluble and particulate ligands (Supplementary Fig. 1). Binding of particulate β -glucans (such as yeast) to Dectin-1 triggers phagocytosis, a process that involves massive reorganization of membrane proteins and membrane movement coordinated by the actin cytoskeleton. We have shown that during this process membrane tyrosine phosphatases, which are well characterized regulators of ITAM signalling¹⁶, are excluded from the particle contact site. In a manner analogous to the formation of immunological synapses between APCs and T cells, 'phagocytic synapses' are required for activation of Dectin-1 when myeloid cells encounter β -glucan-containing microbes. In contrast, it seems that upon detection of soluble β -glucans, the inhibitory activity of membrane tyrosine phosphatases cannot be sufficiently isolated from the crosslinked receptors, and thus Dectin-1 signalling is aborted.

Future studies are required to determine whether the unique nature of the Dectin-1 hemITAM underlies its requirement for ligand immobilization. We suspect that the C-type lectin family member CLEC1B (also known as CLEC2) may be similarly regulated. CLEC2 also contains a hemITAM and signals in an apparently identical manner to Dectin-1 (ref. 20). In addition to having a role in platelet aggregation, CLEC2 is expressed on the surface of neutrophils and is capable of inducing phagocytosis of anti-CLEC2-coated beads²¹. Interestingly, Dectin-1–CLEC2 chimaeras can be activated to induce TNF- α production by zymosan, but this activation is blocked by soluble β -glucans²¹ (which presumably bind but fail to activate the receptor), indicating that activation of CLEC2 signalling may be dependent on the formation of a phagocytic synapse.

It is widely accepted that the nature of an innate immune response to a microbe is defined by the types of pattern-recognition receptors

that detect it. Thus receptors that detect viral nucleotides induce responses appropriate for killing viruses. Activation of phagocytic receptors is only appropriate when they bind intact microbes. Although models exist for the detection of soluble stimuli (for example, receptor dimerization, induction of conformational changes), we are currently lacking good models for the discrimination of soluble versus particulate ligands. Our data present the phagocytic synapse as a mechanistic model for the specific detection of ligands associated with a microbial surface, as opposed to those released from distantly located organisms.

METHODS SUMMARY

β -glucan preparations. Particulate *S. cerevisiae* β -glucans (zymosan; Sigma) and whole glucan particles (Wellmune WGP; Biothera) were used as described previously^{8,22}. Soluble *S. cerevisiae* β -glucans were prepared by acid hydrolysis of WGP and fractionated by preparative gel permeation chromatography. The molecular weight distribution of each soluble β -glucan was determined by gel permeation chromatography (GPC) with multi-angle laser light scattering photometry (MALLS); the polydispersities of the LMW, MMW, MHMW and HMW (low, medium, medium-high, and high molecular weight, respectively) soluble β -glucans were 1.5, 1.6, 1.2 and 1.4, respectively. All β -glucan preparations were endotoxin-free and used at 50 $\mu\text{g ml}^{-1}$ unless otherwise stated.

Confocal microscopy. Cells were plated on glass coverslips overnight before addition of β -glucan particles, centrifuged briefly to ensure particle contact with the cells, and incubated at 37 °C for the times indicated. Cells were washed to remove unbound particles, fixed with 10% formalin, permeabilized with ice-cold acetone, blocked and stained with primary and secondary antibodies. Coverslips were mounted and examined using a Leica TCS SP5 confocal microscope. Image analysis was performed using Leica LAS AF software, as well as ImageJ and ImageSurfer²³.

β -glucan immobilization and CD45 co-immobilization. Soluble β -glucans were immobilized on tissue culture plates or polystyrene latex beads by incubation with PBS/EDTA containing 100 $\mu\text{g ml}^{-1}$ soluble β -glucan for 1 h at 37 °C. Plates or beads were then washed to remove unbound β -glucans, and blocked with media containing 10% fetal calf serum before use. For CD45 co-immobilization assays, HMW soluble β -glucan (20 $\mu\text{g ml}^{-1}$) and LPS (100 ng ml^{-1}) were immobilized on tissue culture plates in PBS/EDTA in the presence or absence of 10 ng ml^{-1} anti-CD45 or rat IgG for 1 h at 37 °C. Plates were washed and blocked as above before addition of BMDC.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

Received 8 July 2010; accepted 22 March 2011.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank K. Wawrowsky for help with confocal microscopy, and G. D. Brown for Dectin-1-deficient mice. This study was funded by grants from the NIH (AI071116 and AI066120 to D.M.U. and A.W., respectively) and the American Heart Association (D.M.U.). H.S.G. held a Research Fellowship Award from the Crohn's and Colitis Foundation of America. D.M.U. holds the Janis and William Wetsman Family Chair in Inflammatory Bowel Disease at Cedars-Sinai Medical Center.

Author Contributions H.S.G. and D.M.U. designed the study; H.S.G., C.N.R., C.A.B., J.M., A.J.W., N.B., A.S.H.C. and D.M.U. performed the experiments; A.S.M., M.E.D. and J.P.V. purified, characterized and provided the β -glucans; T.R.K. and A.W. provided knockout mice and an antibody; T.R.K., A.W. and J.P.V. gave technical support and conceptual advice; H.S.G. and D.M.U. wrote the paper.

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METHODS

β -glucan preparations. Particulate *S. cerevisiae* β -glucans (zymosan; Sigma) and WGP (Wellmune WGP; Biothera) were used as described previously^{8,22}. Soluble *S. cerevisiae* β -glucans were prepared by acid hydrolysis of WGP and fractionated by preparative gel permeation chromatography. The molecular weight distribution of each soluble β -glucan was determined by gel permeation chromatography (GPC) with multi-angle laser light scattering photometry (MALLS); the polydispersities of the LMW, MMW, MHMW and HMW soluble β -glucans were 1.5, 1.6, 1.2 and 1.4, respectively. All β -glucan preparations were endotoxin-free and used at $50 \mu\text{g ml}^{-1}$ unless otherwise stated.

Live yeast. Live *Saccharomyces cerevisiae* and *Candida albicans* yeast were grown in Sabouraud Dextrose Broth. *Aspergillus fumigatus* conidia were prepared from mature colonies grown on potato dextrose agar by flushing with PBS containing 0.05% Tween-80, and incubated in RPMI for 4 h to generate swollen conidia or 12 h to induce germ tube formation.

Cell culture and functional and biochemical assays. RAW264.7 macrophages stably expressing SBPc-tagged Dectin-1 or the ELAM-luciferase reporter have been described previously⁸. Dectin-1-deficient mice were provided by G. D. Brown. Culture of primary mouse macrophages and dendritic cells was performed as in previous studies²². Human monocytes were obtained from peripheral blood, and macrophages were derived by 7-day culture with 50 ng ml^{-1} rhM-CSF. Cytokine and reactive oxygen species production, Syk phosphorylation, MAP kinase and NF- κ B activation, and Egr2/3 induction were assessed as previously described^{11,13,22}. Active phospho-Syk (Y525/Y526), active phospho-Src family kinases (Y416) and inactive phospho-Lyn (Y507) antibodies were from Cell Signalling Technology.

β -glucan immobilization and CD45 co-immobilization. Soluble β -glucans were immobilized on tissue culture plates or large polystyrene latex beads (0.8 and 3 μm ; Sigma) by incubation with PBS/EDTA containing $100 \mu\text{g ml}^{-1}$ soluble β -glucan for 1 h at 37°C . Plates or beads were then washed three times with PBS/EDTA to remove unbound β -glucans, and blocked with media containing 10% FCS for 30 min before use. For CD45 co-immobilization assays, soluble HMW β -glucan ($20 \mu\text{g ml}^{-1}$) and LPS (100 ng ml^{-1}) were immobilized on tissue culture plates in PBS/EDTA in the presence/absence of 10 ng ml^{-1} anti-CD45 or rat IgG for 1 h at 37°C , and washed and blocked as above before the addition of BMDC. For assays using small polystyrene latex beads (0.05, 0.2 and 0.5 μm ; Polysciences), coating was achieved by incubating beads in PBS/EDTA containing $50 \mu\text{g ml}^{-1}$ 5-(4,6-dichlorotriazinyl)aminofluorescein (DTAF)-labelled soluble HMW β -glucan, and beads were diluted to a final concentration of $0.05 \mu\text{g ml}^{-1}$ β -glucan for stimulation, a dose at which the soluble β -glucans are too dilute to block Dectin-1 signalling by β -glucan particles (data not shown). Beads were fed to cells at a dose that achieves presentation of an equivalent total β -glucan-coated surface area per cell (approximately 40:1 0.5 μm beads:cell, 250:1 0.2 μm beads:cell, and 4,000:1 0.05 μm beads:cell).

Soluble β -glucan binding to Dectin-1. Parental RAW264.7 macrophages or RAW264.7 macrophages stably expressing SBPc-tagged-Dectin-1 were incubated

for 2 h at 37°C with $100 \mu\text{g ml}^{-1}$ unlabelled MMW soluble β -glucan, and binding was detected by flow cytometry using a mouse IgM monoclonal antibody specific for β -(1,3)-linked glucan (BfD IV; clone 10C6; ref. 24) and a FITC-conjugated goat anti-mouse secondary antibody. Dectin-1 expression by the macrophages was assessed by flow cytometry using a FITC-conjugated anti-Dectin-1 antibody (2A11) from Serotec. Macrophages and dendritic cells incubated with DTAF-labelled soluble β -glucans were washed and fixed before analysis by flow cytometry.

Anti-Dectin-1 competition assay. Cells were incubated on ice in media containing $0.4 \mu\text{g ml}^{-1}$ FITC-anti-Dectin-1 and the indicated concentrations of unlabelled soluble β -glucans for 30 min, and then washed and fixed before assessment of anti-Dectin-1 binding by flow cytometry.

Confocal microscopy of fixed cells. Cells were plated on glass coverslips overnight before the addition of β -glucan particles (unlabelled zymosan or AlexaFluor647-labelled WGP), brief centrifugation to ensure particle contact with the cells, and incubation for 1 min at 37°C . Cells were then washed with ice-cold PBS to remove unbound particles, fixed with 10% formalin for 20 min, and permeabilized with ice-cold acetone for 30 s. Nonspecific binding was blocked by incubation with TBS + 5% FCS for 10 min. Cells were stained with unconjugated primary antibodies for 1 h as follows. SBPc-tagged Dectin-1: mouse anti-protein C tag (HPC4; Amersham Biosciences); pSyk: rabbit anti-pSyk (Y525/Y526); pSrc: rabbit anti-pSrc (Y416) (Cell Signalling Technology); pTyr: mouse anti-pTyr (Cell Signalling Technology); CD45: rat anti-CD45 (AbD Serotec); and CD148: hamster anti-CD148 (ref. 17). Cells were then washed and incubated with secondary antibodies for 30 min as follows. Dectin-1 and pTyr: AlexaFluor488-conjugated anti-mouse; pSyk and pSrc: FITC-conjugated anti-rabbit; CD45: AlexaFluor568-conjugated anti-rat; and CD148: AlexaFluor568-conjugated anti-hamster (Invitrogen). AlexaFluor647-conjugated cholera toxin (Invitrogen) was used to stain the plasma membrane of unpermeabilized cells. Coverslips were mounted and examined using a Leica TCS SP5 confocal microscope. Image analysis was performed using Leica LAS AF software, ImageJ and ImageSurfer²³, and Volocity (Perkin Elmer).

Confocal microscopy of live cells. RAW264.7 macrophages stably expressing Dectin-1 tagged with green fluorescent protein (GFP) at the carboxy terminus and CD45-tagged with DsRed at the carboxy terminus were plated on chamber slides overnight before stimulation and maintained at 37°C during confocal imaging. For assessment of cell contact with β -glucan-coated surfaces, chamber slides were incubated with PBS/EDTA (control) or $100 \mu\text{g ml}^{-1}$ β -glucan in PBS/EDTA for 1 h at 37°C and washed three times with PBS/EDTA before macrophage addition.

Statistics. Statistical significance was assessed using Student's *t*-test.

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