Supporting Information

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SI Results

Tagged Dectin-1 Expressed on Mammalian Cells Is Functional. We engineered the sequence encoding the LPETG motif followed by an HA epitope tag at the C terminus, the extracellular domain of dectin-1. This construct, dectin-1-LPETG-3xHA, was cloned into a retrovirus expression vector and used to obtain dectin-1–expressing HEK 293T and RAW 264.7 cells. Both cell lines expressed in stable fashion readily detectable levels of HA-tagged dectin-1 on the cell surface. After sorting by FACS, we obtained a population in which >90% of cells expressed tagged dectin-1 (Fig. S1*A*).

The function of dectin-1 was assessed in stably transduced HEK 293T and RAW 264.7 cells by incubating them with particles of the yeast cell wall preparation zymosan. Both HEK 293T and RAW 264.7 cells stably expressing dectin-1 but not the empty vector controls showed zymosan binding, as shown by fluorescence microscopy (Fig. S1*B*) and cytofluorimetry (Fig. S1*C*). Although the tags installed on dectin-1 were located at its carbohydrate recognition domain, the binding of zymosan suggests that the presence of the small tag does not interfere with dectin-1's function.

Mass Spectrometry Analysis of Surface-Exposed Dectin-1 Uncovers an Association with Galectin-3 in RAW 264.7 Macrophages. We explored the possibility of uncovering unique associations between dectin-1 and other potential interacting proteins by using the sortagging technique to install an affinity handle. RAW 264.7 cells stably expressing dectin-1 were incubated with sortase A and a biotinylated probe to label the fraction of tagged dectin-1 located on the cell surface. The cells were lysed, and the lysates were then incubated with streptavidin-coated beads. Bound proteins were removed from beads and separated by SDS gel electrophoresis (Fig. S2). These proteins were extracted from the gel and subjected to analysis by mass spectrometry. A protein of ≈28 kDa identified as galectin-3 by mass spectrometry was detected in those samples from cells expressing tagged dectin-1. No trace of this protein was observed in any lane corresponding to control cells expressing similarly tagged CD74, an unrelated type II membrane protein.

SI Materials and Methods

Reagents. Zymosan and protease inhibitor mixture were from Sigma-Aldrich. The anti-HA antibody conjugated with R-phycoerythrin (R-PE) and the IgG isotype control antibody were from Columbia Biosciences. The anti-HA antibody conjugated with peroxidase was from Roche Applied Science. The streptavidin conjugated with HRP was from GE Healthcare. The polyclonal antibody against galectin-3 was from Santa Cruz Biotechnology. Anti-FLAG antibody conjugated with HRP and anti-FLAG–coated magnetic beads were from Sigma-Aldrich. The monoclonal antibody to detect β-actin was from Abcam. Immunoprecipitation Kit-Dynabeads Protein G and Dynabeads Streptavidin C1 were purchased from Invitrogen. EndoH, PNGase F, and calf intestinal alkaline phosphatase (CIP) were from New England BioLabs. Cycloheximide was from EMD Biosciences. Alexa Fluor 488/555/647 carboxylic acid succinimidyl esters were from Invitrogen Molecular Probes. Transient transfections were done with FuGENE 6 Transfection Reagent from Roche Applied Science. For all experiments quantitation of protein was performed using the BCA protein assay kit from Thermo Fisher Scientific.

Synthesis of GGGK-A647, GGGK-A488, and Biotinylated Probes. The GGGK peptide was synthesized manually by standard Fmoc-based solid phase peptide synthesis on Rink amide resin (Novabiochem). The Fmoc-protected peptide was cleaved from the resin by treatment with 2.5 mL of 95:3:2 trifluoroacetic acid (TFA)-triisopropylsilane (TIPS)/H_2O (5×, 15 min each), which also removed the 4-methyltrityl protecting group on the lysine residue. The combined cleavage solutions were concentrated, dissolved in methanol, and precipitated with cold diethyl ether.

Coupling of the Fmoc-protected peptide to the amine-reactive Alexa Fluor dyes was accomplished in solution. One equivalent of Fmoc-GGGK peptide was mixed with 0.5 equivalents of either Alexa Fluor 647 carboxylic acid succinimidyl ester or Alexa Fluor 488 carboxylic acid succinimidyl ester, and four equivalents of N,N-Diisopropylethylamine (DIPEA) (Sigma-Aldrich) in anhydrous DMSO and incubated at room temperature for 6 h. Under these conditions, Fmoc deprotection also occurred.

The Alexa 488 peptide was purified by reverse-phase HPLC on a Waters Delta Pak 15 mm, 100 Å C18 column (7.8 × 300 mm, MeCN:H_2O gradient mobile phase containing 0.1% TFA, 3 mL/min), and the Alexa 647 peptide was purified on a Waters 5PE column (8 × 250 mm, MeCN:H_2O gradient mobile phase with 0.1% TFA, 3 mL/min). Two peaks were collected for the Alexa 488 peptide, presumably representing different isomers. Fractions were lyophilized and dissolved in water.

Peptide identity was confirmed for the Alexa 488 peptide by MALDI-TOF MS (matrix sinapinic acid), [M+] = 831.16, obs = 832.862; both HPLC peaks contained this mass. We were unable to observe the Alexa 647 peptide by MALDI-TOF, so we inferred the molecular weight and activity as a nucleophile by setting up a test transpeptidation reaction with sortase A and an LPETG-tagged GFP substrate as previously described (1) and observing the mass change in the transpeptidation product by ESI-MS on a Micromass LCT mass spectrometer (Micromass MS Technologies) and a Paradigm MG4 HPLC system equipped with an HTC PAL autosampler (Michrom BioResources) and a Waters Symmetry 5-mm C8 column [2.1 × 50 mm, MeCN:H_2O (0.1% formic acid) gradient mobile phase, 150 mL/min]. The predicted molecular weight for the Alexa647 peptide is 1155.06, obs = 1155.0.

The biotinylated probe was synthesized as previously described (2), except the peptide scaffold consisted of GGGK.

Stable Cell Lines and siRNA. The coding region for murine dectin-1 was cloned into the retroviral vector pLHCX (Clontech Laboratories) and tagged at the C terminus with additional coding region for the following residues: GGGGSGGGGSLPETG. This tag includes a flexible linker and the recognition site for sortase A. A 3xHA epitope tag was also added C-terminal to the sortase recognition site. Retrovirus was prepared by transfecting HEK 293T cells with plasmids encoding VSV-G and Gag-Pol, as well as the dectin-1 pLHCX construct. Medium was collected 24 and 48 h after infection, filtered through a 0.45-μm membrane, and added to RAW264.7 and HEK 293T cells with 8 μg/mL polybrene. Cells were spun at 1000 × *g* for 90 min. Media were changed, and cells were allowed to recover for 24 h before reinfection and selection to obtain hygromycin-resistant populations. Cells were sorted on a FACS ARIA cell sorter (BD Biosciences) to typically >90% purity on postsort analysis. A subset of RAW 264.7 and HEK 293T cells was transduced with empty retroviral plasmid. The hygromycin-resistant population obtained was used as a control for further experiments. Cells were cultured under hygromycin B selection (250 μg/mL in RAW 264.7 cells and 125 μg/mL in HEK 293T cells) in complete DMEM/10% FBS supplemented with penicillin 100 units/mL, and streptomycin 100 mg/mL.

To generate galectin-3 knockdowns, we used the lentiviral vector pLKO.1. We used three different shRNAs with the sequences 5′-AGAGTCATTGTGTGTAACACG-3′, 5′-AACCATCGGATG-AAGAACCTC-3′, and 5′-AGCTGCCTGTCTTTATATGCC-3′. RAW 264.7 cells with stable dectin-1 expression and bone marrow-derived macrophages (BMDMs) were infected with the shRNAs as described previously (3), and puromycin-resistant clones were selected. A subset of cells were infected with shRNA directed against GFP mRNA (5′GCCACAACATCGAGGAC-GGCA-3′) and used as a control for further experiments. Cells were cultured under puromycin selection (8 μg/mL).

Isolation of Mouse Macrophages. BMDMs were generated from C57BL/6 and Dectin-1$^{-/-}$ mice as previously described (4), with slight modifications. Briefly, bone marrow cells from 8- to 10-wk-old mice were cultured in DMEM/10%FBS conditioned with 2 mM L-glutamine and 5 ng/mL of recombinant macrophage colony-stimulating factor (M-CSF) (Sigma-Aldrich) for 1 to 2 d. Nonadherent cells were collected and then cultured in the presence of 30 ng/mL of M-CSF. Adherent cells were harvested after 6 d for transduction and further experiments. Knockout mice had been backcrossed to a C57BL/6 background for 8–12 generations before use. Mice were specific pathogen free, and all animal procedures were conducted under a protocol approved by the Massachusetts Institute of Technology/Whitehead Institute for Biomedical Research Committee on Animal Care.

Cell Sortagging. Cells were cultured in complete DMEM/10% FBS and incubated in a six-well plate in a 5% CO_2 humidified incubator at 37 °C. The cells were harvested by scraping in ice-cold PBS. After centrifugation, the cell pellet was resuspended in complete DMEM. Tagged dectin-1 was labeled by incubating the cells with sortase A (200 μM) and the probe to be installed: biotinylated probe (500 μM), Alexa 488 probe (10 μM), or Alexa 647 probe (10 μM). The cells were incubated at 37 °C for 30 min. As a control, a subset of cells was incubated with each probe but without sortase enzyme.

For the sortagging of cell lysates, cells were lysed by addition of PBS supplemented with 1% Nonidet P-40 and protease inhibitor mixture. The lysate (10 μg in a volume of 10 μL) was incubated with sortase A (200 μM) and the biotinylated probe (500 μM) in sortase buffer [50 mM Tris (pH 7.5), 150 mM NaCl, and 10 mM $CaCl_2$] at 37 °C for 30 min. The reaction products were analyzed by immunoblot. A fraction of the lysate was incubated with no enzyme and used as a control.

Glycosidase and Phosphatase Treatment. Intact cells were sortagged with a biotinylated probe and centrifuged. The cell pellet was lysed with PBS supplemented with 1% Nonidet P-40 and protease inhibitor mixture. Glycosidase digestions were performed by incubating cell lysates (10 μg) with 1,500 U of endoglycosidase H (EndoH) or *N*-Glycosidase F (PNGase F) as previously described (5, 6), with slight modifications. The alkaline phosphatase digestion was carried out as previously described (7). Thirty units of CIP were used to digest 10 μg from cell lysates at 37 °C for 1 h. HEK 293T cells transfected with CD74 were used as a positive control. The CD74 protein contained two amionoacid substitutions (L7A, L17A) to prevent recycling of the invariant chain.

Ligand Binding Assays. HEK 293T cells were plated onto a poly-lysine–coated chambered coverglass at a density of 3×10^5 cells per well (0.7 cm^2) and allowed to attach overnight. Zymosan was labeled with Alexa Fluor 488, Alexa Fluor 555, or Alexa Fluor 647 carboxylic acid succinimidyl ester according to the manufacturer's instructions. Cells were incubated with labeled zymosan (10 particles per cell) 30 min at 37 °C and then washed thoroughly with PBS to remove unbound particles. For binding assays on sortagged cells, cells were sortagged on the chambered coverglass in DMEM/10% FBS media and then washed thoroughly with PBS. Labeled cells were incubated with zymosan in DMEM/10% FBS media 30 min at 37 °C and then washed with PBS. If a second fluorophore was installed by sortagging, at that stage cells were labeled again on the coverglass with the second fluorophore and then washed with PBS. For the binding assay on a mixed population of HEK 293T cells, cells were cultured overnight on a six-well plate and transfected with 2 μg of pCAG-GFP vector (8). Cells were incubated with the transfection reagent overnight at 37 °C. After the incubation, 3×10^3 GFP-transfected cells and 3×10^3 stably dectin-transduced cells were transferred to each of the wells of a poly-lysine–coated chambered coverglass. The ligand binding assay was performed as previously described.

Protein Degradation Assay. The half-life of dectin-1 was determined in HEK 293T cells cultured on 12-well plates and sortagged with a biotinylated probe. Cycloheximide (50 μg/mL) was added to medium immediately after sortagging, and the cells were harvested at different time points (0, 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, and 12 h). A fraction of the cell lysate was digested with EndoH for 2 h. All cell lysates in SDS sample buffer (crude lysate and EndoH-treated fraction) were boiled and then analyzed by immunoblotting using streptavidin, anti-HA antibody, or anti–β-actin antibody. Samples were obtained from cells maintained under two different conditions: with and without zymosan exposure. Ten particles of zymosan per cell were added to the culture and incubated for 30 min at 37 °C. The quantification of the levels of protein from the immunoblots was performed with ImageJ software (National Institutes of Health).

Streptavidin Beads Affinity Precipitation and Coimmunoprecipitation. RAW 264.7 macrophages expressing either tagged dectin-1 or tagged CD74 (with two amino acid substitutions, L7A and L17A) were sortagged with a biotinylated probe. Cells were divided in two fractions: one sample received zymosan (10 particles per cell) for 30 min at 37 °C, the other one did not. Cells were lysed with 0.5% Nonidet P-40 and a protease inhibitor mix and precleared with magnetic beads (without streptavidin) for 1 h at 4 °C and then incubated with magnetic streptavidin Dynabeads overnight at 4 °C. Beads were washed three times in PBS with 0.5% Nonidet P-40, and bound protein was eluted with SDS/PAGE sample buffer. The samples were then separated by SDS/PAGE. The gels were immunoblotted with streptavidin or the appropriate antibody or stained with silver stain, and bands of interest were excised for analysis by mass spectrometry.

For the coimmunoprecipitation assay, RAW 264.7 or HEK 293T cells stably expressing tagged dectin-1 or tagged mutated CD74 were lysed and the lysates precleared with IgG and Dynabeads/protein G for 1 h at 4 °C. Anti-galectin-3 antibody (5 μg) was immobilized on 50 mL Dynabeads/protein G and incubated with 500 μg of cell extract supernatant overnight at 4 °C. The immunoprecipitates were washed three times in PBS with 0.5% Nonidet P-40 and eluted in 30 μL of gel sample buffer containing SDS. For anti-FLAG immunoprecipitation, HEK 293T cells stably expressing tagged dectin-1 were cultured overnight on a six-well plate and transfected with a vector encoding 3xFLAG-tagged galectin-3. Cell lysates were precleared with Dynabeads (without streptavidin or antibody attached) and incubated overnight at 4 °C with anti-FLAG–coated magnetic beads. For immunoblotting, the appropriate antibodies or streptavidin were used.

RNA Extraction and Quantitative RT-PCR. Total RNA was extracted from cells using RNeasy (Qiagen). To measure the relative mRNA levels of the different genes, quantitative RT-PCR (qPCR) was done using SYBR Green (Applied Biosystems) and a 7500 real-time PCR system (Applied Biosystems), according to the manufacturing protocol. GAPDH was used as the invariant

control. The following primer sets were used to detect dectin-1, galectin-3, and mTNF-α. Dectin-1: 5′-ACCACAAGCCCACAGAATCATC-3′ (forward), 5′-CATGGTCCAATTAGGAAGGCAA-3′ (reverse); galectin-3: 5′-TATCCTGCTGCTGGCCCTTAT-3′ (forward), 5′-CACTGTGCCCATGATTGTGATC-3′ (reverse); mTNF-α: 5′-ATGGCCTCCCTCTCATCAGTTC-3′ (forward), 5′-TTGGTGGTTTGCTACGACGTG-3′ (reverse). For determination of TNF-α mRNA levels, cells were incubated with UV-inactivated wild-type *Saccharomyces cerevisiae* (BY4742) or *Candida albicans* (SC5314) at a 1:5 (macrophage:yeast) ratio or without fungi for 4 h at 37 °C. The UV inactivation of yeast was performed as previously described (9). The equivalent of 2.5×10^7 cells resuspended in 1 mL of PBS was exposed to four doses of 100,000 μjoules/cm^2 in a CL-1000 UV-crosslinker (UVP).

Cytokine Production by Macrophages Stimulated with Yeast. UV-treated yeast were added to macrophages at a 1:5 (macrophage/yeast) ratio and incubated for 12 h at 37 °C. TNF-α was measured in cell-free collected supernantants with a commercial ELISA kit (BD Biosciences). ELISAs were performed according to the manufacturer's instructions.

Western Blotting. When live sortagged cells were analyzed, cells were centrifuged, and the pellet was lysed with PBS supplemented with 1% SDS or 0.5–1% Nonidet P-40 and protease inhibitor mixture. For Western blotting, a fixed amount of total protein was mixed with sample buffer [50 mM Tris (pH 7.5), 2% SDS, 10% glycerol, 2.5% 2-mercaptoethanol, and 0.02% Bromophenol Blue] and resolved by Tris-glycine SDS/PAGE. After transferring to PVDF membrane, proteins were analyzed by immunoblotting. HRP-conjugated antibodies or streptavidin were used at a 1:10,000 dilution.

Fluorescence Microscopy and Flow Cytometry (FACS). Cells were sortagged and/or incubated with Alexa Fluor 647-labeled or Alexa Fluor 555-labeled zymosan and fixed with 3% paraformaldehyde. Samples were visualized on chambered coverglass with an inverted Nikon TE2000-s microscope equipped with a Spot RT Camera (Diagnostic Instruments). The imaging camera was set to capture 8-bit images that were subsequently processed with Photoshop (Adobe Systems). Samples were examined with a 40×/0.75 M/N2 dry objective lens at room temperature.

For the cytofluorometry analysis, cells were fixed with 3% paraformaldehyde. Fluorescence was quantified on a FACScalibur cytometer (BD Biosciences). Cells were gated by forward and side scatter according to wild-type cell size and shape, and mean fluorescence intensity of 10,000 labeled cells was calculated using Cellquest software (BD Biosciences).

Statistical Analysis. Data from RT-qPCR and ELISA were acquired from three independent experiments. Statistical significance was determined with two-tailed Student's *t* test. Values of $P < 0.05$ were considered significant.

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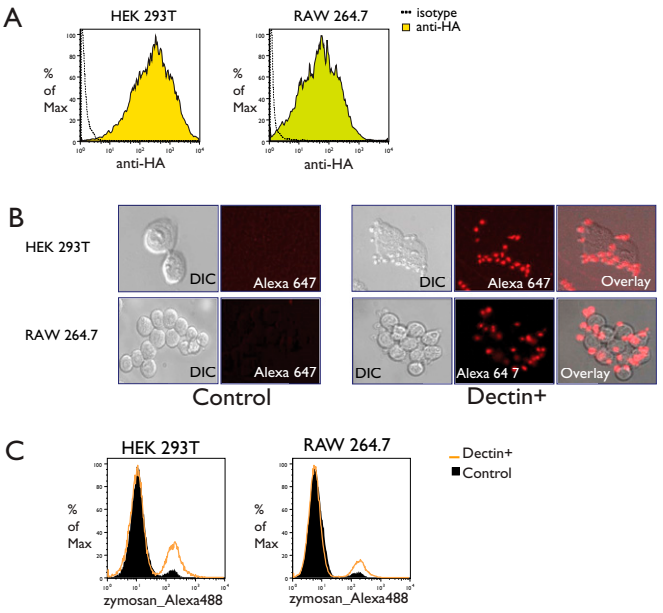

Fig. S1. Tagged dectin-1 is expressed on the cell surface of mammalian cells and is functional. (*A*) HEK 293T and RAW 264.7 cells were transduced with a retrovirus expression vector containing murine tagged dectin-1 (with LPETG motif and 3xHA tag at the C terminus). Cells were then sorted by FACS to obtain homogeneous populations of cells expressing readily detectable levels of the tagged protein. Dectin-1-LPETG-3xHA was detected with anti-HA antibodies conjugated with R-PE (anti-HA). A sample of cells was incubated with IgG isotype control antibody (isotype). (*B* and *C*) Stably transduced HEK 293T and RAW 264.7 cells (Dectin+) were incubated with Alexa Fluor 647 (red) or Alexa Fluor 488 (green)-labeled particles of zymosan (10 particles per cell, 30 min). Tagged dectin-1 recognized β-glucan and bound zymosan particles as shown by fluorescence microscopy (*B*) and cytofluorometry (*C*). Cells transduced with an empty retrovirus plasmid (Control) were used as a control.

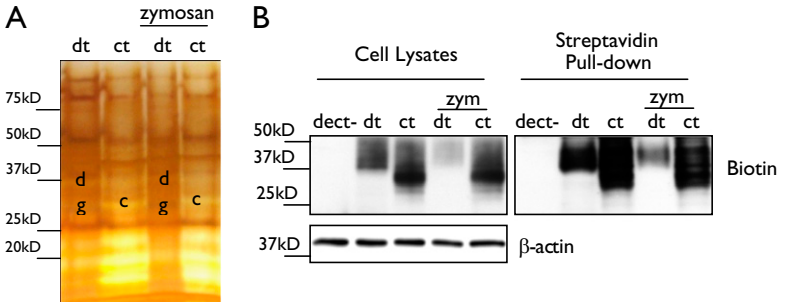

Fig. S2. Mass spectrometry analysis of surface-exposed biotinylated dectin-1 uncovers the association with galectin-3. Dectin-1 from stably transduced RAW 264.7 cells was labeled with a biotinylated probe. A fraction of these cells was incubated with zymosan particles (10 particles per cell, 30 min). Cell lysates were incubated with magnetic streptavidin Dynabeads, and bound protein was eluted with SDS/PAGE sample buffer. The samples were then separated by SDS/PAGE. (*A*) The gel was incubated with silver stain and the bands extracted for analysis by MS. The site where dectin-1 (d), galectin-3 (g), and the control CD74 (c) were localized in the gel after MS analysis is indicated. (*B*) Western analysis with HRP-conjugated streptavidin reveals biotinylated dectin-1 on sortagged cells before and after precipitation with streptavidin beads (Biotin). RAW 264.7 cells expressing similarly tagged CD74 were used as a control. dect-, cells with no expression of tagged dectin-1 or tagged CD74; dt, cells expressing labeled dectin-1; ct, control cells expressing labeled CD74; zym, zymosan.