The C-type Lectin Receptor Dectin-2 is a receptor for *Aspergillus fumigatus* galactomannan

Jennifer L. Reedy[1,2], Arianne J. Crossen[1], Paige E. Negoro[1], Hannah E. Brown[1], Rebecca A. Ward[1], Diego A. Vargas Blanco[1], Kyle D. Timmer[1], Michael K. Mansour[1,2], Marcel Wüthrich[3], Thierry Fontaine[4], Jean-Paul Latgé[5], and Jatin M. Vyas[1,2*].

Division of Infectious Diseases, Department of Medicine, Massachusetts General Hospital, Boston, Massachusetts 02114[1], Harvard Medical School, Department of Medicine, Boston, Massachusetts 02115[2], Department of Pediatrics, University of Wisconsin Medical School, University of Wisconsin Hospital and Clinics, Madison, WI, 53792[3], Institut Pasteur, Université de Paris, INRAE, USC2019, Unité Biologie et Pathogénicité Fongiques, F-75015, Paris[4], Institute of Molecular Biology and Biotechnology (IMBBFORTH), University of Crete, Heraklion, Greece[5].

*Corresponding Author: Massachusetts General Hospital, Division of Infectious Diseases, Gray-Jackson, Room 504, Boston, MA 02114. Phone: (617) 643-6444. Fax: (617) 643-6443. Email: jvyas@mgh.harvard.edu

Conflict of Interest: The authors have declared that no conflict of interest exists.

Keywords: *Aspergillus fumigatus*, galactomannan, Dectin-2, innate immunity, C-type Lectin Receptor

Abstract:

Aspergillus fumigatus is a ubiquitous environmental mold that causes significant mortality particularly amongst immunocompromised patients. The detection of the *Aspergillus*-derived carbohydrate galactomannan in patient sera and bronchoalveolar lavage fluid is the major biomarker used to detect *A. fumigatus* infection in clinical medicine. Despite the clinical relevance of this carbohydrate, we lack a fundamental understanding of how galactomannan is recognized by the innate immune system. Galactomannan is composed of a linear mannan backbone with galactofuranose sidechains and is found both attached to the cell surface of *Aspergillus* and as a soluble carbohydrate in the extracellular milieu. In this study, we utilized fungal-like particles composed of purified *Aspergillus* galactomannan to identify a C-type lectin host receptor responsible for binding this fungal carbohydrate. We identified a novel interaction between *Aspergillus* galactomannan and the C-type lectin receptor Dectin-2. We demonstrate that galactomannan bound to Dectin-2 and induced Dectin-2 dependent signaling including activation of spleen tyrosine kinase and potent TNFa production. Deficiency of Dectin-2 increased neutrophil recruitment to the lungs but was dispensable for survival in a mouse model of pulmonary aspergillosis. Our results identify a novel interaction between galactomannan and Dectin-2 and demonstrate that Dectin-2 is a receptor for *Aspergillus* galactomannan which leads to a pro-inflammatory response.

SUMMARY:

The *Aspergillus* carbohydrate galactomannan is the primary clinical biomarker for diagnosis of invasive aspergillosis. In this manuscript, we identify a novel interaction between galactomannan and the C-type lectin receptor Dectin-2. We demonstrate that galactomannan induces Dectin-2 dependent proinflammatory signaling in macrophages.

55 **INTRODUCTION**

56 *Aspergillus fumigatus* is a saprophytic mold that causes a wide spectrum of clinical manifestations
57 ranging from allergic disease to invasive infections, including pneumonia and disseminated
58 disease. While the majority of patients that develop invasive aspergillosis are
59 immunocompromised due to transplantation, inherited immune deficiencies, or use of
60 immunosuppressant therapies, invasive aspergillosis also occurs in otherwise healthy individuals
61 in association with preceding influenza or SARS-CoV2 infection (1-3). The mortality rate for
62 invasive aspergillosis remains unacceptably high with approximately 65 – 80% of patients
63 succumbing to disease despite antifungal therapy (4), and is a major cause of morbidity,
64 particularly among patients with compromised immune systems.

65 Humans are infected with *A. fumigatus* through inhalation of conidia deposited within
66 small airways and alveoli. Conidia are eliminated by mechanical expulsion through the action of
67 mucus and ciliated cells, as well as killing by macrophages and neutrophils (5, 6). Upon
68 encountering a fungal pathogen, innate immune cells synchronously engage multiple fungal cell
69 wall antigens through a diverse array of pattern recognition receptors including Toll-like receptors,
70 scavenger receptors, complement receptors, and C-type lectin receptors (CLRs), and integrate
71 those signals to generate a coordinated immune response (7-11). The majority of the fungal cell
72 wall antigens are polysaccharides, as > 90% of the fungal cell wall is composed of complex
73 carbohydrates. The cell wall is commonly composed of an inner chitin and middle b-1,3 glucan
74 layer with additional carbohydrate constituents like an outer mannan layer depending upon the
75 genus of fungus (12, 13). In *A. fumigatus*, the mannan content is primarily exists within the
76 galactomannan polymer. Although specific cell wall carbohydrate–pattern recognition receptor
77 interaction pairings have been defined for a few cell wall polysaccharides, such as b-1,3 glucan
78 and the CLR Dectin-1 (8, 14), we lack a fundamental understanding of the specific receptors that
79 engage many other fungal carbohydrates and how these ligand-receptor cognate pairs drive the
80 innate immune response.

81 The carbohydrate galactomannan was identified approximately 40 years ago as an
82 important clinical biomarker for the diagnosis of invasive aspergillosis, as it can be detected in
83 serum and bronchial fluid from infected patients (15-18). However, the biological and
84 immunological responses to this carbohydrate remain poorly defined. Galactomannan is
85 composed of a linear mannan core composed of a-1,2-linked mannotetraose units attached via
86 a-1,6-linkage with side changes composed of four to five b-1,5-linked galactofuranose residues
87 (19, 20). Galactomannan can be found in both the conidial and mycelial stages of the *Aspergillus*
88 lifecycle. It is bound to the cell membrane through a GPI-anchor, covalently attached to b-1,3-
89 glucan in the cell wall and released as a soluble polysaccharide into the extracellular milieu (21,
90 22). Most studies have utilized soluble galactomannan, since strains of *Aspergillus* that lack
91 galactomannan have altered growth morphology and kinetics (23, 24). Stimulation of peripheral
92 blood mononuclear cells (PBMCs) with soluble galactomannan suppressed LPS-induced cytokine
93 production. Furthermore, an *Aspergillus* vaccine protection model demonstrated that treatment
94 with soluble galactomannan induced a non-protective Th2/Th17 immunity (25, 26). Dendritic cell
95 and macrophages internalization of *A. fumigatus* conidia by the C-type Lectin Receptor DC-SIGN
96 could be inhibited through the addition of soluble galactomannan (27), suggesting that DC-SIGN
97 could play a role in recognizing this carbohydrate. These observations indicated that *Aspergillus*
98 subverts the immune response by shedding galactomannan, but the innate immune response to
99 cell-associated galactomannan has yet to be determined.

100 In this study, we identified Dectin-2 as a receptor for cell wall-associated galactomannan
101 by using fungal-like particles (FLPs), a novel technique previously developed in our laboratory
102 (28). To determine how the immune system recognizes galactomannan in the cell-associated
103 state, we isolated galactomannan from *A. fumigatus* and created FLPs consisting of purified
104 galactomannan. Using these particles to screen a library of CLR reporter cells, we identified
105 Dectin-2 as a receptor for galactomannan. We confirmed the binding of Dectin-2 to
106 galactomannan FLPs, using a solubilized receptor and demonstrated galactomannan FLPs

107 stimulate production of TNFα by murine macrophages in a Dectin-2 dependent manner.
108 Furthermore, we determined that immunocompetent mice lacking Dectin-2 recruit more
109 neutrophils to lungs compared with wild-type mice in a model of pulmonary aspergillosis, but
110 Dectin-2 is dispensable for survival. Taken together, our results demonstrate a novel and
111 important role for Dectin-2 in innate immune recognition of *Aspergillus* galactomannan.

112 **MATERIALS AND METHODS**

113 **Animals**

114 Mice were maintained in specific-pathogen-free barrier facilities at Massachusetts General
115 Hospital (MGH; Boston, MA) according to Institutional Animal Care and Use Committee (IACUC)
116 Guidelines. Wildtype C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME)
117 and Dectin-2$^{-/-}$ mice (29) were from the University of Wisconsin-Madison. All mice used for bone
118 marrow harvests and *in vivo* experiments were 8 – 20 weeks of age. Wildtype and Dectin-2$^{-/-}$
119 mice were both age and sex-matched for each experiment. Unless noted, an equal distribution
120 of male and female mice was used.

121

122 ***Cell lines and cell culture***

123 Immortalized C57BL/6 bone marrow-derived macrophages (BMDM) were a gift from Doug
124 Golenbock (University of Massachusetts Medical School, Worcester, MA). Macrophages were
125 cultured in complete RPMI media (RPMI 1650, L-glutamine, 10% heat-inactivated fetal bovine
126 serum (FBS; Gibco, Thermo Fisher Scientific, Rockford, IL), 1% penicillin/streptomycin, 1%
127 HEPES buffer, and 50 μM of β-mercaptoethanol). Puromycin and blastocidin were added to a
128 final concentration of 5 μg/mL as needed for the selection of transduced cells. Primary BMDMs
129 from C57BL/6 and Dectin-2$^{-/-}$ mice were harvested as previously described (30). After harvest,
130 primary BMDMs were grown for 7 days in complete RPMI media containing 20 ng/mL of
131 recombinant MCSF (Peprotech, Rocky Hill, NJ) prior to use in assays. HEK293T cells were
132 purchased from the ATCC (American Type Cell Collection, Manassas, VA) and were grown in
133 complete DMEM (DMEM, L-glutamine, 10% heat-inactivated FBS, 1% penicillin/streptomycin, 1%
134 HEPES buffer). All cell lines were grown at 37^{0}C in the presence of 5% CO_2.

135

136 ***Lentiviral transduction of cells with Dectin-2 and FCRy***

137 Murine Dectin-2 was amplified from plasmid pFB-dectin2-IRES-eGFP from Caetano Reis e Sousa
138 using a 5' primer containing NotI digestion site with sequence
139 TATTGCGGCCGCCATGGTGCAGGAAAGACAATCCCAAGG and a 3' primer containing a BglII
140 restriction site with sequence
141 TCGACGATAGATCTTCATAGGTAAATCTTCTTCATTTCACATATTGAATTG. Primers were
142 synthesized by Integrated DNA technologies (IDT, Research Triangle Park, NC). The gamma
143 chain protein (FcRg) of the Fc-gamma receptor (FcgR) was PCR amplified from plasmid pSVL-
144 mo FceRI gamma (plasmid was a gift from Jean Kinet, Addgene plasmid #8372), using 5' primer
145 containing a NotI digestion site with sequence
146 TATTGCGGCCGCCATGATCTCAGCCGTGATCTTGTTCTTG and 3' primer containing a BamHI
147 digestion site TCGACGATGGATCCCTACTGGGGTGGTTTTTCATGCTTC. The constructs were
148 cloned into a pHAGEII lentiviral vector containing either puromycin or blasticidin selectable
149 markers using NotI and BamHI digestion and sequence verified. Generation of lentivirus in
150 HEK293T cells and transduction was performed as previously described (31). Cells were
151 selected for uptake of the lentivirus vector using puromycin or blasticidin at 5 µg/mL. After
152 selection, expression of the desired gene was confirmed using western blot. Surface localization
153 of Dectin-2 was confirmed using flow cytometry using anti-murine Dectin-2-APC (R&D Systems,
154 Minneapolis, MN, FAB1515A) or anti-human Dectin-2 polyclonal goat antibody (R&D, AF3114)
155 with Donkey anti-Goat AF488 secondary antibody (Invitrogen, Thermo Fisher Scientific, Rockford,
156 IL, A11055).
157
158 ***Fungal culture***
159 *A. fumigatus* strains Af293 and CEA10 were grown at 37℃ for 5 days on Sabouraud's Dextrose
160 Agar (Difco, Fisher Scientific, Rockford, IL) or for 3 days on glucose minimal media slants (GMM),
161 respectively. Slants were seeded from frozen stocks of conidia maintained at -80℃. To harvest
162 conidia, a sterile solution of purified deionized water (Milli-Q, Millipore Sigma, Burlington, MA)

163 containing 0.01% Tween 20 was added to each slant and spores were liberated using gentle
164 shaking and surface agitation with a sterile cotton tip swab. The solution containing liberated
165 spores was passed through a 40 µm cell strainer to separate hyphal debris. Spores were washed
166 three times with sterile PBS and then counted on a LUNA™ automated cell counter (Logos
167 Biosystems, Annandale, VA) and diluted to the appropriate concentration. To generate *A.*
168 *fumigatus* germlings with strain Af293, conidia were incubated in complete RPMI media at 37℃
169 for 4-6 hours and visually inspected under the microscope for the presence of emerging germ
170 tubes. Germlings were then washed, counted, and resuspended in PBS at the desired inoculum.
171 For heat-killing studies, *A. fumigatus* germlings were incubated at 95℃ for 30 minutes.
172
173 ***Purification of galactomannan from* Aspergillus fumigatus**
174 The galactomannan was isolated as lipogalactomannan from the cellular membrane of mycelium
175 that had been grown in Sabouraud's Dextrose medium using previously described methods (21).
176 The lipid moiety was removed by nitrous deamination and the polysaccharide moiety was purified
177 by gel filtration chromatography on Superdex 75 column (10/300 GL, GE-Healthcare) (21).
178
179 ***Generation and quality control of fungal FLP***
180 Carbohydrate coated-FLP were creating using previously published methods (28). Briefly, 300
181 ml of amine-coated polystyrene beads (Polysciences, Warrington, PA) were washed three times
182 in sterile anhydrous dimethylsulfoxide (DMSO, MilliporeSigma,Burlington, MA) using 0.45 um
183 PTFE centrifugal filters (MilliporeSigma, Burlington, MA) to remove any aqueous solution. The
184 beads were then resuspended in either DMSO (adsorbed beads) or DMSO containing 0.5 M 1,1'-
185 carbonyldiimidazole (CDI) and incubated at room temperature for 1 hour with shaking. Beads
186 were then rinsed three times with DMSO using centrifugal filters. Beads were resuspended in
187 200 ml of purified carbohydrates dissolved in DMSO. Soluble b-1,3 glucan (Wellmune, Biothera,
188 *Saccharomyces cerevisiae*, Eagan, MN) was used at 10mg/ml, mannan (*S. cerevisiae*, Sigma-

189 Aldrich, M704-1G) at 2.5 mg/ml, and galactomannan at 1mg/ml. Unmodified beads were
190 resuspended just in DMSO without additional carbohydrates. The mixture was incubated for 1
191 hour at room temperature with shaking and centrifuged using centrifugal filters to remove the
192 DMSO and excess carbohydrates. Beads were then washed three times in PBS, resuspended
193 in PBS, counted on a Luna™ automated cell counter, and stored at 4°C.

194 To confirm the association of carbohydrates with the FLPs and ensure rigor, a subset of
195 each newly created batch of FLPs was analyzed by flow cytometry. Briefly, 3 ml of beads were
196 blocked overnight in PBS containing 2% BSA at 4℃. To analyze mannan binding to the surface,
197 10 ml of 20 mg/ml stock fluorescein labeled Concanavalin A (Con A; Invitrogen, Thermo Fisher
198 Scientific, Carlsbad, CA; C827) was added to each sample and incubated for 1 hour at room
199 temperature while shaking. Samples were washed and resuspended in FACS buffer (PBS
200 containing 2% BSA) and then analyzed on a BD FACSCalibur™ or BD FACSCelesta™ Flow
201 Cytometer (BD, Franklin Lakes, NJ). For analysis of b-1,3 glucan, 20 ng of primary monoclonal
202 anti-b-1,3 glucan antibody (Biosupplies Australia Pty LTD, Victoria, Australia; 400-2) was added
203 to each sample and incubated for 1 hour at room temperature. FLPs were washed and then
204 incubated for 30 minutes with a 1:100 dilution of secondary rabbit anti-mouse IgG conjugated with
205 Alexa Fluor 488 (Invitrogen, Carlsbad, CA; A11059). FLPs were then washed, resuspended in
206 FACS buffer, and analyzed by flow cytometry. For galactomannan-coated beads, FLPs were
207 centrifuged, and the blocking buffer was removed. FLPs were resuspended in 50 ml of component
208 R6 from the Platelia *Aspergillus* Ag kit (Bio-Rad, Hercules, CA) containing rat anti-galactomannan
209 IgM antibody and were incubated for 1 hour at room temperature. FLPs were then washed and
210 resuspended in a 1:200 dilution of secondary goat anti-rat IgM conjugated with Alexa Fluor 488
211 (Invitrogen, Carlsbad, CA; A-21212) in FACs Buffer. FLPs were incubated for 30 minutes at room
212 temperature and then washed and resuspended in FACS buffer.

213 To assess the stability of carbohydrate association, 10 ml of FLP were incubated in 800
214 ml of either 3% SDS, 1X NP40, 1% Triton X-100 for 1 hour at room temperature with constant

215 shaking, or 3% SDS at 95^0C for 1 hour. The FLPs were centrifuged, washed with detergent, and
216 washed three times with FACS buffer. FLPs were then subjected to antibody staining to assess
217 for carbohydrate association as detailed above.

218 For fluorescent imaging of carbohydrate coated FLPs, FLPs were fluorescently labeled in
219 the same manner as for flow cytometry and then placed onto glass slides. FLPs were imaged
220 using a Nikon Ti-E inverted microscope with a CSU-X1 confocal spinning-disk head (Yokogawa,
221 Sugarland, TX) with a Coherent 4-Watt laser (Coherent, Santa Clara, CA) as the excitation
222 source. Images were acquired through an EM-CCD camera (Hamamatsu, C9100-13,
223 Bridgewater, NJ) and performed using MetaMorph® software (Molecular Devices, Downington,
224 PA). Image data files were processed using Adobe Photoshop 2021 and assembled in Adobe
225 Illustrator 2022 (Adobe Systems, San Jose, CA).

226

227 ***ELISA***

228 1 x10^5 macrophages (immortalized or BMDM) were plated in triplicate in tissue culture treated 48-
229 well plates and stimulated overnight with FLP or fungal germlings at the indicated target-to-
230 effector ratios. Supernatants were collected for cytokine analysis using ELISA (Duoset, R&D
231 Systems, Minneapolis, MN) per the manufacturer's instructions and read using an i3X
232 Spectrophotometer (Molecular Devices, LLC, San Jose, CA). Results were analyzed using
233 PRISM9 software (GraphPad Software, San Diego, CA).

234

235 ***FLP binding assays***

236 FLPs were blocked overnight in PBS containing 2% BSA. FLPs were washed and resuspended
237 in 100 ml of binding buffer (20 mM Tris-HCL, pH 7.4, 150 mM NaCl, 10 mM $CaCl_2$, 0.05% Tween-
238 20). Dectin-2(murine)- IgG1 Fc (human) fusion protein (ENZO Life Sciences, Inc, Farmingdale,
239 NY) or IgG1 Fc (human) protein (Thermo Fisher Scientific, Rockford, IL) was added to a final
240 concentration of 10 mg/ml and samples were incubated with gentle mixing for 2 hours at room

241 temperature. Following washing, samples were resuspended in binding buffer containing a 1:100
242 dilution of mouse anti-human IgG1 Fc conjugated to Alexa Fluor 488 (Thermo Fisher Scientific,
243 Rockford, IL; A-10631) and incubated for 30 minutes at room temperature in the dark. FLPs were
244 washed with binding buffer, resuspended in FACS buffer (PBS containing 2% BSA), examined
245 on a BD FACSCalibur™ or BD FACSCelesta™- Flow Cytometer (BD, Franklin Lakes, NJ), and
246 data analyzed using FlowJo 10. For neutralizing antibody experiments, mouse anti-Dectin-2
247 antibody (R&D Systems, MAB1525) was preincubated with the Dectin-2-Fc fusion protein at a
248 concentration of 50 mg/ml for 30 minutes prior to adding the mixture to FLPs.
249
250 ***CLR ligand reporter cell assay***
251 Reporter cells were used based on previously published protocols (32). B3Z and BWZ cells
252 carrying an NFAT-lacZ reporter construct have been previously described (33, 34). B3Z cells
253 expressing Dectin-2 or a wildtype FCRg-chain as well as BWZ cells expressing Dectin-1CD3z
254 (the extracellular domain of mouse Dectin-1 fused to CD3z intracellular tail) were constructed by
255 Dr. Caetano Reis e Sousa (London Research Institute, London, U.K) (35). Mincle and Dectin-3
256 expressing cells were created as previously described (32). Briefly, cells were seeded at 1 x 10^5
257 cells per well in 96-well plates followed by stimulation with FLPs or media alone. Cells were
258 incubated for 18 hours in a tissue culture incubator at 37℃ with 5% CO_2. Cells were pelleted and
259 the supernatant was removed. Cells were lysed with triton lysis buffer (1% Triton X-100, 9 mM
260 KH_2PO_4, 90 mM K_2HPO_4) on ice for 30 minutes. Plates were centrifuged to pellet cellular debris
261 and 40 ml of lysate was transferred to a new 96-well plate. Assay buffer containing chlorophenol
262 red-b-D-galactopyranoside (CPRG) was added to each well and plates were incubated for 6 hours
263 at 37℃ protected from light and then absorbance at 560 nm and 620 nm was measured using an
264 i3x Spectrophotometer (Molecular Devices LLC, San Jose, CA).
265
266 ***In vivo Aspergillus survival model***

267 Wildtype C57BL/6 mice or Dectin-2$^{-/-}$ mice were immunosuppressed with 40 mg/kg subcutaneous
268 triamcinolone acetonide (Kenalog®-10, Bristol-Myers Squibb, New York, NY) starting the day prior
269 to infection and every 7 days thereafter if needed. The mice were infected with 2.4 x10^4 CEA10
270 conidia intranasally under isoflurane anesthesia. Mice were then monitored twice daily for the
271 first 5 days of the experiment and then daily thereafter. Mice were assessed based upon a 12-
272 point scale to determine when an individual animal should be euthanized due to illness and
273 removed from the study. This was developed in conjunction with the MGH IACUC and includes
274 the following symptoms and points assigned to each in parentheses: hunched posture (3), ruffled
275 and/or matted fur (3), shivering (3), abnormal breathing (increased respiratory rate) (12), 75%
276 reduction in activity compared to controls (3), inactivity leading to inability to acquire food or water
277 (12), and barrel rolling (12). If any animal reaches 12 points, they are humanely euthanized and
278 counted as a death for the purposes of survival analysis. All animal experiments were approved
279 under the MGH IACUC protocol #2008N000078.
280
281 ***Immunophenotyping of pulmonary infiltrates***
282 Wildtype or Dectin-2$^{-/-}$ mice were infected with 4 x 10^7 conidia of *A. fumigatus* strain CEA10
283 through oropharyngeal inhalation under isoflurane anesthesia. Mice were monitored daily for the
284 duration of the experiment and euthanized at 48 hours. Immunophenotyping was performed
285 based on previously published methods (36). The lungs were perfused with PBS through cardiac
286 puncture to flush intravascular blood cells and the lungs were carefully dissected and placed into
287 Digestion Solution (38 mg Collagenase Type I and 100 U DNase I in 100 ml RPMI 1640). Lung
288 tissue was finely minced using scissors and incubated for 1 hour with shaking at 37℃. After 1
289 hour, 800 ml of 0.5 M EDTA was added to each sample to stop the collagenase activity. Lungs
290 samples were passed through a 70 mM filter, pelleted, resuspended in a 40% Percoll-RPMI
291 solution, and under-layered with a 67% Percoll-RPMI solution. Samples were centrifuged at 650
292 x g for 20 minutes and leukocytes were collected at the interface between the two layers and

293 washed twice with FACS buffer (PBS with 2% FBS). Cells were pelleted, resuspended in FACS
294 buffer (PBS containing 2% BSA), counted, and 1 – 2 x 10^6 cells were stained for flow cytometry
295 analysis. Fc block (anti-Mouse CD16/CD32, Thermo Fisher Scientific, Rockford, IL, 14-0161-85)
296 was added to cells at 1:100 dilution and incubated at room temperature for 15 minutes. Cells
297 were then labeled with a mixture of the following fluorophore-conjugated antibodies for 1 hour at
298 4℃ (all antibodies are from Biolegend, San Diego, CA unless otherwise noted): anti-CD45 BV605
299 (Biolegend, 103139), anti-CD90.2 BV786 (Biolegend, 105331), anti-CD19 PE-Dazzle (Biolegend,
300 115553), anti-CD4 BV510 (Biolegend, 100559), anti-CD8 APC FIRE (Biolegend, 100765), anti-
301 Siglec-F APC (Biolegend, 155507), anti-Ly6G AF 488 (Biolegend, 127625), anti-Ly6C BV510
302 (Biolegend, 128033), anti-I-A/I-E AF700 (Biolegend, 107622), anti-NCR1 BV421 (Biolegend,
303 137611), anti-CD64 PE (Biolegend, 139303), anti-CD103 BV421 (Biolegend, 121421), anti-
304 CD11b APC FIRE (Biolegend, 101261), anti-CD11c BV650 (Biolegend, 117339), anti-
305 B220/CD45R PE (Biolegend, 103207), anti-TCRγδ APC (Biolegend, 118115), and anti-TCRβ
306 BV650 (Biolegend, 109251). Cells were washed with FACS buffer, and 7-AAD (Stem Cell
307 Technologies, 75001.1) was added for live-dead cell staining just prior to flow cytometry. Flow
308 cytometry was performed using BD FACSCelesta™ and analysis was performed using FlowJo 10
309 software (BD, Ashland, OR).
310

311 **Quantification of Fungal Burden in Lungs**

312 Wildtype or Dectin-$2^{-/-}$ mice were infected with 4 x 10^7 conidia of *A. fumigatus* strain CEA10
313 through oropharyngeal inhalation under isoflurane anesthesia**.** Each experiment consisted of 5
314 wild-type and 5 Dectin-$2^{-/-}$ mice infected with CEA10 *Aspergillus* and one mock infection (PBS
315 only) control per mouse line. After 48 hours lungs were extracted, frozen at -80°C, and lyophilized
316 overnight. Freeze-dried tissue was homogenized using 0.5 mm glass beads with a TissueLyser
317 LT homogenizer (Qiagen, Germantown, MD) to create a fine powder. DNA was extracted from
318 the homogenized tissue using the E.Z.N.A Fungal DNA Extraction Kit (Omega Bio-tek, Norcross,

319 GA). The total quantity of DNA extracted from each sample was quantified using a NanoDrop™
320 One Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific, Rockford, IL). Fungal
321 DNA in each sample was quantified using qPCR. A standard curve was created using *A.*
322 *fumigatus* genomic DNA and consisted of 10-fold serial dilutions from 1 pg to 100 ng. 500 ng of
323 sample DNA was analyzed per reaction and each sample was analyzed in triplicate. The
324 sequences for the amplification primers and dual-labeled fluorogenic hybridization probe for the
325 *A. fumigatus* 18S rRNA gene were previously published(37). All three probes were synthesized
326 by Integrated DNA Technologies (IDT, Research Triangle Park, NC). Each 20 μl qPCR contained
327 TaqMan® Fast Advanced Master Mix (Thermo Fisher Scientific, Rockland, IL), 500 ng of sample
328 DNA and 1 μM each of forward primer, reverse primer and probe. Samples were run and
329 analyzed using the Applied Biosystems™ 7500 Fast Real-Time PCR System (Thermo Fisher
330 Scientific, Rockland, IL). The percent of fungal DNA in each sample was determined by dividing
331 the quantified fungal DNA in each PCR by 500 ng (input DNA per sample). The total DNA
332 obtained from each lung sample was multiplied by the percent fungal DNA to obtain the total
333 fungal DNA/lung.
334
335 **Western blot**
336 Cells were placed on ice and lysed with 1% NP40 lysis buffer containing protease and
337 phosphatase inhibitors. Proteins were denatured using NuPAGE LDS Sample Buffer (Thermo
338 Fisher Scientific, Rockford, IL). The proteins were resolved by SDS-PAGE on 4-12% NuPAGE
339 gels using NuPAGE MOPS buffer (NuPage gels, Thermo Fisher Scientific, Rockford, IL), and
340 transferred to methanol-activated PVDF membrane (Perkin Elmer, Waltham, MA) using transfer
341 buffer (0.025M Tris, 0.192 M glycine, 20% methanol) and electrophoretic transfer at 100V for 1
342 hour. For detection of proteins, PVDF membranes were blocked for 1 hour at room temperature
343 in 5% BSA in PBS-0.01% Tween 20 (PBST). Blots were incubated with anti-phospho spleen

344 tyrosine kinase (Syk) antibody (Cell Signaling Technologies, Danvers, MA; 2710S) or anti-total
345 Syk antibody (Cell Signaling Technologies, 13198S), in 5% BSA in PBST overnight at 4℃. The
346 membranes were washed and incubated with secondary swine anti-rabbit HRP conjugated
347 antibody (Agilent DAKO, Santa Clara, CA, P0399) in 1% milk in PBST for 1 hour at room
348 temperature. Membranes were washed and then visualized using Western Lightning Plus ECL
349 chemiluminescent substrate (Perkin Elmer, Waltham, MA) on Kodak BioMax XAR film
350 (MilliporeSigma, Burlington, MA). Films were then scanned and processed using Adobe
351 Photoshop 2021. Any contrast adjustments were applied evenly to the entire image and adheres
352 to standards set forth by the scientific community (38).
353

354 **RESULTS**

355 ***Screening of a C-type lectin library identifies Dectin-2 as a receptor for galactomannan***

356 The fungal cell wall is a dynamic structure that can change in both structure and composition
357 depending on the morphological stage, environmental milieu, and upon engagement with immune
358 cells (13, 39). Therefore, probing the role of a single carbohydrate entity is challenging. Standard
359 techniques have relied upon solubilized carbohydrates or genetic deletion mutants, however
360 these techniques have limitations including pleiotropic changes that occur in genetic deletion
361 strains, such as compensatory changes in cell wall composition or structure, growth defects,
362 alterations in antigen exposure (40), and known differences in immune response to soluble versus
363 particulate (or cell-bound) carbohydrates. Most fungal cell wall carbohydrates remain bound to
364 the cell wall, however galactomannan and β-1,3 glucan are unique as they exist as both cell wall-
365 associated and free/soluble carbohydrates. β-1,3 glucan has been extensively studied revealing
366 differences in how soluble and cell wall β-1,3 glucan interact with immune cells (41-43). Cell wall
367 associated β-1,3 glucan signals via both the CLR Dectin-1 and complement receptor 3, whereas
368 soluble β-1,3 glucan is Dectin-1 independent (41-43). Although both cell wall β-1,3 glucan and
369 soluble β-1,3 glucan bind Dectin-1, only cell wall β-1,3 glucan activates signaling (41). In contrast,
370 receptors such as TLRs signal in response to both soluble and cell associated ligands. Activation
371 of Dectin-1 requires receptor clustering into phagocytic synapses which exclude regulatory
372 phosphatases licensing activation of Dectin-1 (41, 44). Whether the difference in response to cell
373 wall and soluble carbohydrates is unique to β-1,3 glucan and Dectin-1 or extends to other fungal
374 carbohydrates and CLRs remains unknown. We hypothesize that the innate immune system
375 distinguishes between soluble and cell-associated fungal carbohydrates to tailor the immune
376 response. Most studies to date have been conducted using soluble galactomannan. Thus, we
377 sought to characterize the immune response to cell-associated galactomannan to understand
378 how innate immune cells interact with *Aspergillus* at the site of infection.

379 Since CLRs are known to be involved in sensing of fungal pathogens and bind to
380 carbohydrates, we examined if any of the known CLRs were potential receptors for
381 galactomannan in the *Aspergillus* cell wall. We first screened a CLR library using *A. fumigatus*
382 germlings to determine which CLRs were stimulated by the whole organism and confirm utility of
383 the assay. T cell hybridomas containing an NFAT-lacZ reporter were engineered to express
384 individual or combinations of CLRs: Dectin-1, Dectin-2, Dectin-3, or Mincle. Dectin-2, Dectin-3,
385 and Mincle require interaction with the gamma subunit of the Fc gamma receptor (FcRg) for both
386 cell surface localization and signaling, thus this protein was co-expressed. We stimulated the
387 CLR reporter cells for 18 hours at 37℃ 5% CO_2 with live and heat-killed *Aspergillus* germlings
388 and observed robust activation of Dectin-1, Dectin-2, and Dectin-2/Dectin-3 co-expressing cells
389 (Figure 1A). Since cells that express Dectin-3 alone were not activated, the activation of the
390 Dectin-2/Dectin-3 co-expressing cells is likely due to either activation of Dectin-2 alone or possible
391 Dectin-2/Dectin-3 in combination (Figure 1A). Activation of Dectin-1 is known to occur through
392 binding of cell-wall associated b-1,3 glucan, but the Dectin-2 ligand on the surface of *Aspergillus*
393 has not been established. We hypothesized that galactomannan could be a potential Dectin-2
394 ligand.

395 To dissect individual carbohydrate contributions to CLR reporter cell activation, we
396 employed a technique developed in our laboratory for creating FLPs that consist of polystyrene
397 beads coated with a discrete, purified fungal carbohydrates (28). We have previously generated
398 FLPs made from purified b-1,3 glucan and demonstrated that carbohydrate-coated FLPs mimic
399 cell-associated or particulate carbohydrates. These b-1,3 glucan FLPs can activate Dectin-1
400 signaling and elicit the formation of a phagocytic synapse (28, 30, 44). Generation of
401 galactomannan FLPs was performed using methods previously described for the creation of b-
402 1,3 glucan and mannan FLPs (28) (Supplemental Methods). All generated FLPs underwent
403 quality control using flow cytometry to demonstrate the stable attachment of carbohydrates to the
404 FLP surface (Figure S1). We stimulated the reporter cell library with unmodified beads, b-1,3

405 glucan FLP, mannan FLP, and galactomannan FLP. Activation of CLR signaling resulted in
406 expression of a LacZ reporter and was quantified using a CPRG assay to measure β-
407 galactosidase activity. As expected, b-1,3 glucan FLPs activated Dectin-1 expressing reporter
408 cells and *S. cerevisiae* mannan FLPs stimulated Dectin-2 expressing reporter cells. The
409 galactomannan FLPs also stimulated Dectin-2 (Figure 1B) and Dectin-2/Dectin-3 co-expressing
410 cells (data not shown), suggesting that not only did galactomannan bind to Dectin-2, but also
411 triggered signaling by the CLR. None of our FLPs stimulated Mincle or Dectin-3 signaling alone,
412 however these cells were activated by incubation with trehalose-6,6-dibehenate (TDB) (data not
413 shown) demonstrating that the receptors are functional, and that absence of observed stimulation
414 is due to lack of CLR activation by the galactomannan FLPs. These data suggested that Dectin-
415 2, but not Dectin-3 or Mincle, is a potential receptor for galactomannan.
416
417 ***Soluble Dectin-2 binds to galactomannan FLPs***
418 After identifying Dectin-2 as a receptor for galactomannan using the CLR reporter cell screen, we
419 next assessed whether Dectin-2 binds directly to galactomannan FLP. To do this, we used
420 soluble murine Dectin-2-human IgG1 Fc fusion protein and a control human IgG1 Fc protein
421 (lacking Dectin-2). Flow cytometry using an anti-human IgG1 Fc antibody and an AF488 labeled
422 secondary antibody was used to detect adherence of Dectin-2-Fc to the surface of FLP (Figure
423 2A). Dectin-2-Fc was specifically bound to both *Aspergillus* galactomannan FLPs and
424 *Saccharomyces* mannan FLPs but did not bind to either unmodified or b-1,3 glucan FLPs. As
425 expected, human IgG1 Fc protein did not bind to any FLP. As an additional control to demonstrate
426 that binding to the galactomannan FLPs was Dectin-2 specific, we incubated the Dectin-2-Fc
427 protein with anti-Dectin-2 neutralizing antibody or isotype control for 30 minutes prior to incubation
428 with galactomannan FLPs. The Dectin-2 neutralizing antibody, but not the isotype control,
429 blocked binding of Dectin-2-Fc to galactomannan FLP demonstrating a specific interaction (Figure
430 2B).

431

432 ***Galactomannan FLPs induce Dectin-2 dependent Syk activation***

433 The CLR reporter assay and binding studies demonstrated that Dectin-2 is capable of binding
434 *Aspergillus* galactomannan. Next, we investigated whether this binding results in downstream
435 signaling. Dectin-2 does not possess an intrinsic signaling domain within its short intracellular tail
436 and signaling occurs through partnering of Dectin-2 with the gamma subunit (FcRg) of the Fc
437 gamma receptor (FcgR) (35). Activation of Dectin-2 through ligand binding leads to
438 phosphorylation of the ITAM motif of FcRg and subsequent signaling through Syk. FcRg is also
439 required for localization of Dectin-2 to the cell surface (35). Macrophages are known to express
440 Dectin-2, however immortalized C57BL/6 macrophages have low levels of Dectin-2 surface
441 expression at baseline (data not shown). Therefore, we transduced macrophages with lentivirus
442 containing either the murine Dectin-2 receptor or the human Dectin-2 receptor. As macrophages
443 produce abundant FCRg, we did not observe any difference in Dectin-2 surface expression
444 between macrophages transduced with only Dectin-2 or those transduced with both Dectin-2 and
445 FCRg. To determine if galactomannan is sufficient to trigger Dectin-2 dependent Syk activation,
446 we stimulated macrophages with galactomannan FLPs for 1 hour as well as unmodified FLPs, b-
447 1,3 glucan FLPs, mannan FLPs, and *C. albicans.* We used *C. albicans* as a positive control in
448 these experiments since it is a strong activator of Syk phosphorylation in wild-type macrophages.
449 Lysates from stimulated cells were immunoblotted for phosphorylated and total Syk. As expected,
450 we observed phosphorylation of Syk when wildtype macrophages were stimulated by *Candida*
451 *albicans* and b-1,3 glucan FLPs (positive controls for Syk stimulation) (Figure 3A). There was
452 increased phosphorylation of Syk in response to both galactomannan and mannan in Dectin-2
453 expressing macrophages demonstrating that galactomannan is sufficient to drive Dectin-2
454 dependent Syk phosphorylation (Figure 3A).

455

456 ***Dectin-2 mediates TNFa production by murine macrophages in response to both* A.**
457 **fumigatus *and galactomannan FLPs***
458 Having demonstrated that galactomannan is sufficient to activate Dectin-2 dependent Syk
459 signaling, we next interrogated the downstream effects of this activation. Specifically, we aimed
460 to determine if galactomannan stimulation of Dectin-2 is both sufficient and necessary for cytokine
461 production. To determine if galactomannan stimulation of Dectin-2 is sufficient to trigger cytokine
462 production, Dectin-2 expressing macrophages were stimulated overnight with unmodified FLPs,
463 b-1,3 glucan FLPs, *A. fumigatus* galactomannan FLPs, or *S. cerevisiae* mannan FLPs (positive
464 control) and TNFa production was measured by ELISA. Galactomannan and mannan FLPs
465 enhanced TNFa production in Dectin-2 expressing macrophages (Figure 3A). To demonstrate
466 that this effect was specific to Dectin-2, we pre-incubated the macrophages with a Dectin-2
467 neutralizing antibody or an isotype control for 30 minutes prior to stimulation with FLPs. TNFa
468 production was blocked by the Dectin-2 specific antibody, but not by the isotype control,
469 demonstrating a specific role for Dectin-2 in mediating the TNFa production in response to
470 galactomannan (Figure 3B).
471 To examine if Dectin-2 is required for primary BMDMs to respond to galactomannan FLPs,
472 primary BMDM from wildtype or Dectin-2 deficient mice were stimulated by galactomannan FLPs,
473 unmodified FLPs (negative control), or LPS (positive control). There was a significant decrease
474 in TNFa production in response to galactomannan FLPs (Figure 3C), indicating that
475 galactomannan triggers the secretion of pro-inflammatory cytokines in BMDMs in a Dectin-2
476 dependent manner.
477
478 ***Dectin-2 is dispensable for survival in a murine model of infection***
479 Our data suggest that Dectin-2 is a receptor for *A. fumigatus* galactomannan, and that recognition
480 of galactomannan by Dectin-2 results in Syk phosphorylation and cytokine production. To
481 determine if this pathway is required for survival during *Aspergillus* infection, we intranasally

482 infected corticosteroid immunosuppressed C57BL/6 mice and Dectin-2 deficient mice with *A.*
483 *fumigatus* (CEA10 strain) and monitored the mice for signs of infection. There was no difference
484 in survival between wild-type and Dectin-2 deficient mice, suggesting that in this model, Dectin-2
485 is dispensable for survival (Figure 4A).
486
487 ***Deletion of Dectin-2 results in increased recruitment of neutrophils, but does not alter***
488 ***fungal burden***
489 Although Dectin-2 did not affect the overall survival of the mice, we sought to determine if the
490 immune cell influx in response to infection was altered in the absence of Dectin-2. Since treatment
491 with steroids affects immune cell function, we chose to perform this experiment in an
492 immunocompetent, rather than a steroid-induced immunosuppression mouse model.
493 Immunocompetent mice were administered either *A. fumigatus* strain CEA10 or PBS via
494 oropharyngeal aspiration. After 48 hours of infection lungs were harvested, digested, and the
495 resulting single cell suspension was enriched for leukocytes using a Percoll gradient. A multi-
496 channel flow cytometry experiment was performed to allow simultaneous assessment of multiple
497 cell lines including neutrophils, eosinophils, T cells, B cells, dendritic cells (including cDC1, cDC2,
498 monocyte derived DCs, and plasmacytoid dendritic cells [pDCs]), NK cells, monocytes, and
499 alveolar macrophages. All infected mice demonstrated a marked increase in cellular infiltrates
500 compared to the PBS control mice, however only neutrophil influx was altered by Dectin-2
501 deficiency. We infected mice with 1×10^7, 2×10^7 and 4×10^7 conidia of CEA10 *A. fumigatus* and
502 noted a dose-dependent increase in immune cell infiltrate. At doses higher than 4×10^7 most
503 mice succumbed within 24 – 48 hours, presumably due to an exuberant inflammatory response.
504 While at all doses, there was a trend toward increased neutrophil influx in Dectin-2 deficient mice,
505 this only reached the level of significance in mice infected with 4×10^7 conidia. Dectin-2 deficient
506 mice had increased neutrophil infiltration of lungs compared to wild-type (Figure 4B), suggesting

507 that Dectin-2 is involved in the coordination of neutrophil response in this airway model of
508 infection.

509 To determine whether the increased influx of neutrophils could be the result of difference
510 in fungal burden between wild-type and Dectin-2 deficient mice, we quantified fungal burden in
511 the lungs of mice infected with 4 x 10^7 conidia at 48 hours post-infection using qPCR to detect
512 and quantify *Aspergillus* DNA(37). *Aspergillus fumigatus* DNA was detected in all samples from
513 infected mice, but not in PBS controls. There was no difference in *Aspergillus* DNA between wild-
514 type and Dectin-2 deficient mice (Figure 4C). CFU assays were also performed as a confirmatory
515 test to ensure that there was no difference in live *Aspergillus* fungal burden, since fungal DNA
516 testing cannot distinguish between live versus dead organisms. Lungs from both wild-type and
517 Dectin-2 deficient mice still contained live *Aspergillus fumigatus* at 48 hours, and there was no
518 difference in CFU between strains (data not shown). Taken together these results suggest that
519 the enhanced neutrophil infiltrate in Dectin-2 deficient mice is not due to a difference in fungal
520 burden.

521

522 **DISCUSSION**

523 Galactomannan is a clinically important polysaccharide that is released into patients' blood and
524 bronchial lavage fluid and used to diagnose invasive aspergillosis infections. However, we do not
525 understand the immunological implications of the wide-scale release of this carbohydrate into
526 patient tissue, or the role that it plays in mediating direct immunological interactions between *A.*
527 *fumigatus* and innate immune cells at the site of infection. Here, we utilized FLPs coated with
528 purified *A. fumigatus* galactomannan to identify Dectin-2 as a receptor for galactomannan. Our
529 results demonstrate that cell-associated galactomannan binds Dectin-2 inducing phosphorylation
530 of Syk and TNFa production.

531 Although BMDMs from Dectin-2 deficient mice had decreased cytokine production in
532 response to galactomannan FLPs, there was no difference in overall survival of

533 immunosuppressed Dectin-2 deficient mice compared with wild-type mice when challenged
534 intranasally with *A. fumigatus*. There is significant redundancy in CLRs signaling, therefore it is
535 possible that the lack of Dectin-2 is compensated for through Dectin-1 or other CLR-ligand
536 interactions in the context of the whole organism. However, publications have suggested a critical
537 role for Dectin-2 in the immune response to *A. fumigatus(45, 46)*. A recent case report identified
538 a patient without previously recognized immunodeficiency that developed invasive aspergillosis
539 and was found to have a mutation in Dectin-2 that led to decreased responsiveness to *Aspergillus*
540 (45). Previous work has also demonstrated a role for Dectin-2 in mediating plasmacytoid dendritic
541 cell (pDC) responses to *A. fumigatus* (46). These observations, coupled with this study, suggest
542 that while Dectin-2 may not be required for survival in a murine model, it may contribute to human
543 disease through modulating interactions with discrete cell populations.

544 Interestingly, immunocompetent mice that lacked Dectin-2 have increased recruitment of
545 neutrophils into the lung tissue upon infection with *Aspergillus*, which is in sharp contrast to
546 galactosaminogalactan (GAG) from *A. fumigatus* which led to a reduction of neutrophil infiltration
547 (47). The difference in neutrophil infiltrates was not due to difference in the fungal burden within
548 the lungs, suggesting that it is a direct result of Dectin-2 deficiency. This result is interesting, as
549 our data also demonstrated that galactomannan-induced Dectin-2 signaling in macrophages
550 leads to pro-inflammatory TNFα release. We anticipated that the absence of Dectin-2 would
551 result in decreased pulmonary inflammation, but surprisingly observed increased neutrophil
552 recruitment. Our results suggest that Dectin-2 may normally temper neutrophil recruitment to the
553 lungs, or that Dectin-2 signaling normally leads to increased neutrophil turnover. There are
554 multiple potential hypotheses to explain these findings which will provide fertile ground for future
555 studies. In the absence of Dectin-2, expression of Dectin-1 and/or other C-type Lectin Receptors
556 may be upregulated and thus these receptors may play a more substantial role in driving the
557 immune response. Additionally, Dectin-2 is expressed not just by macrophages, but also by many
558 immune cells and other lung resident cells, including epithelial cells. Thus, a cell type other than

559 macrophages, may be responsible for mediating the difference in neutrophil influx that we
560 observed. For instance, studies by Loures, *et al* (46) demonstrated that plasmacytoid dendritic
561 cell production of type I IFNs in response to *Aspergillus fumigatus* is dependent upon Dectin-2
562 mediated recognition of *Aspergillus fumigatus*. While we did not observe a difference in pDC
563 recruitment to the lungs in wild-type compared to Dectin-2 deficient mice, these cells are likely
564 impaired in their ability to produce type I IFNs during infection. While the increased PMN influx
565 observed in Dectin-2 deficient mice did not affect fungal clearance, whether the increased PMN
566 influx seen in Dectin-2 deficient mice could have detrimental effects on the host, such as
567 increasing lung damage during infection is unknown.

568 Our current work addresses the mechanism by which cell-wall associated galactomannan
569 is recognized by Dectin-2. Galactomannan and β-1,3 glucan are both fungal cell wall
570 carbohydrates that exists in both soluble and cell-wall associated states. B-1,3 glucan is known
571 to interact with the immune system differently depending upon its form. Whether galactomannan-
572 induced immune activation is triggered only at sites of infection or more systemically and how this
573 could impact the outcome of infection remain critically important questions. Whether circulating
574 soluble galactomannan can stimulate recognition by Dectin-2 will be the subject of future studies.
575 Dectin-2 has also been implicated in allergic disease(48-51), suggesting that galactomannan from
576 *Aspergillus* could play a role in stimulating allergic manifestations to this pathogen. Overall, our
577 data demonstrate that galactomannan is recognized by Dectin-2 and lays the exciting groundwork
578 for dissecting the role of galactomannan in an important clinical disease.
579
580
581

582 **ACKNOWLEDGEMENTS**

583 We thank all the members of the Vyas and Mansour lab at MGH and Dr. Vishukumar Aimanianda,

584 Institut Pasteur, for useful discussions and reading of the manuscript.

585

586 **FINANCIAL SUPPORT:** J.L.R is supported by NIH/NIAID grant 1K08AI14755, NIH/NIAID PCTAS

587 supplement, and KL2/Catalyst Medical Research Investigator Training award (an appointed KL2

588 award) from Harvard Catalyst| The Harvard Clinical Translational Science Center (National

589 Center for Research Resources and the National Center for Advancing Translational Sciences,

590 National Institutes of Health [NIH], award KL2 TR001100. J.M.V is supported by R01AI150181

591 and R01AI136529. M.K.M. is supported by RO1AI132638.

592

593

594 **FIGURE LEGENDS**

595

596 **Figure 1.** *A. fumigatus* and galactomannan activate Dectin-2 expressing CLR reporter cells.
597 Reporter cells were stimulated for 18 hours using (A) either live or heat killed germlings of *A.*
598 *fumigatus* Af293 at MOI 20:1 or (B) unmodified FLPs, b-1,3 glucan (BG) FLPs, *S. cerevisiae*
599 mannan FLPs, and *A. fumigatus* galactomannan (GALM) FLPs at an effector to target ration of
600 30:1. LacZ activity was measured in total cell lysates using CPRG as a substrate. Error bars
601 represent the SEM of duplicate wells, and each experiment was repeated three times.

602

603 **Figure 2.** Recombinant Dectin-2 Fc protein binds galactomannan FLPs. (A) Unmodified FLPs, b-
604 1,3 glucan FLPs, and galactomannan FLPs were blocked overnight in PBS/2% BSA then washed
605 and resuspended in binding buffer. FLP were incubated with soluble recombinant murine Dectin-
606 2- human IgG1 Fc protein conjugate, control IgG1-Fc protein, or in buffer only, incubated with
607 anti-human IgG1 conjugated to Alexa Fluor 488 and analyzed by flow cytometry. Dectin-2-Fc
608 bound to galactomannan FLPs, but not to either unmodified or b-1,3 glucan FLPs. (B) FLPs were
609 incubated for 2 hours in binding buffer alone or containing Dectin-2-Fc protein or Dectin-2-Fc pre-
610 incubated for 30 minutes with a Dectin-2 neutralizing antibody. Dectin-2 neutralizing antibody
611 disrupted the association of Dectin-2-Fc with galactomannan FLPs.

612

613 **Figure 3.** Galactomannan FLPs activate Dectin-2 dependent signaling in macrophages. (A)
614 Immunoblot of immortalized wildtype C57BL/6 macrophages or C57BL/6 macrophages
615 expressing Dectin-2 stimulated with live *C. albicans* (SC5314), unmodified FLPs, galactomannan
616 FLPs, b-1,3 glucan FLPs, or mannan FLPs at MOI 10:1 for 1 hour at 37°C and probed for both
617 total Syk and phosphorylated (active) Syk. Overexpression of Dectin-2 increased phosphorylation
618 of Syk in response to *C. albicans*, galactomannan and mannan FLPs, but not to unmodified or b-
619 1,3 glucan FLPs. (B) Immortalized wildtype C57BL/6 macrophages or C57BL/6 macrophages

620 expressing Dectin-2 were stimulated overnight with unmodified (UM), galactomannan (GALM), or
621 mannan (MAN) FLPs at a target to effector ratio of 10:1. Prior to FLPs stimulation, macrophages
622 were pre-treated for 30 minutes with either a Dectin-2 neutralizing antibody or an isotype control
623 antibody as indicated. Supernatants were analyzed for TNFa using ELISA. Both galactomannan
624 and mannan FLPs induced robust TNFa production by macrophages expressing Dectin-2, which
625 was blocked by Dectin-2 neutralizing antibody, but not the isotype control. The significance
626 indicated is in relation to the wild-type cell stimulated with the same FLPs. (C) BMDMs from wild-
627 type C57BL/6 and Dectin-2$^{-/-}$ mice were stimulated with LPS (500ng/ml), and unmodified (UM) or
628 galactomannan (GALM) FLPs at an effector to target ration of 20:1 for 18 hours. Supernatants
629 were analyzed for TNFa using ELISA. Dectin-2$^{-/-}$ BMDM produced less TNFa when stimulated
630 with galactomannan FLP compared with wildtype BMDM. Error bars represent standard error of
631 three biological replicates, statistics were calculated with two-way ANOVA using PRISM 9
632 software (not significant, ns, *** $p<0.001$, **** $p<0.0001$).
633
634 **Figure 4.** Dectin-2 is dispensible for survival, however deficiency of Dectin-2 leads to increased
635 neutrophil influx. (A) Wild-type C57BL/6 and Dectin-2$^{-/-}$ mice were immunosuppressed with
636 corticosteroids and infected intranasally with 2.5 x 10^4 conidia of *A. fumigatus* strain CEA10.
637 There was no significant difference in survival between wildtype and Dectin-2$^{-/-}$ mice as
638 determined based upon Kaplan-Meyer and Cox Analysis using PRISM 9 software (B)
639 Immunocompetent wild-type and Dectin-2$^{-/-}$ mice were infected via oropharyngeal inhalation of 4
640 x 10^7 conidia of *A. fumigatus* CEA10. Lung tissue was harvested 48 hours post-infection and
641 digested to obtain a single cell suspension that was then analyzed by flow cytometry. The
642 neutrophil fraction was identified as live (7-AAD low), CD45+, CD90.2-, CD19-, SiglecF-, Ly6G+
643 cells. Each point represents an individual animal and horizontal lines represent the median
644 neutrophil burden in the lungs. One-way ANOVA was performed using PRISM9 software to
645 determine statistical significance (**; $p<0.01$). (C) Immunocompetent wild-type and Dectin-2$^{-/-}$

646 mice were infected with 4 x 10^7 CEA10. After 48 hours lungs were extracted, and fungal burden
647 was quantified using qPCR. There was no difference in fungal burden between wild-type and
648 Dectin-$2^{-/-}$ mice. Each point represents an individual animal and horizontal lines represent the
649 median fungal burden in the lungs. Each experiment was repeated at least twice, and concordant
650 results were obtained in each experiment.
651

652 **REFERENCES**

653 1. Arastehfar A, Carvalho A, van de Veerdonk FL, Jenks JD, Koehler P, Krause R, Cornely
654 OA, D SP, Lass-Florl C, Hoenigl M. 2020. COVID-19 Associated Pulmonary Aspergillosis
655 (CAPA)-From Immunology to Treatment. J Fungi (Basel) 6:91-108.

656 2. Schauwvlieghe A, Rijnders BJA, Philips N, Verwijs R, Vanderbeke L, Van Tienen C,
657 Lagrou K, Verweij PE, Van de Veerdonk FL, Gommers D, Spronk P, Bergmans D,
658 Hoedemaekers A, Andrinopoulou ER, van den Berg C, Juffermans NP, Hodiamont CJ,
659 Vonk AG, Depuydt P, Boelens J, Wauters J, Dutch-Belgian Mycosis study g. 2018.
660 Invasive aspergillosis in patients admitted to the intensive care unit with severe influenza:
661 a retrospective cohort study. Lancet Respir Med 6:782-792.

662 3. van de Veerdonk FL, Wauters J, Verweij PE. 2020. Invasive *Aspergillus* Tracheobronchitis
663 Emerging as a Highly Lethal Complication of Severe Influenza. Am J Respir Crit Care Med
664 202:646-648.

665 4. Wirk B, Wingard JR. 2009. Current approaches in antifungal prophylaxis in high risk
666 hematologic malignancy and hematopoietic stem cell transplant patients. Mycopathologia
667 168:299-311.

668 5. Schaffner A, Douglas H, Braude A. 1982. Selective protection against conidia by
669 mononuclear and against mycelia by polymorphonuclear phagocytes in resistance to
670 *Aspergillus*. Observations on these two lines of defense *in vivo* and *in vitro* with human
671 and mouse phagocytes. J Clin Invest 69:617-31.

672 6. Ibrahim-Granet O, Philippe B, Boleti H, Boisvieux-Ulrich E, Grenet D, Stern M, Latge JP.
673 2003. Phagocytosis and intracellular fate of *Aspergillus fumigatus* conidia in alveolar
674 macrophages. Infect Immun 71:891-903.

675 7. Ariizumi K, Shen GL, Shikano S, Ritter R, 3rd, Zukas P, Edelbaum D, Morita A, Takashima
676 A. 2000. Cloning of a second dendritic cell-associated C-type lectin (dectin-2) and its
677 alternatively spliced isoforms. J Biol Chem 275:11957-63.

678 8. Brown GD, Taylor PR, Reid DM, Willment JA, Williams DL, Martinez-Pomares L, Wong
679 SY, Gordon S. 2002. Dectin-1 is a major beta-glucan receptor on macrophages. J Exp
680 Med 196:407-12.
681 9. LeibundGut-Landmann S, Wuthrich M, Hohl TM. 2012. Immunity to fungi. Curr Opin
682 Immunol 24:449-58.
683 10. Levitz SM. 2010. Innate recognition of fungal cell walls. PLoS Pathog 6:e1000758.
684 11. Willment JA, Brown GD. 2008. C-type lectin receptors in antifungal immunity. Trends
685 Microbiol 16:27-32.
686 12. Latge JP. 2010. Tasting the fungal cell wall. Cell Microbiol 12:863-72.
687 13. Latge JP. 2016. 30 years of battling the cell wall. Med Mycol 55:4-9.
688 14. Brown GD, Gordon S. 2001. Immune recognition. A new receptor for beta-glucans. Nature
689 413:36-7.
690 15. Sarfati J, Boucias DG, Latge JP. 1995. Antigens of *Aspergillus fumigatus* produced *in vivo*.
691 J Med Vet Mycol 33:9-14.
692 16. Stynen D, Sarfati J, Goris A, Prevost MC, Lesourd M, Kamphuis H, Darras V, Latge JP.
693 1992. Rat monoclonal antibodies against *Aspergillus* galactomannan. Infect Immun
694 60:2237-45.
695 17. Lehmann PF, Reiss E. 1978. Invasive aspergillosis: antiserum for circulating antigen
696 produced after immunization with serum from infected rabbits. Infect Immun 20:570-2.
697 18. Reiss E, Lehmann PF. 1979. Galactomannan antigenemia in invasive aspergillosis. Infect
698 Immun 25:357-65.
699 19. Fontaine T, Latge JP. 2020. Galactomannan Produced by *Aspergillus fumigatus*: An
700 Update on the Structure, Biosynthesis and Biological Functions of an Emblematic Fungal
701 Biomarker. J Fungi (Basel) 6:283.

702 20. Latge JP, Kobayashi H, Debeaupuis JP, Diaquin M, Sarfati J, Wieruszeski JM, Parra E,
703 Bouchara JP, Fournet B. 1994. Chemical and immunological characterization of the
704 extracellular galactomannan of *Aspergillus fumigatus*. Infect Immun 62:5424-33.
705 21. Costachel C, Coddeville B, Latge JP, Fontaine T. 2005. Glycosylphosphatidylinositol-
706 anchored fungal polysaccharide in *Aspergillus fumigatus*. J Biol Chem 280:39835-42.
707 22. Fontaine T, Simenel C, Dubreucq G, Adam O, Delepierre M, Lemoine J, Vorgias CE,
708 Diaquin M, Latge JP. 2000. Molecular organization of the alkali-insoluble fraction of
709 A*spergillus fumigatus* cell wall. J Biol Chem 275:41528.
710 23. Engel J, Schmalhorst PS, Routier FH. 2012. Biosynthesis of the fungal cell wall
711 polysaccharide galactomannan requires intraluminal GDP-mannose. J Biol Chem
712 287:44418-24.
713 24. Lamarre C, Beau R, Balloy V, Fontaine T, Wong Sak Hoi J, Guadagnini S, Berkova N,
714 Chignard M, Beauvais A, Latge JP. 2009. Galactofuranose attenuates cellular adhesion
715 of *Aspergillus fumigatus*. Cell Microbiol 11:1612-23.
716 25. Bozza S, Clavaud C, Giovannini G, Fontaine T, Beauvais A, Sarfati J, D'Angelo C,
717 Perruccio K, Bonifazi P, Zagarella S, Moretti S, Bistoni F, Latge JP, Romani L. 2009.
718 Immune sensing of *Aspergillus fumigatus* proteins, glycolipids, and polysaccharides and
719 the impact on Th immunity and vaccination. J Immunol 183:2407-14.
720 26. Chai LY, Vonk AG, Kullberg BJ, Verweij PE, Verschueren I, van der Meer JW, Joosten
721 LA, Latge JP, Netea MG. 2011. *Aspergillus fumigatus* cell wall components differentially
722 modulate host TLR2 and TLR4 responses. Microbes Infect 13:151-9.
723 27. Serrano-Gomez D, Dominguez-Soto A, Ancochea J, Jimenez-Heffernan JA, Leal JA,
724 Corbi AL. 2004. Dendritic cell-specific intercellular adhesion molecule 3-grabbing
725 nonintegrin mediates binding and internalization of Aspergillus fumigatus conidia by
726 dendritic cells and macrophages. J Immunol 173:5635-43.

727 28. Tam JM, Mansour MK, Khan NS, Yoder NC, Vyas JM. 2012. Use of fungal derived
728 polysaccharide-conjugated particles to probe Dectin-1 responses in innate immunity.
729 Integr Biol (Camb) 4:220-7.
730 29. Saijo S, Ikeda S, Yamabe K, Kakuta S, Ishigame H, Akitsu A, Fujikado N, Kusaka T, Kubo
731 S, Chung SH, Komatsu R, Miura N, Adachi Y, Ohno N, Shibuya K, Yamamoto N,
732 Kawakami K, Yamasaki S, Saito T, Akira S, Iwakura Y. 2010. Dectin-2 recognition of
733 alpha-mannans and induction of Th17 cell differentiation is essential for host defense
734 against Candida albicans. Immunity 32:681-91.
735 30. Tam JM, Mansour MK, Khan NS, Seward M, Puranam S, Tanne A, Sokolovska A, Becker
736 CE, Acharya M, Baird MA, Choi AM, Davidson MW, Segal BH, Lacy-Hulbert A, Stuart LM,
737 Xavier RJ, Vyas JM. 2014. Dectin-1-dependent LC3 recruitment to phagosomes enhances
738 fungicidal activity in macrophages. J Infect Dis 210:1844-54.
739 31. Mansour MK, Tam JM, Khan NS, Seward M, Davids PJ, Puranam S, Sokolovska A, Sykes
740 DB, Dagher Z, Becker C, Tanne A, Reedy JL, Stuart LM, Vyas JM. 2013. Dectin-1
741 activation controls maturation of beta-1,3-glucan-containing phagosomes. J Biol Chem
742 288:16043-54.
743 32. Wang H, LeBert V, Hung CY, Galles K, Saijo S, Lin X, Cole GT, Klein BS, Wuthrich M.
744 2014. C-type lectin receptors differentially induce th17 cells and vaccine immunity to the
745 endemic mycosis of North America. J Immunol 192:1107-19.
746 33. Karttunen J, Sanderson S, Shastri N. 1992. Detection of rare antigen-presenting cells by
747 the lacZ T-cell activation assay suggests an expression cloning strategy for T-cell
748 antigens. Proc Natl Acad Sci U S A 89:6020-4.
749 34. Fiering S, Northrop JP, Nolan GP, Mattila PS, Crabtree GR, Herzenberg LA. 1990. Single
750 cell assay of a transcription factor reveals a threshold in transcription activated by signals
751 emanating from the T-cell antigen receptor. Genes Dev 4:1823-34.

752 35. Robinson MJ, Osorio F, Rosas M, Freitas RP, Schweighoffer E, Gross O, Verbeek JS,
753 Ruland J, Tybulewicz V, Brown GD, Moita LF, Taylor PR, Reis e Sousa C. 2009. Dectin-
754 2 is a Syk-coupled pattern recognition receptor crucial for Th17 responses to fungal
755 infection. J Exp Med 206:2037-51.
756 36. Wiesner DL, Merkhofer RM, Ober C, Kujoth GC, Niu M, Keller NP, Gern JE, Brockman-
757 Schneider RA, Evans MD, Jackson DJ, Warner T, Jarjour NN, Esnault SJ, Feldman MB,
758 Freeman M, Mou H, Vyas JM, Klein BS. 2020. Club Cell TRPV4 Serves as a Damage
759 Sensor Driving Lung Allergic Inflammation. Cell Host Microbe 27:614-628 e6.
760 37. Bowman JC, Abruzzo GK, Anderson JW, Flattery AM, Gill CJ, Pikounis VB, Schmatz DM,
761 Liberator PA, Douglas CM. 2001. Quantitative PCR assay to measure Aspergillus
762 fumigatus burden in a murine model of disseminated aspergillosis: demonstration of
763 efficacy of caspofungin acetate. Antimicrob Agents Chemother 45:3474-81.
764 38. Rossner M, Yamada KM. 2004. What's in a picture? The temptation of image
765 manipulation. J Cell Biol 166:11-5.
766 39. Latge JP, Beauvais A. 2014. Functional duality of the cell wall. Curr Opin Microbiol 20:111-
767 7.
768 40. Gravelat FN, Beauvais A, Liu H, Lee MJ, Snarr BD, Chen D, Xu W, Kravtsov I, Hoareau
769 CM, Vanier G, Urb M, Campoli P, Al Abdallah Q, Lehoux M, Chabot JC, Ouimet MC,
770 Baptista SD, Fritz JH, Nierman WC, Latge JP, Mitchell AP, Filler SG, Fontaine T,
771 Sheppard DC. 2013. *Aspergillus* galactosaminogalactan mediates adherence to host
772 constituents and conceals hyphal beta-glucan from the immune system. PLoS Pathog
773 9:e1003575.
774 41. Goodridge HS, Reyes CN, Becker CA, Katsumoto TR, Ma J, Wolf AJ, Bose N, Chan AS,
775 Magee AS, Danielson ME, Weiss A, Vasilakos JP, Underhill DM. 2011. Activation of the
776 innate immune receptor Dectin-1 upon formation of a 'phagocytic synapse'. Nature
777 472:471-5.

778 42. Huang H, Ostroff GR, Lee CK, Agarwal S, Ram S, Rice PA, Specht CA, Levitz SM. 2012.
779 Relative contributions of dectin-1 and complement to immune responses to particulate
780 beta-glucans. J Immunol 189:312-7.
781 43. Qi C, Cai Y, Gunn L, Ding C, Li B, Kloecker G, Qian K, Vasilakos J, Saijo S, Iwakura Y,
782 Yannelli JR, Yan J. 2011. Differential pathways regulating innate and adaptive antitumor
783 immune responses by particulate and soluble yeast-derived beta-glucans. Blood
784 117:6825-36.
785 44. Tam JM, Reedy JL, Lukason DP, Kuna SG, Acharya M, Khan NS, Negoro PE, Xu S, Ward
786 RA, Feldman MB, Dutko RA, Jeffery JB, Sokolovska A, Wivagg CN, Lassen KG, Le Naour
787 F, Matzaraki V, Garner EC, Xavier RJ, Kumar V, van de Veerdonk FL, Netea MG, Miranti
788 CK, Mansour MK, Vyas JM. 2019. Tetraspanin CD82 Organizes Dectin-1 into Signaling
789 Domains to Mediate Cellular Responses to *Candida albicans*. J Immunol 202:3256-3266.
790 45. Griffiths JS, White PL, Czubala MA, Simonazzi E, Bruno M, Thompson A, Rizkallah PJ,
791 Gurney M, da Fonseca DM, Naglik JR, Ingram W, Wilson K, van de Veerdonk FL, Barnes
792 R, Taylor PR, Orr SJ. 2021. A Human Dectin-2 Deficiency Associated With Invasive
793 Aspergillosis. J Infect Dis 224:1219-1224.
794 46. Loures FV, Rohm M, Lee CK, Santos E, Wang JP, Specht CA, Calich VL, Urban CF,
795 Levitz SM. 2015. Recognition of *Aspergillus fumigatus* hyphae by human plasmacytoid
796 dendritic cells is mediated by dectin-2 and results in formation of extracellular traps. PLoS
797 Pathog 11:e1004643.
798 47. Fontaine T, Delangle A, Simenel C, Coddeville B, van Vliet SJ, van Kooyk Y, Bozza S,
799 Moretti S, Schwarz F, Trichot C, Aebi M, Delepierre M, Elbim C, Romani L, Latge JP.
800 2011. Galactosaminogalactan, a new immunosuppressive polysaccharide of Aspergillus
801 fumigatus. PLoS Pathog 7:e1002372.

802 48. Barrett NA, Maekawa A, Rahman OM, Austen KF, Kanaoka Y. 2009. Dectin-2 recognition
803 of house dust mite triggers cysteinyl leukotriene generation by dendritic cells. J Immunol
804 182:1119-28.
805 49. Clarke DL, Davis NH, Campion CL, Foster ML, Heasman SC, Lewis AR, Anderson IK,
806 Corkill DJ, Sleeman MA, May RD, Robinson MJ. 2014. Dectin-2 sensing of house dust
807 mite is critical for the initiation of airway inflammation. Mucosal Immunol 7:558-67.
808 50. Norimoto A, Hirose K, Iwata A, Tamachi T, Yokota M, Takahashi K, Saijo S, Iwakura Y,
809 Nakajima H. 2014. Dectin-2 promotes house dust mite-induced T helper type 2 and type
810 17 cell differentiation and allergic airway inflammation in mice. Am J Respir Cell Mol Biol
811 51:201-9.
812 51. Parsons MW, Li L, Wallace AM, Lee MJ, Katz HR, Fernandez JM, Saijo S, Iwakura Y,
813 Austen KF, Kanaoka Y, Barrett NA. 2014. Dectin-2 regulates the effector phase of house
814 dust mite-elicited pulmonary inflammation independently from its role in sensitization. J
815 Immunol 192:1361-71.
816
817

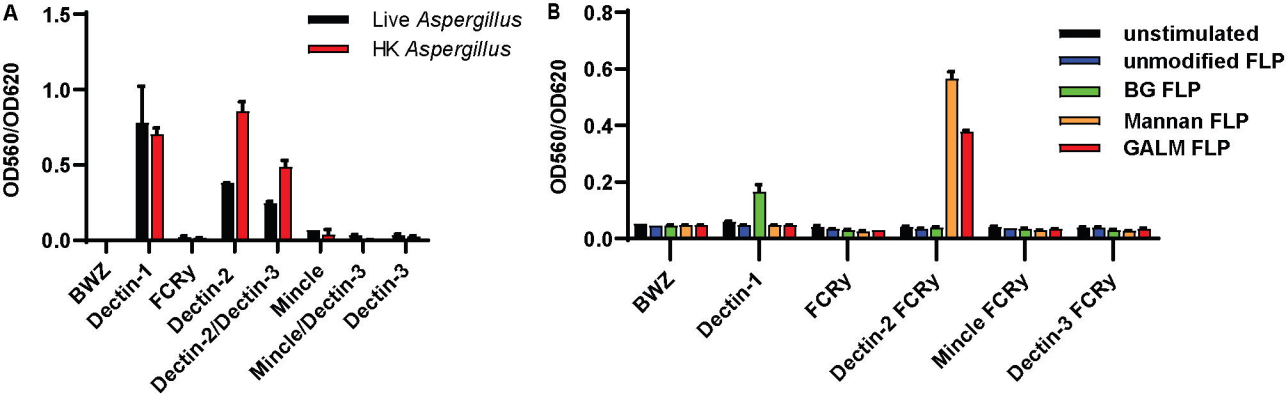

A
OD560/OD620
1.5
1.0
0.5
0.0
Live Aspergillus
HK Aspergillus
BWZ
Dectin-1
FCRγ
Dectin-2
Dectin-2/Dectin-3
Mincle
Mincle/Dectin-3
Dectin-3
B
OD560/OD620
0.8
0.6
0.4
0.2
0.0
unstimulated
unmodified FLP
BG FLP
Mannan FLP
GALM FLP
BWZ
Dectin-1
FCRγ
Dectin-2 FCRγ
Mincle FCRγ
Dectin-3 FCRγ

Figure 1

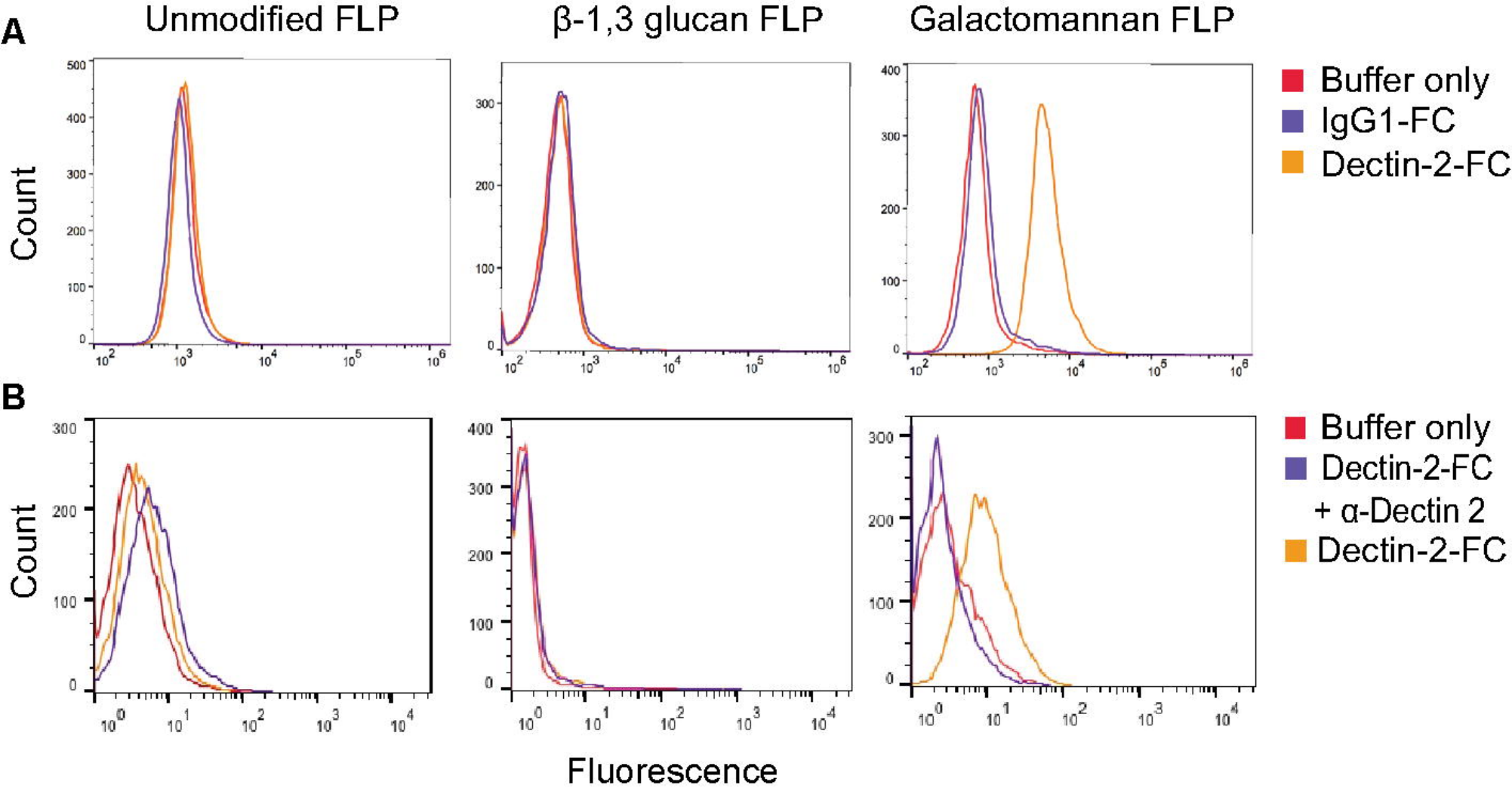
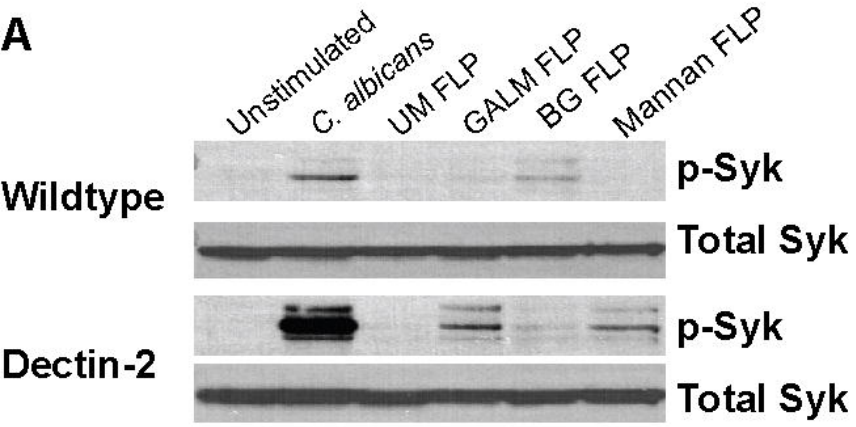
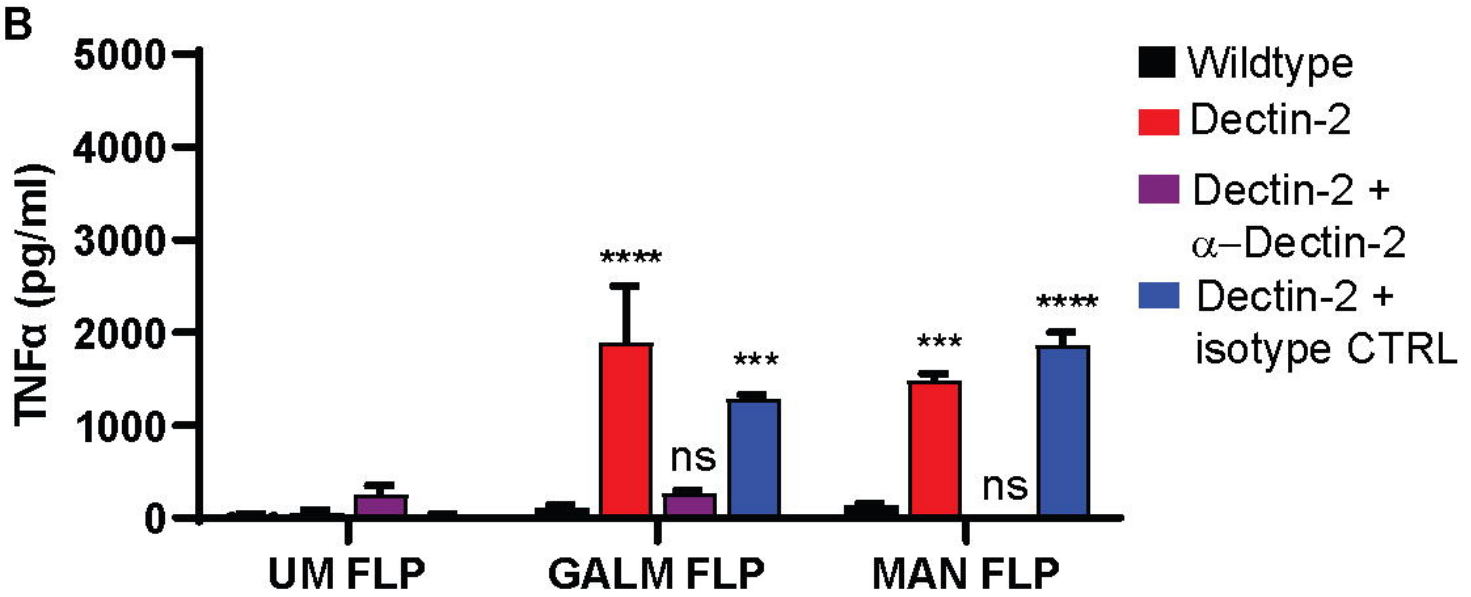

A
Unmodified FLP
β-1,3 glucan FLP
Galactomannan FLP
Count
Buffer only
IgG1-FC
Dectin-2-FC
B
Count
Buffer only
Dectin-2-FC
+ α-Dectin 2
Dectin-2-FC
Fluorescence

Figure 2


A
Unstimulated
C. albicans
UM FLP
GALM FLP
BG FLP
Mannan FLP
Wildtype
p-Syk
Total Syk
Dectin-2
p-Syk
Total Syk


B
TNFα (pg/ml)
5000
4000
3000
2000
1000
0
****
***
ns
***
ns
****
UM FLP
GALM FLP
MAN FLP
Wildtype
Dectin-2
Dectin-2 + α–Dectin-2
Dectin-2 + isotype CTRL

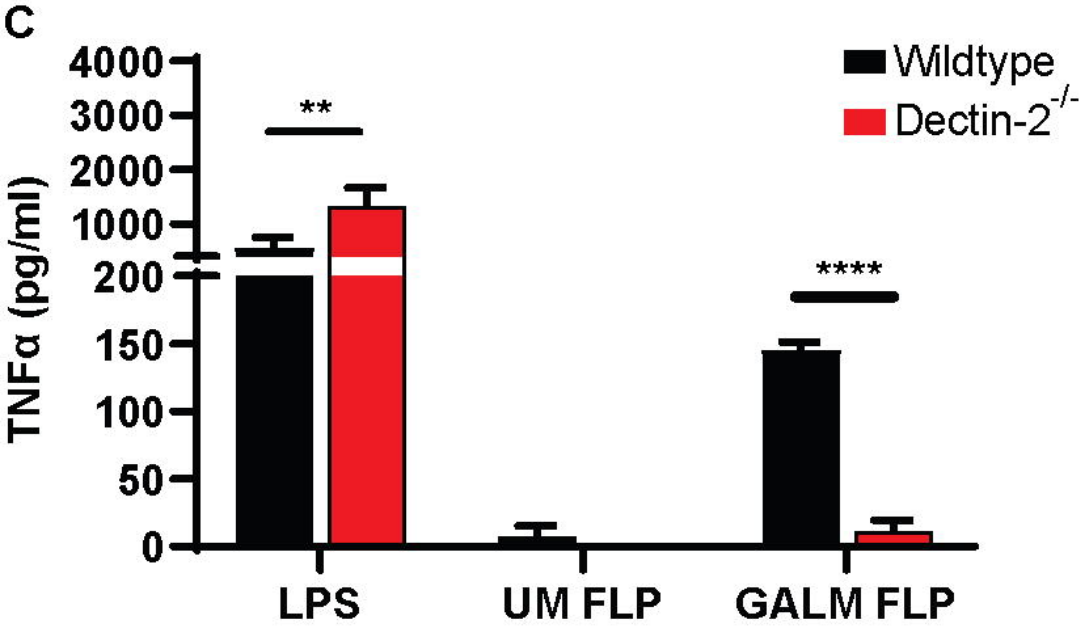
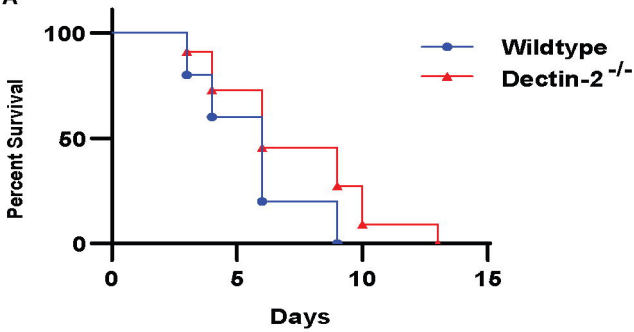
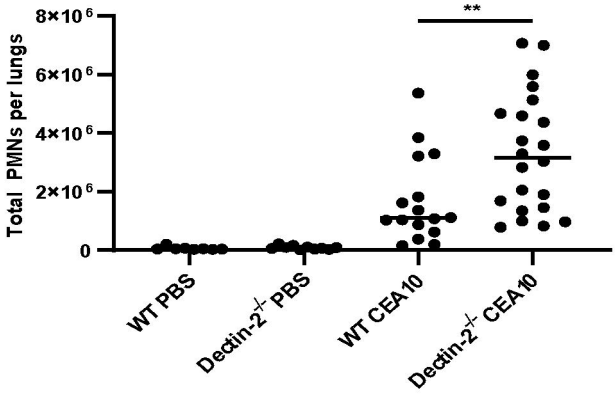

C
TNFα (pg/ml)
4000
3000
2000
1000
200
150
100
50
0
**
****
LPS
UM FLP
GALM FLP
Wildtype
Dectin-2-/-

Figure 3

A


100
50
0
Percent Survival
0
5
10
15
Days
Wildtype
Dectin-2 -/-

B


8×10 6
6×10 6
4×10 6
2×10 6
0
Total PMNs per lungs
**
WT PBS
Dectin-2 -/- PBS
WT CEA10
Dectin-2 -/- CEA10

C

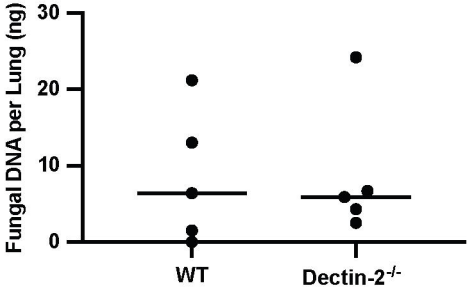

30
20
10
0
Fungal DNA per Lung (ng)
WT
Dectin-2 -/-

Figure 4