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

Jennifer L. Reedy[a,b], Arianne J. Crossen[a], Paige E. Negoro[a]*, Hannah E. Brown[a], Rebecca A. Ward[a], Diego A. Vargas Blanco[a], Kyle D. Timmer[a], Michael K. Mansour[a,b], Marcel Wüthrich[c], Thierry Fontaine[d], Jean-Paul Latgé[e], and Jatin M. Vyas[a,b,#].

[a]Division of Infectious Diseases, Department of Medicine, Massachusetts General Hospital, Boston, Massachusetts 02114

[b]Harvard Medical School, Department of Medicine, Boston, Massachusetts 02115

[c]Department of Pediatrics, University of Wisconsin Medical School, University of Wisconsin Hospital and Clinics, Madison, WI, 53792

[d]Institut Pasteur, Université de Paris, INRAE, USC2019, Unité Biologie et Pathogénicité Fongiques, F-75015, Paris

[e]Institute of Molecular Biology and Biotechnology (IMBBFORTH), University of Crete, Heraklion, Greece

Running Headline: Dectin-2 is a receptor for *Aspergillus* galactomannan

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

*Present address: Loyola University Chicago, Stritch School of Medicine, Maywood, IL

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

**Word Count: Abstract: 210 words, Importance: 148 Text: 4798**

**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 immune system and its consequences. 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 highly purified *Aspergillus* galactomannan to identify a C-type lectin host receptor for this fungal carbohydrate. We identified a novel and specific 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 TNF$\alpha$ production. Deficiency of Dectin-2 increased immune cell 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 galactomannan which leads to a pro-inflammatory immune response in the lung.

**IMPORTANCE:**

*Aspergillus fumigatus* is a fungal pathogen that causes serious and often fatal disease in humans. The surface of *Aspergillus* is composed of complex sugar molecules. Recognition of these carbohydrates by immune cells by carbohydrate lectin receptos can lead to clearance of the infection or, in some cases, benefit the fungus by dampening the host response. Galactomannan is a carbohydrate that is part of the cell surface of *Aspergillus* but is also released during infection and is found in patient lungs as well as their bloodstreams. The significance of our research is that we have identified a mammalian immune cell receptor that recognizes, binds, and signals in response to galactomannan. These results enhance our understanding of how this carbohydrate interacts with the immune system at the site of infection and will lead to broader understanding of how release of galactomannan by *Aspergillus* effects the immune response in infected patients.

58 **INTRODUCTION**

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

69 Humans are infected with *A. fumigatus* through inhalation of conidia deposited
70 within small airways and alveoli. Conidia are eliminated in part by mechanical expulsion
71 through the action of mucus and ciliated cells, as well as killing by macrophages and
72 neutrophils (5, 6). Upon encountering a fungal pathogen, innate immune cells
73 synchronously engage multiple fungal cell wall antigens through a diverse array of
74 pattern recognition receptors including Toll-like receptors, scavenger receptors,
75 complement receptors, and C-type lectin receptors (CLRs), and integrate those signals
76 to generate a coordinated immune response (7-11). The majority of the fungal cell wall
77 antigens are polysaccharides, as > 90% of the fungal cell wall is composed of complex
78 carbohydrates. The cell wall is commonly composed of an inner chitin and middle β-1,3
79 glucan layer with additional carbohydrate constituents like an outer mannan layer
80 depending upon the genus of fungus (12, 13). In *A. fumigatus*, the mannan content is

81 primarily exists within the galactomannan polymer. Although specific cell wall
82 carbohydrate–pattern recognition receptor interaction pairings have been defined for a
83 few cell wall polysaccharides, such as β-1,3 glucan and the CLR Dectin-1 (8, 14), we
84 lack a fundamental understanding of the specific receptors that engage many other
85 fungal carbohydrates and how these ligand-receptor cognate pairs drive the innate
86 immune response.

87 The fungal cell wall is a highly dynamic structure that changes in both structure
88 and composition depending on the morphological stage, environmental milieu, and upon
89 engagement with immune cells (13, 15). Therefore, probing the role of a single
90 carbohydrate entity using whole pathogens is challenging. Standard techniques have
91 relied upon solubilized carbohydrates or genetic deletion mutants, however these
92 techniques have limitations including pleiotropic changes that occur in genetic deletion
93 strains, such as compensatory changes in cell wall composition or structure, growth
94 defects, alterations in antigen exposure (16), and known differences in immune
95 response to soluble versus particulate (or cell-bound) carbohydrates. Most fungal cell
96 wall carbohydrates remain bound to the cell wall, however galactomannan and β-1,3
97 glucan are unique as they exist as both cell wall-associated and free/soluble
98 carbohydrates. β-1,3 glucan has been extensively studied revealing differences in how
99 soluble and cell wall β-1,3 glucan interact with immune cells (17-19). Cell wall
100 associated β-1,3 glucan signals via both the CLR Dectin-1 and complement receptor 3,
101 whereas soluble β-1,3 glucan is Dectin-1 independent (17-19). Although both cell wall
102 β-1,3 glucan and soluble β-1,3 glucan bind Dectin-1, only cell wall β-1,3 glucan
103 activates signaling (17). In contrast, receptors such as TLRs signal in response to both

104 soluble and cell associated ligands. Activation of Dectin-1 requires receptor clustering
105 into phagocytic synapses which exclude regulatory phosphatases licensing activation of
106 Dectin-1 (17, 20). Whether the difference in response to cell wall and soluble
107 carbohydrates is unique to β-1,3 glucan and Dectin-1 or extends to other fungal
108 carbohydrates and CLRs remains unknown. We hypothesize that the innate immune
109 system distinguishes between soluble and cell-associated fungal carbohydrates to tailor
110 the immune response. Most studies to date have been conducted using soluble
111 galactomannan. Thus, we sought to characterize the immune response to cell-
112 associated galactomannan to understand how innate immune cells interact with
113 *Aspergillus* at the site of infection.

114 The carbohydrate galactomannan was identified approximately 40 years ago as
115 an important clinical biomarker for the diagnosis of invasive aspergillosis, as it can be
116 detected in serum and bronchial fluid from infected patients (21-24). However, the
117 biological and immunological responses to this carbohydrate remain poorly defined.
118 Galactomannan is composed of a linear mannan core composed of α-1,2-linked
119 mannotetraose units attached via a-1,6-linkage with side changes composed of four to
120 five β-1,5-linked galactofuranose residues (25, 26). Galactomannan can be found in
121 both the conidial and mycelial stages of the *Aspergillus* lifecycle. It is bound to the cell
122 membrane through a GPI-anchor, covalently attached to β-1,3-glucan in the cell wall
123 and also released as a soluble polysaccharide into the extracellular milieu (27, 28).
124 Most studies have utilized soluble galactomannan, since strains of *Aspergillus* that lack
125 galactomannan have altered growth morphology and kinetics (29, 30). Stimulation of
126 peripheral blood mononuclear cells (PBMCs) with soluble galactomannan suppressed

127 LPS-induced cytokine production. Furthermore, an *Aspergillus* vaccine protection model
128 demonstrated that treatment with soluble galactomannan induced a non-protective
129 Th2/Th17 immunity (31, 32). Dendritic cell and macrophages internalization of *A.*
130 *fumigatus* conidia by the C-type Lectin Receptor DC-SIGN could be inhibited through
131 the addition of soluble galactomannan (33), suggesting that DC-SIGN could play a role
132 in recognizing this carbohydrate. These observations indicated that *Aspergillus* subverts
133 the immune response by shedding galactomannan, but the innate immune response to
134 cell-associated galactomannan has yet to be determined.

135 In this study, we identified Dectin-2 as a receptor for cell wall-associated
136 galactomannan by using fungal-like particles (FLPs), a novel technique previously
137 developed in our laboratory (34). To determine how the immune system recognizes
138 galactomannan in the cell-associated state, we isolated galactomannan from *A.*
139 *fumigatus* and created FLPs consisting of purified galactomannan. Using these particles
140 to screen a library of CLR reporter cells, we identified Dectin-2 as a receptor for
141 galactomannan. We confirmed the binding of Dectin-2 to galactomannan FLPs, using a
142 solubilized receptor and demonstrated galactomannan FLPs stimulate production of
143 TNF$\alpha$ by murine macrophages in a Dectin-2 dependent manner. Furthermore, we
144 determined that immunocompetent mice lacking Dectin-2 recruit increased numbers of
145 immune cells to lungs compared with wild-type mice in a model of pulmonary
146 aspergillosis, but Dectin-2 is dispensable for survival. Taken together, our results
147 demonstrate a novel and important role for Dectin-2 in innate immune recognition of
148 *Aspergillus* galactomannan.

149 **RESULTS**

150 ***Dectin-2 is a receptor for galactomannan***

151 Since CLRs are known to be involved in sensing of fungal pathogens and bind to
152 carbohydrates, we examined if any of the known CLRs were potential receptors for
153 galactomannan in the *Aspergillus* cell wall. We first screened a murine CLR library using
154 *A. fumigatus* germlings to determine which CLRs were stimulated by the whole
155 organism and confirm utility of the assay. T cell hybridomas containing an NFAT-lacZ
156 reporter were engineered to express individual or combinations of CLRs: Dectin-1,
157 Dectin-2, Dectin-3 (MCL), or Mincle. Dectin-2, Dectin-3, and Mincle require interaction
158 with the gamma subunit of the Fc gamma receptor (FcR$\gamma$) for both cell surface
159 localization and signaling, thus this protein was co-expressed. We stimulated the CLR
160 reporter cells for 18 hours with live and heat-killed *Aspergillus* germlings and observed
161 robust activation of Dectin-1, Dectin-2, and Dectin-2/Dectin-3 co-expressing cells
162 (Figure 1A). Since cells that express Dectin-3 alone were not activated, the activation of
163 the Dectin-2/Dectin-3 co-expressing cells is likely due to either activation of Dectin-2
164 alone or possible Dectin-2/Dectin-3 in combination (Figure 1A). Activation of Dectin-1 is
165 known to occur through binding of cell-wall associated $\beta$-1,3 glucan, but the Dectin-2
166 ligand on the surface of *Aspergillus* has not been established. We hypothesized that
167 galactomannan could be the potential Dectin-2 ligand.

168 To dissect individual carbohydrate contributions to CLR reporter cell activation,
169 we employed a technique developed in our laboratory for creating FLPs that consist of
170 polystyrene beads coated with a discrete, purified fungal carbohydrates (34). We have
171 previously generated FLPs made from purified $\beta$-1,3 glucan and demonstrated that

172 carbohydrate-coated FLPs mimic cell-associated or particulate carbohydrates. These β-
173 1,3 glucan FLPs can activate Dectin-1 signaling and elicit the formation of a phagocytic
174 synapse (20, 34, 35). Generation of galactomannan FLPs was performed using
175 methods previously described for the creation of β-1,3 glucan and mannan FLPs (34)
176 (Supplemental Methods). All generated FLPs underwent rigorous quality control using
177 flow cytometry to demonstrate the stable attachment of carbohydrates to the FLP
178 surface (Figure S1). As an added control, multiple independent purifications of the
179 galactomannan carbohydrate were used for FLP creation for all experiments and
180 yielded similar results. We stimulated the reporter cell library with unmodified beads, β-
181 1,3 glucan FLP, mannan FLP, and galactomannan FLP. Activation of CLR signaling
182 resulted in expression of a LacZ reporter and was quantified using a CPRG assay to
183 measure β-galactosidase activity. As expected, β-1,3 glucan FLPs activated Dectin-1
184 expressing reporter cells and *S. cerevisiae* mannan FLPs stimulated Dectin-2
185 expressing reporter cells. The galactomannan FLPs also stimulated Dectin-2 (Figure
186 1B) and Dectin-2/Dectin-3 co-expressing cells (data not shown), suggesting that not
187 only did galactomannan bind to Dectin-2, but also triggered signaling by the CLR. None
188 of our FLPs stimulated Mincle or Dectin-3 signaling alone, however these cells were
189 activated by incubation with trehalose-6,6-dibehenate (TDB) (Figure S2) demonstrating
190 that the receptors are functional, and that absence of observed stimulation is due to lack
191 of CLR activation by the galactomannan FLPs. These data suggested that Dectin-2, but
192 not Dectin-3 or Mincle, is a receptor for *Aspergillus*-derived galactomannan.
193
194 ***Soluble Dectin-2 binds to galactomannan FLPs***

195 After identifying Dectin-2 as a receptor for galactomannan using the CLR reporter cell
196 screen, we next assessed whether Dectin-2 binds directly to galactomannan FLP. To
197 evaluate this, we used soluble murine Dectin-2-human IgG1 Fc fusion protein and a
198 control human IgG1 Fc protein (lacking Dectin-2). Flow cytometry using an anti-human
199 IgG1 Fc antibody and an AF488 labeled secondary antibody was used to detect
200 adherence of Dectin-2-Fc to the surface of FLP (Figure 2A). Dectin-2-Fc was
201 specifically bound to both *Aspergillus* galactomannan FLPs and *Saccharomyces*
202 mannan FLPs but did not bind to either unmodified or β-1,3 glucan FLPs. As expected,
203 human IgG1 Fc protein did not bind to any FLP. As an additional control to demonstrate
204 that binding to the galactomannan FLPs was Dectin-2 specific, we incubated the Dectin-
205 2-Fc protein with anti-Dectin-2 neutralizing antibody or isotype control for 30 minutes
206 prior to incubation with galactomannan FLPs. The Dectin-2 neutralizing antibody, but
207 not the isotype control, potently blocked binding of Dectin-2-Fc to galactomannan FLP
208 demonstrating a specific interaction (Figure 2B).
209
210 ***Galactomannan FLPs induce Dectin-2 dependent Syk activation***
211 The CLR reporter assay and binding studies demonstrated that Dectin-2 is capable of
212 binding *Aspergillus* galactomannan. Next, we investigated whether this binding results
213 in downstream signaling. Activation of Dectin-2 through ligand binding triggers
214 phosphorylation of the ITAM motif of FcRγ and subsequent signaling through Syk. FcRγ
215 is also required for localization of Dectin-2 to the cell surface (36). Macrophages
216 express Dectin-2, however immortalized C57BL/6 murine macrophages have low levels
217 of Dectin-2 surface expression at baseline (data not shown). Therefore, we transduced

218 macrophages with lentivirus containing the murine Dectin-2 receptor (Supplemental
219 Methods). As macrophages produce abundant FCRγ, we did not observe any difference
220 in Dectin-2 surface expression between macrophages transduced with only Dectin-2 or
221 those transduced with both Dectin-2 and FCRγ. To determine if galactomannan is
222 sufficient to trigger Dectin-2 dependent Syk activation, we stimulated macrophages with
223 galactomannan FLPs for 1 hour as well as unmodified FLPs, β-1,3 glucan FLPs,
224 mannan FLPs, and *C. albicans.* We used *C. albicans* as a positive control in these
225 experiments since it is a strong activator of Syk phosphorylation in wild-type
226 macrophages. Lysates from stimulated cells were immunoblotted for phosphorylated
227 and total Syk. As expected, we observed phosphorylation of Syk when wildtype
228 macrophages were stimulated by *Candida albicans* and β-1,3 glucan FLPs (positive
229 controls for Syk stimulation) (Figure 3A). There was increased phosphorylation of Syk in
230 response to both galactomannan and mannan in Dectin-2 expressing macrophages
231 demonstrating that *Aspergillus* galactomannan is sufficient to drive Dectin-2 dependent
232 Syk phosphorylation (Figure 3A).
233
234 ***Dectin-2 mediates TNFα production by murine macrophages in response to both***
235 **A. fumigatus *and galactomannan FLPs***
236 Having demonstrated that galactomannan is sufficient to activate Dectin-2 dependent
237 Syk signaling, we next interrogated the downstream effects of this activation.
238 Specifically, we sought to determine if galactomannan stimulation of Dectin-2 is both
239 sufficient and necessary for cytokine production. To determine if galactomannan
240 stimulation of Dectin-2 is sufficient to trigger cytokine production, Dectin-2 expressing

241 murine macrophages were stimulated overnight with unmodified FLPs, β-1,3 glucan
242 FLPs, *A. fumigatus* galactomannan FLPs, or *S. cerevisiae* mannan FLPs (positive
243 control) and TNFα production was measured by ELISA. Galactomannan and mannan
244 FLPs enhanced TNFα production in Dectin-2 expressing macrophages (Figure 3A). To
245 demonstrate that this effect was specific to Dectin-2, we pre-incubated the
246 macrophages with a Dectin-2 neutralizing antibody or an isotype control for 30 minutes
247 prior to stimulation with FLPs. TNFα production was blocked by the Dectin-2 specific
248 antibody, but not by the isotype control, demonstrating a specific role for Dectin-2 in
249 mediating the TNFα production in response to galactomannan (Figure 3B).
250 To examine if Dectin-2 is required for primary BMDMs to respond to
251 galactomannan FLPs, primary BMDM from wildtype or Dectin-2 deficient mice were
252 stimulated by galactomannan FLPs, unmodified FLPs (negative control), or LPS
253 (positive control). There was near complete reduction of TNFα production in response
254 to galactomannan FLPs (Figure 3C), indicating that galactomannan triggers the
255 secretion of pro-inflammatory cytokines in BMDMs in a Dectin-2 dependent manner.
256

257 ***Deletion of Dectin-2 results in increased recruitment of immune cells, but does***
258 ***not alter fungal burden***

259 We sought to determine if the immune cell influx in response to infection was altered in
260 the absence of Dectin-2. Since treatment with steroids affects immune cell function, we
261 chose to perform this experiment in an immunocompetent, rather than a steroid-induced
262 immunosuppression mouse model. Immunocompetent mice were administered either *A.*
263 *fumigatus* strain CEA10 or PBS via oropharyngeal aspiration. After 48 hours of infection

264 lungs were harvested, digested, and the resulting single cell suspension was enriched
265 for leukocytes using a Percoll gradient. A multi-channel flow cytometry experiment was
266 performed to allow simultaneous assessment of multiple cell lines including neutrophils,
267 eosinophils, T cells, B cells, dendritic cells (including cDC1, cDC2, monocyte derived
268 DCs, and plasmacytoid dendritic cells [pDCs]), NK cells, monocytes, and alveolar
269 macrophages. All infected mice demonstrated a marked increase in $CD45^+$ cellular
270 infiltrates compared to the PBS control mice. We infected mice with $1 \times 10^7$, $2 \times 10^7$ and
271 $4 \times 10^7$ conidia of CEA10 *A. fumigatus* and noted a dose-dependent increase in immune
272 cell infiltrate. At doses higher than $4 \times 10^7$ most mice succumbed within 24 – 48 hours,
273 presumably due to an exuberant inflammatory response. Infected Dectin-$2^{-/-}$ mice
274 recruited more $CD45^+$ cells to the lungs than did infected wild-type mice. Additionally,
275 Dectin-$2^{-/-}$ infected mouse lungs had a significant increase in innate cells (Figure 4B,
276 Supplemental Figure S3), as well as neutrophils compared to wild-type (Figure 4C).
277 Notably, the proportion of the cellular infiltrate comprised of innate cells (Figure 4D) and
278 neutrophils (Figure 4E) was not significantly different between infected wild-type and
279 Dectin-$2^{-/-}$ mice suggesting that there is increased immune cell recruitment to the lungs
280 during infection in Dectin-$2^{-/-}$ mice, but no change in the overall distribution of innate cell
281 and neutrophil populations. Our results suggest that Dectin-2 is involved in the
282 coordination of inflammatory responses in this airway model of infection.

283 To determine whether the increased influx of $CD45^+$ cells could be the result of
284 difference in fungal burden between wild-type and Dectin-2 deficient mice, we quantified
285 fungal burden in the lungs of mice infected with $4 \times 10^7$ conidia at 48 hours post-
286 infection using qPCR to detect and quantify *Aspergillus* DNA(37). *Aspergillus fumigatus*

287 DNA was detected in all samples from infected mice, but not in PBS controls. There
288 was no difference in *Aspergillus* DNA between wild-type and Dectin-2 deficient mice
289 (Figure 4F). CFU assays were also performed as a confirmatory test to ensure that
290 there was no difference in live *Aspergillus* fungal burden, since fungal DNA testing
291 cannot distinguish between live versus dead organisms. Lungs from both wild-type and
292 Dectin-2 deficient mice still contained live *Aspergillus fumigatus* at 48 hours, and there
293 was no difference in CFU between strains (Figure 4G). Taken together these results
294 suggest that the enhanced immune cell infiltration in Dectin-2 deficient mice is not due
295 to a difference in fungal burden.
296
297 ***Dectin-2 is dispensable for survival in a murine model of infection***
298 Our data suggest that Dectin-2 is a receptor for *A. fumigatus* galactomannan, and that
299 recognition of galactomannan by Dectin-2 results in Syk phosphorylation, cytokine
300 production and increased inflammatory cell recruitment in Dectin-2$^{-/-}$ mice infected with
301 *A. fumigatus*. To determine if this pathway is required for survival during *Aspergillus*
302 infection, we intranasally infected corticosteroid immunosuppressed Dectin-2$^{-/-}$ and wild-
303 type C57BL/6 mice with *A. fumigatus* (CEA10 strain) and monitored the mice for signs
304 of infection. There was no difference in survival between wild-type and Dectin-2
305 deficient mice, suggesting that in this model, Dectin-2 is dispensable for survival (Figure
306 4H).
307

308 **DISCUSSION**

309 Galactomannan is a clinically important polysaccharide released into patients' blood and
310 bronchial lavage fluid and used to diagnose invasive aspergillosis infections. However,
311 we do not understand the immunological implications of the wide-scale release of this
312 carbohydrate into patient tissue, or the role that it plays in mediating direct
313 immunological interactions between *A. fumigatus* and innate immune cells at the site of
314 infection. Here, we utilized FLPs coated with purified *A. fumigatus* galactomannan to
315 identify Dectin-2 as a receptor for galactomannan. Our results demonstrate that cell-
316 associated galactomannan binds Dectin-2 inducing phosphorylation of Syk and TNF$\alpha$
317 production.

318 Although BMDMs from Dectin-2 deficient mice had decreased cytokine
319 production in response to galactomannan FLPs, there was no difference in overall
320 survival of immunosuppressed Dectin-2 deficient mice compared with wild-type mice
321 when challenged intranasally with *A. fumigatus*. There is significant redundancy in CLRs
322 signaling, therefore it is possible that the lack of Dectin-2 is compensated for through
323 Dectin-1 or other CLR-ligand interactions in the context of the whole organism.
324 However, publications have suggested a critical role for Dectin-2 in the immune
325 response to *A. fumigatus(38, 39)*. A recent case report identified a patient without
326 previously recognized immunodeficiency that developed invasive aspergillosis and was
327 found to have a mutation in Dectin-2 that led to decreased responsiveness to
328 *Aspergillus* (38). Previous work has also demonstrated a role for Dectin-2 in mediating
329 plasmacytoid dendritic cell (pDC) responses to *A. fumigatus* (39). These observations,
330 coupled with this study, suggest that while Dectin-2 may not be required for survival in a

331 murine model, it may contribute to human disease through modulating interactions with
332 discrete immune cell populations.
333 Interestingly, immunocompetent mice that lacked Dectin-2 have increased
334 recruitment of immune cells into the lung tissue upon infection with *Aspergillus*, which is
335 in sharp contrast to galactosaminogalactan (GAG) from *A. fumigatus* which led to a
336 reduction of neutrophil infiltration (40). The difference in inflammatory cell infiltrates was
337 not due to difference in the fungal burden within the lungs, suggesting that it is a direct
338 result of Dectin-2 deficiency. This result is interesting, as our data also demonstrated
339 that galactomannan-induced Dectin-2 signaling in macrophages leads to pro-
340 inflammatory TNF$\alpha$ release. We anticipated that the absence of Dectin-2 would result in
341 decreased pulmonary inflammation, but surprisingly observed increased immune cell
342 recruitment. Our results suggest that Dectin-2 may normally temper inflammatory cell
343 recruitment to the lungs, or that Dectin-2 signaling normally leads to increased
344 inflammatory cell turnover. There are multiple potential hypotheses to explain these
345 findings which will provide fertile ground for future studies. In the absence of Dectin-2,
346 expression of Dectin-1 and/or other C-type Lectin Receptors may be upregulated and
347 thus these receptors may play a more substantial role in driving the immune response.
348 Additionally, Dectin-2 is expressed not just by macrophages, but also by many immune
349 cells and other lung resident cells, including epithelial cells. Thus, a cell type other than
350 macrophages, may be responsible for mediating the difference in immune cell influx that
351 we observed. For instance, studies by Loures, *et al* (39) demonstrated that
352 plasmacytoid dendritic cell production of type I IFNs in response to *Aspergillus*
353 *fumigatus* is dependent upon Dectin-2 mediated recognition of *Aspergillus fumigatus*.

354 While we did not observe a difference in pDC recruitment to the lungs in wild-type
355 compared to Dectin-2 deficient mice, these cells are likely impaired in their ability to
356 produce type I IFNs during infection. Interestingly, the increased immune cell influx
357 observed in Dectin-2 deficient mice did not affect fungal burden suggesting, that the
358 increased immune cells infiltrate did not result in more rapid clearance of infection.
359 Whether the increased inflammatory influx seen in Dectin-2 deficient mice could have
360 detrimental effects on the host, such as increasing lung damage during infection is
361 unknown.

362 Our current work addresses the mechanism by which cell-wall associated
363 galactomannan is recognized by Dectin-2. Galactomannan and β-1,3 glucan are both
364 fungal cell wall carbohydrates that exists in both soluble and cell-wall associated states.
365 β-1,3 glucan is known to interact with the immune system differently depending upon its
366 form. Whether galactomannan-induced immune activation is triggered only at sites of
367 infection or more systemically and how this could impact the outcome of infection
368 remain critically important questions. Whether circulating soluble galactomannan can
369 stimulate recognition by Dectin-2 will be the subject of future studies. Dectin-2 has also
370 been implicated in allergic disease (41-44), suggesting that galactomannan from
371 *Aspergillus* could play a role in stimulating allergic manifestations to this pathogen.
372 Overall, our data demonstrate that galactomannan is recognized by Dectin-2 and lays
373 the exciting groundwork for dissecting the role of galactomannan in an important clinical
374 disease.

375 **MATERIALS AND METHODS**

376 ***Animals***

377 Mice were maintained in specific-pathogen-free barrier facilities at Massachusetts
378 General Hospital (MGH; Boston, MA) according to Institutional Animal Care and Use
379 Committee (IACUC) Guidelines. Wildtype C57BL/6 mice were obtained from Jackson
380 Laboratories (Bar Harbor, ME) and Dectin-2$^{-/-}$ mice (45) were from the University of
381 Wisconsin-Madison. All mice used for bone marrow harvests and *in vivo* experiments
382 were 8 – 20 weeks of age and were co-housed prior to experiments. Mice were both
383 age and sex-matched for each experiment and an equal distribution of male and female
384 mice used.

385

386 ***Cell lines and culture***

387 Immortalized murine C57BL/6 bone marrow-derived macrophages (BMDM) were a gift
388 from Doug Golenbock (University of Massachusetts Medical School, Worcester, MA).
389 Macrophages were cultured in complete RPMI media (cRMPI; RPMI 1650, L-glutamine,
390 10% heat-inactivated fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific,
391 Rockford, IL), 1% penicillin/streptomycin, 1% HEPES buffer, and 50 μM of β-
392 mercaptoethanol). Puromycin and blastocidin were added to a final concentration of 5
393 μg/mL as needed for the selection of transduced cells. Primary BMDMs from C57BL/6
394 and Dectin-2$^{-/-}$ mice were harvested as previously described (35). After harvest, primary
395 BMDMs were grown for 7 days in complete RPMI media containing 20 ng/mL of
396 recombinant MCSF (Peprotech, Rocky Hill, NJ) prior to use in assays. HEK293T cells
397 were purchased from the ATCC (American Type Cell Collection, Manassas, VA) and

398 were grown in complete DMEM (DMEM, L-glutamine, 10% heat-inactivated FBS, 1%
399 penicillin/streptomycin, 1% HEPES buffer). All cell lines were grown at $37^0$C in the
400 presence of 5% $CO_2$.
401
402 ***Fungal culture***
403 *A. fumigatus* strains Af293 and CEA10 were grown at 37℃ for 5 days on Sabouraud's
404 Dextrose Agar (SBD; Difco, Fisher Scientific, Rockford, IL) or for 3 days on glucose
405 minimal media slants (GMM), respectively. Slants were seeded from frozen stocks of
406 conidia maintained at -80℃. To harvest conidia, a sterile solution of deionized water
407 (Milli-Q, Millipore Sigma, Burlington, MA) containing 0.01% Tween 20 was added to
408 each slant and spores were liberated using gentle surface agitation with a sterile swab.
409 The spore solution was passed through a 40 μm cell strainer to separate hyphal debris.
410 Spores were washed three times with sterile PBS and counted on a LUNA™ automated
411 cell counter (Logos Biosystems, Annandale, VA) and diluted appropriately. To generate
412 *A. fumigatus* germlings, conidia were incubated in cRPMI media at 37℃ for 4-6 hours
413 and visually inspected for the presence of emerging germ tubes. Germlings were then
414 washed, counted, and resuspended in PBS at the desired inoculum. For heat-killing, *A.*
415 *fumigatus* germlings were incubated at 95℃ for 30 minutes.
416
417 ***Purification of galactomannan***
418 The galactomannan was isolated as lipogalactomannan from the cellular membrane of
419 *A. fumigatus* mycelium that had been grown in SBD medium using previously described
420 methods (27). The lipid moiety was removed by nitrous deamination and the

421 polysaccharide moiety was purified by gel filtration chromatography on Superdex 75
422 column (10/300 GL, GE-Healthcare) (27). Three independent preparations of
423 galactomannan were used to create FLP and multiple batches were used per
424 experiment to confirm that results were not batch dependent.
425
426 ***FLP binding assays***
427 FLPs were blocked overnight in PBS containing 2% BSA. FLPs were washed and
428 resuspended in 100 mL of binding buffer (20 mM Tris-HCL, pH 7.4, 150 mM NaCl, 10
429 mM $CaCl_2$, 0.05% Tween-20). Dectin-2(murine)- IgG1 Fc (human) fusion protein (ENZO
430 Life Sciences, Inc, Farmingdale, NY) or IgG1 Fc (human) protein (Thermo Fisher
431 Scientific, Rockford, IL) was added to a final concentration of 10 mg/mL and samples
432 were incubated with gentle mixing for 2 hours at room temperature. Following washing,
433 samples were resuspended in binding buffer containing a 1:100 dilution of mouse anti-
434 human-IgG1-Fc conjugated to Alexa Fluor 488 (Thermo Fisher Scientific, Rockford, IL;
435 A-10631) and incubated for 30 minutes at room temperature in the dark. FLPs were
436 washed with binding buffer, resuspended in FACS buffer (PBS containing 2% BSA),
437 examined on a BD FACSCalibur™ or BD FACSCelesta™- Flow Cytometer (BD,
438 Franklin Lakes, NJ), and data analyzed using FlowJo 10. For neutralizing antibody
439 experiments, mouse anti-Dectin-2 antibody (R&D Systems, MAB1525) was
440 preincubated with the Dectin-2-Fc fusion protein at a concentration of 50 mg/ml for 30
441 minutes prior to adding the mixture to FLPs.
442
443 ***In vivo Aspergillus survival model***

444 Wild-type C57BL/6 mice or Dectin-2$^{-/-}$ mice were immunosuppressed with 40 mg/kg
445 subcutaneous triamcinolone acetonide (Kenalog®-10, Bristol-Myers Squibb, New York,
446 NY) starting the day prior to infection and every 7 days thereafter. The mice were
447 infected with 2.4 x$10^4$ CEA10 conidia intranasally under isoflurane anesthesia. For each
448 experiment 10 mice were infected with CEA10 and 3 mice were treated with PBS only
449 per genotype. Mice were monitored twice daily for the first 5 days of the experiment and
450 then daily thereafter. Mice were assessed based upon a 12-point scale to determine
451 when an individual animal should be euthanized due to illness and removed from the
452 study. This was developed in conjunction with the MGH IACUC and includes the
453 following symptoms and points assigned to each in parentheses: hunched posture (3),
454 ruffled and/or matted fur (3), shivering (3), abnormal breathing (increased respiratory
455 rate) (12), 75% reduction in activity compared to controls (3), inactivity leading to
456 inability to acquire food or water (12), and barrel rolling (12). If any animal reaches 12
457 points, they are humanely euthanized and counted as a death for the purposes of
458 survival analysis. All animal experiments were approved under the MGH IACUC
459 protocol #2008N000078.
460
461 ***Western blot***
462 Cells were placed on ice and lysed with 1% NP40 lysis buffer containing protease and
463 phosphatase inhibitors. Proteins were denatured using NuPAGE LDS Sample Buffer
464 (Thermo Fisher Scientific, Rockford, IL). The proteins were resolved by SDS-PAGE on
465 4-12% NuPAGE gels using NuPAGE MOPS buffer (NuPage gels, Thermo Fisher
466 Scientific, Rockford, IL), and transferred to methanol-activated PVDF membrane (Perkin

467 Elmer, Waltham, MA) using transfer buffer (0.025M Tris, 0.192 M glycine, 20%
468 methanol) and electrophoretic transfer at 100V for 1 hour. For detection of proteins,
469 PVDF membranes were blocked for 1 hour at room temperature in 5% BSA in PBS-
470 0.01% Tween 20 (PBST). Blots were incubated with anti-phospho spleen tyrosine
471 kinase (Syk) antibody (Cell Signaling Technologies, Danvers, MA; 2710S) or anti-total
472 Syk antibody (Cell Signaling Technologies, 13198S), in 5% BSA in PBST overnight at
473 4℃. The membranes were washed and incubated with secondary swine anti-rabbit HRP
474 conjugated antibody (Agilent DAKO, Santa Clara, CA, P0399) in 1% milk in PBST for 1
475 hour at room temperature. Membranes were washed and then visualized using Western
476 Lightning Plus ECL chemiluminescent substrate (Perkin Elmer, Waltham, MA) on Kodak
477 BioMax XAR film (MilliporeSigma, Burlington, MA). Films were then scanned and
478 processed using Adobe Photoshop 2021. Any contrast adjustments were applied evenly
479 to the entire image and adheres to standards set forth by the scientific community (46).

480 **ACKNOWLEDGEMENTS**

481 We thank all the members of the Vyas and Mansour lab at MGH and Dr. Vishukumar
482 Aimanianda, Institut Pasteur, for useful discussions and reading of the manuscript.

483

484 **AUTHOR CONTRIBUTIONS**

485 Conceptualization – Jennifer Reedy and Jatin Vyas. Investigation – Jennifer Reedy,
486 Arianne Crossen, Paige Negoro, Marcel Wuethrich, Kyle Timmer, and Hannah Brown.
487 Writing – Jennifer Reedy and Jatin Vyas, Manuscript review and revision – Arianne
488 Crossen, Paige Negoro, Kyle Timmer, Hannah Brown, Diego Vargas Blanco, Rebecca
489 Ward, Thierry Fontaine, Marcel Wüthrich, Michael Mansour, Jatin Vyas. Resources -
490 Thierry Fontaine, Marcel Wuethrich, Michael K. Mansour, and Jatin Vyas.

491

492

493 **FINANCIAL SUPPORT:** J.L.R is supported by NIH/NIAID grant 1K08AI14755,
494 NIH/NIAID PCTAS supplement, and KL2/Catalyst Medical Research Investigator
495 Training award (an appointed KL2 award) from Harvard Catalyst| The Harvard Clinical
496 Translational Science Center (National Center for Research Resources and the
497 National Center for Advancing Translational Sciences, National Institutes of Health
498 [NIH], award KL2 TR001100. J.M.V is supported by R01AI150181 and R01AI136529.
499 M.K.M. is supported by R01AI132638.

500

501

502

503 **SUPPLEMENTAL MATERIALS**

504 **Supplemental Methods:** Generation and quality control of FLP, Lentiviral transduction
505 of cells with Dectin-2 and FcRγ, CLR ligand reporter cell assay, ELISA,
506 Immunophenotyping of pulmonary infiltrates, quantification of fungal burden in lungs,
507 Colony forming unit (CFU)

508 **Figure S1:** Generation and validation of galactomannan FLP

509 **Figure S2:** CLR reporter cell activation using CLR control agonists

510 **Figure S3:** Flow cytometry gating strategy for lung immunophenotyping to identify
511 neutrophils.

512 REFERENCES
513


514 1. Arastehfar A, Carvalho A, van de Veerdonk FL, Jenks JD, Koehler P, Krause R,
515 Cornely OA, D SP, Lass-Florl C, Hoenigl M. 2020. COVID-19 Associated
516 Pulmonary Aspergillosis (CAPA)-From Immunology to Treatment. J Fungi (Basel)
517 6:91-108.

518 2. Schauwvlieghe A, Rijnders BJA, Philips N, Verwijs R, Vanderbeke L, Van Tienen
519 C, Lagrou K, Verweij PE, Van de Veerdonk FL, Gommers D, Spronk P,
520 Bergmans D, Hoedemaekers A, Andrinopoulou ER, van den Berg C, Juffermans
521 NP, Hodiamont CJ, Vonk AG, Depuydt P, Boelens J, Wauters J, Dutch-Belgian
522 Mycosis study g. 2018. Invasive aspergillosis in patients admitted to the intensive
523 care unit with severe influenza: a retrospective cohort study. Lancet Respir Med
524 6:782-792.

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

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

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

535 6. Ibrahim-Granet O, Philippe B, Boleti H, Boisvieux-Ulrich E, Grenet D, Stern M,
536 Latge JP. 2003. Phagocytosis and intracellular fate of *Aspergillus fumigatus*
537 conidia in alveolar macrophages. Infect Immun 71:891-903.
538 7. Ariizumi K, Shen GL, Shikano S, Ritter R, 3rd, Zukas P, Edelbaum D, Morita A,
539 Takashima A. 2000. Cloning of a second dendritic cell-associated C-type lectin
540 (dectin-2) and its alternatively spliced isoforms. J Biol Chem 275:11957-63.
541 8. Brown GD, Taylor PR, Reid DM, Willment JA, Williams DL, Martinez-Pomares L,
542 Wong SY, Gordon S. 2002. Dectin-1 is a major beta-glucan receptor on
543 macrophages. J Exp Med 196:407-12.
544 9. LeibundGut-Landmann S, Wuthrich M, Hohl TM. 2012. Immunity to fungi. Curr
545 Opin Immunol 24:449-58.
546 10. Levitz SM. 2010. Innate recognition of fungal cell walls. PLoS Pathog
547 6:e1000758.
548 11. Willment JA, Brown GD. 2008. C-type lectin receptors in antifungal immunity.
549 Trends Microbiol 16:27-32.
550 12. Latge JP. 2010. Tasting the fungal cell wall. Cell Microbiol 12:863-72.
551 13. Latge JP. 2016. 30 years of battling the cell wall. Med Mycol 55:4-9.
552 14. Brown GD, Gordon S. 2001. Immune recognition. A new receptor for beta-
553 glucans. Nature 413:36-7.
554 15. Latge JP, Beauvais A. 2014. Functional duality of the cell wall. Curr Opin
555 Microbiol 20:111-7.
556 16. Gravelat FN, Beauvais A, Liu H, Lee MJ, Snarr BD, Chen D, Xu W, Kravtsov I,
557 Hoareau CM, Vanier G, Urb M, Campoli P, Al Abdallah Q, Lehoux M, Chabot JC,

558 Ouimet MC, Baptista SD, Fritz JH, Nierman WC, Latge JP, Mitchell AP, Filler SG,
559 Fontaine T, Sheppard DC. 2013. *Aspergillus* galactosaminogalactan mediates
560 adherence to host constituents and conceals hyphal beta-glucan from the
561 immune system. PLoS Pathog 9:e1003575.
562 17. Goodridge HS, Reyes CN, Becker CA, Katsumoto TR, Ma J, Wolf AJ, Bose N,
563 Chan AS, Magee AS, Danielson ME, Weiss A, Vasilakos JP, Underhill DM. 2011.
564 Activation of the innate immune receptor Dectin-1 upon formation of a
565 'phagocytic synapse'. Nature 472:471-5.
566 18. Huang H, Ostroff GR, Lee CK, Agarwal S, Ram S, Rice PA, Specht CA, Levitz
567 SM. 2012. Relative contributions of dectin-1 and complement to immune
568 responses to particulate beta-glucans. J Immunol 189:312-7.
569 19. Qi C, Cai Y, Gunn L, Ding C, Li B, Kloecker G, Qian K, Vasilakos J, Saijo S,
570 Iwakura Y, Yannelli JR, Yan J. 2011. Differential pathways regulating innate and
571 adaptive antitumor immune responses by particulate and soluble yeast-derived
572 beta-glucans. Blood 117:6825-36.
573 20. Tam JM, Reedy JL, Lukason DP, Kuna SG, Acharya M, Khan NS, Negoro PE,
574 Xu S, Ward RA, Feldman MB, Dutko RA, Jeffery JB, Sokolovska A, Wivagg CN,
575 Lassen KG, Le Naour F, Matzaraki V, Garner EC, Xavier RJ, Kumar V, van de
576 Veerdonk FL, Netea MG, Miranti CK, Mansour MK, Vyas JM. 2019. Tetraspanin
577 CD82 Organizes Dectin-1 into Signaling Domains to Mediate Cellular Responses
578 to *Candida albicans*. J Immunol 202:3256-3266.
579 21. Sarfati J, Boucias DG, Latge JP. 1995. Antigens of *Aspergillus fumigatus*
580 produced *in vivo*. J Med Vet Mycol 33:9-14.

581 22. Stynen D, Sarfati J, Goris A, Prevost MC, Lesourd M, Kamphuis H, Darras V,
582 Latge JP. 1992. Rat monoclonal antibodies against *Aspergillus* galactomannan.
583 Infect Immun 60:2237-45.
584 23. Lehmann PF, Reiss E. 1978. Invasive aspergillosis: antiserum for circulating
585 antigen produced after immunization with serum from infected rabbits. Infect
586 Immun 20:570-2.
587 24. Reiss E, Lehmann PF. 1979. Galactomannan antigenemia in invasive
588 aspergillosis. Infect Immun 25:357-65.
589 25. Fontaine T, Latge JP. 2020. Galactomannan Produced by *Aspergillus fumigatus*:
590 An Update on the Structure, Biosynthesis and Biological Functions of an
591 Emblematic Fungal Biomarker. J Fungi (Basel) 6:283.
592 26. Latge JP, Kobayashi H, Debeaupuis JP, Diaquin M, Sarfati J, Wieruszeski JM,
593 Parra E, Bouchara JP, Fournet B. 1994. Chemical and immunological
594 characterization of the extracellular galactomannan of *Aspergillus fumigatus*.
595 Infect Immun 62:5424-33.
596 27. Costachel C, Coddeville B, Latge JP, Fontaine T. 2005.
597 Glycosylphosphatidylinositol-anchored fungal polysaccharide in *Aspergillus*
598 *fumigatus*. J Biol Chem 280:39835-42.
599 28. Fontaine T, Simenel C, Dubreucq G, Adam O, Delepierre M, Lemoine J, Vorgias
600 CE, Diaquin M, Latge JP. 2000. Molecular organization of the alkali-insoluble
601 fraction of A*spergillus fumigatus* cell wall. J Biol Chem 275:41528.

602 29. Engel J, Schmalhorst PS, Routier FH. 2012. Biosynthesis of the fungal cell wall
603 polysaccharide galactomannan requires intraluminal GDP-mannose. J Biol Chem
604 287:44418-24.
605 30. Lamarre C, Beau R, Balloy V, Fontaine T, Wong Sak Hoi J, Guadagnini S,
606 Berkova N, Chignard M, Beauvais A, Latge JP. 2009. Galactofuranose
607 attenuates cellular adhesion of *Aspergillus fumigatus*. Cell Microbiol 11:1612-23.
608 31. Bozza S, Clavaud C, Giovannini G, Fontaine T, Beauvais A, Sarfati J, D'Angelo
609 C, Perruccio K, Bonifazi P, Zagarella S, Moretti S, Bistoni F, Latge JP, Romani L.
610 2009. Immune sensing of *Aspergillus fumigatus* proteins, glycolipids, and
611 polysaccharides and the impact on Th immunity and vaccination. J Immunol
612 183:2407-14.
613 32. Chai LY, Vonk AG, Kullberg BJ, Verweij PE, Verschueren I, van der Meer JW,
614 Joosten LA, Latge JP, Netea MG. 2011. *Aspergillus fumigatus* cell wall
615 components differentially modulate host TLR2 and TLR4 responses. Microbes
616 Infect 13:151-9.
617 33. Serrano-Gomez D, Dominguez-Soto A, Ancochea J, Jimenez-Heffernan JA, Leal
618 JA, Corbi AL. 2004. Dendritic cell-specific intercellular adhesion molecule 3-
619 grabbing nonintegrin mediates binding and internalization of Aspergillus
620 fumigatus conidia by dendritic cells and macrophages. J Immunol 173:5635-43.
621 34. Tam JM, Mansour MK, Khan NS, Yoder NC, Vyas JM. 2012. Use of fungal
622 derived polysaccharide-conjugated particles to probe Dectin-1 responses in
623 innate immunity. Integr Biol (Camb) 4:220-7.

624 35. Tam JM, Mansour MK, Khan NS, Seward M, Puranam S, Tanne A, Sokolovska
625 A, Becker CE, Acharya M, Baird MA, Choi AM, Davidson MW, Segal BH, Lacy-
626 Hulbert A, Stuart LM, Xavier RJ, Vyas JM. 2014. Dectin-1-dependent LC3
627 recruitment to phagosomes enhances fungicidal activity in macrophages. J Infect
628 Dis 210:1844-54.
629 36. Robinson MJ, Osorio F, Rosas M, Freitas RP, Schweighoffer E, Gross O,
630 Verbeek JS, Ruland J, Tybulewicz V, Brown GD, Moita LF, Taylor PR, Reis e
631 Sousa C. 2009. Dectin-2 is a Syk-coupled pattern recognition receptor crucial for
632 Th17 responses to fungal infection. J Exp Med 206:2037-51.
633 37. Bowman JC, Abruzzo GK, Anderson JW, Flattery AM, Gill CJ, Pikounis VB,
634 Schmatz DM, Liberator PA, Douglas CM. 2001. Quantitative PCR assay to
635 measure Aspergillus fumigatus burden in a murine model of disseminated
636 aspergillosis: demonstration of efficacy of caspofungin acetate. Antimicrob
637 Agents Chemother 45:3474-81.
638 38. Griffiths JS, White PL, Czubala MA, Simonazzi E, Bruno M, Thompson A,
639 Rizkallah PJ, Gurney M, da Fonseca DM, Naglik JR, Ingram W, Wilson K, van de
640 Veerdonk FL, Barnes R, Taylor PR, Orr SJ. 2021. A Human Dectin-2 Deficiency
641 Associated With Invasive Aspergillosis. J Infect Dis 224:1219-1224.
642 39. Loures FV, Rohm M, Lee CK, Santos E, Wang JP, Specht CA, Calich VL, Urban
643 CF, Levitz SM. 2015. Recognition of *Aspergillus fumigatus* hyphae by human
644 plasmacytoid dendritic cells is mediated by dectin-2 and results in formation of
645 extracellular traps. PLoS Pathog 11:e1004643.

646 40. Fontaine T, Delangle A, Simenel C, Coddeville B, van Vliet SJ, van Kooyk Y,
647 Bozza S, Moretti S, Schwarz F, Trichot C, Aebi M, Delepierre M, Elbim C,
648 Romani L, Latge JP. 2011. Galactosaminogalactan, a new immunosuppressive
649 polysaccharide of Aspergillus fumigatus. PLoS Pathog 7:e1002372.
650 41. Barrett NA, Maekawa A, Rahman OM, Austen KF, Kanaoka Y. 2009. Dectin-2
651 recognition of house dust mite triggers cysteinyl leukotriene generation by
652 dendritic cells. J Immunol 182:1119-28.
653 42. Clarke DL, Davis NH, Campion CL, Foster ML, Heasman SC, Lewis AR,
654 Anderson IK, Corkill DJ, Sleeman MA, May RD, Robinson MJ. 2014. Dectin-2
655 sensing of house dust mite is critical for the initiation of airway inflammation.
656 Mucosal Immunol 7:558-67.
657 43. Norimoto A, Hirose K, Iwata A, Tamachi T, Yokota M, Takahashi K, Saijo S,
658 Iwakura Y, Nakajima H. 2014. Dectin-2 promotes house dust mite-induced T
659 helper type 2 and type 17 cell differentiation and allergic airway inflammation in
660 mice. Am J Respir Cell Mol Biol 51:201-9.
661 44. Parsons MW, Li L, Wallace AM, Lee MJ, Katz HR, Fernandez JM, Saijo S,
662 Iwakura Y, Austen KF, Kanaoka Y, Barrett NA. 2014. Dectin-2 regulates the
663 effector phase of house dust mite-elicited pulmonary inflammation independently
664 from its role in sensitization. J Immunol 192:1361-71.
665 45. Saijo S, Ikeda S, Yamabe K, Kakuta S, Ishigame H, Akitsu A, Fujikado N,
666 Kusaka T, Kubo S, Chung SH, Komatsu R, Miura N, Adachi Y, Ohno N, Shibuya
667 K, Yamamoto N, Kawakami K, Yamasaki S, Saito T, Akira S, Iwakura Y. 2010.

668 Dectin-2 recognition of alpha-mannans and induction of Th17 cell differentiation
669 is essential for host defense against Candida albicans. Immunity 32:681-91.
670 46. Rossner M, Yamada KM. 2004. What's in a picture? The temptation of image
671 manipulation. J Cell Biol 166:11-5.
672
673
674 **FIGURE LEGENDS**
675
676 **Figure 1.** *A. fumigatus* and galactomannan activate Dectin-2 expressing CLR reporter
677 cells. Reporter cells were stimulated for 18 hours using (A) either live or heat killed
678 germlings of *A. fumigatus* Af293 at MOI 20:1 or (B) unmodified FLPs, β-1,3 glucan (BG)
679 FLPs, *S. cerevisiae* mannan FLPs, and *A. fumigatus* galactomannan (GALM) FLPs at
680 an effector to target ratio of 30:1. LacZ activity was measured in total cell lysates using
681 CPRG as a substrate. Error bars represent the SEM of duplicate wells, and each
682 experiment was repeated three times.
683
684 **Figure 2.** Recombinant Dectin-2 Fc protein binds galactomannan FLPs. (A) Unmodified
685 FLPs, β -1,3 glucan FLPs, and galactomannan FLPs were incubated with soluble
686 recombinant murine Dectin-2- human IgG1 Fc protein conjugate, control IgG1-Fc
687 protein, or in buffer only, incubated with anti-human IgG1 conjugated to Alexa Fluor 488
688 and analyzed by flow cytometry. Dectin-2-Fc bound to galactomannan FLPs, but not to
689 either unmodified or β-1,3 glucan FLPs. (B) FLPs were incubated for 2 hours in binding
690 buffer alone or containing Dectin-2-Fc protein or Dectin-2-Fc pre-incubated for 30

691 minutes with a Dectin-2 neutralizing antibody. Dectin-2 neutralizing antibody disrupted
692 the association of Dectin-2-Fc with galactomannan FLPs.
693
694 **Figure 3.** Galactomannan FLPs activate Dectin-2 dependent signaling in macrophages.
695 (A) Immunoblot of immortalized wildtype C57BL/6 macrophages or C57BL/6
696 macrophages expressing Dectin-2 stimulated with live *C. albicans* (SC5314),
697 unmodified FLPs (UM FLP), galactomannan FLPs (GALM FLP), β-1,3 glucan FLPs (BG
698 FLP), or mannan FLPs at MOI 10:1 for 1 hour at 37°C and probed for both total Syk and
699 phosphorylated (active) Syk. Overexpression of Dectin-2 increased phosphorylation of
700 Syk in response to *C. albicans*, galactomannan and mannan FLPs, but not to
701 unmodified or β-1,3 glucan FLPs. (B) Immortalized wildtype C57BL/6 macrophages or
702 C57BL/6 macrophages expressing Dectin-2 were stimulated overnight with unmodified
703 (UM), galactomannan (GALM), or mannan (MAN) FLPs at a target to effector ratio of
704 10:1. Prior to FLPs stimulation, macrophages were pre-treated for 30 minutes with
705 either a Dectin-2 neutralizing antibody or an isotype control antibody as indicated.
706 Supernatants were analyzed for TNFα using ELISA. Both galactomannan and mannan
707 FLPs induced robust TNFα production by macrophages expressing Dectin-2, which was
708 blocked by Dectin-2 neutralizing antibody, but not the isotype control. The significance
709 indicated is in relation to the wild-type cell stimulated with the same FLPs. (C) BMDMs
710 from wild-type C57BL/6 and Dectin-2$^{-/-}$ mice were stimulated with LPS (500 ng/mL), and
711 unmodified (UM) or galactomannan (GALM) FLPs at an effector to target ration of 20:1
712 for 18 hours. Supernatants were analyzed for TNFα using ELISA. Dectin-2$^{-/-}$ BMDM
713 produced less TNFα when stimulated with galactomannan FLP compared with wildtype

714 BMDM. Error bars represent standard error of three biological replicates, statistics were
715 calculated with two-way ANOVA using PRISM 9 software (not significant, ns, ***
716 $p<0.001$, **** $p<0.0001$).
717
718 **Figure 4.** Deficiency of Dectin-2 leads to increased immune cell recruitment, but is
719 dispensable for survival. (A - E) Immunocompetent wild-type and Dectin-2$^{-/-}$ mice were
720 infected via oropharyngeal inhalation of 4 x $10^7$ conidia of *A. fumigatus* CEA10. Lung
721 tissue was harvested 48 hours post-infection and digested to obtain a single cell
722 suspension that was then analyzed by flow cytometry. Dectin-2 infected mice had (A)
723 increased infiltration of live CD45+ cells compared with wild-type. There was a
724 significant increase in the total number of (B) innate cells (7-AAD$^{lo}$, CD45$^+$, CD90.2$^-$,
725 CD19$^-$) and (C) neutrophils (7-AAD$^{lo}$ , CD45$^+$, CD90.2$^-$, CD19$^-$, SiglecF$^-$, Ly6G$^+$) in
726 Dectin-2$^{-/-}$ mice compared with wild-type. However, there was no difference in the
727 percentage of cells that were innate cells or (E) neutrophils. The data shown is a
728 composite from two independent experiments, consisting of at least 5 PBS control mice
729 and 10 CEA10 infected mice per mouse strain. Each point represents an individual
730 animal and horizontal lines represent the median. One-way ANOVA was performed
731 using PRISM9 software to determine statistical significance (not significant, ns, *
732 $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$). (F,G) Immunocompetent wild-type and
733 Dectin-2$^{-/-}$ mice were infected with 4 x $10^7$ CEA10. After 48 hours lungs were extracted,
734 and fungal burden was quantified using (F) qPCR or (G) CFU. There was no difference
735 in fungal burden between wild-type and Dectin-2$^{-/-}$ mice. Each point represents an
736 individual animal and horizontal lines represent the median fungal burden in the lungs.

737 Data shown are from a single replicated consisting of 5 infected and 1 PBS control per
738 mouse strain. Each experiment was repeated at least twice, and concordant results
739 were obtained in each experiment. (H) Wild-type C57BL/6 and Dectin-2$^{-/-}$ mice were
740 immunosuppressed with corticosteroids and infected intranasally with 2.5 x $10^4$ conidia
741 of *A. fumigatus* strain CEA10. There was no significant difference in survival between
742 wildtype and Dectin-2$^{-/-}$ mice as determined based upon Kaplan-Meyer and Cox
743 Analysis using PRISM 9 software.
744

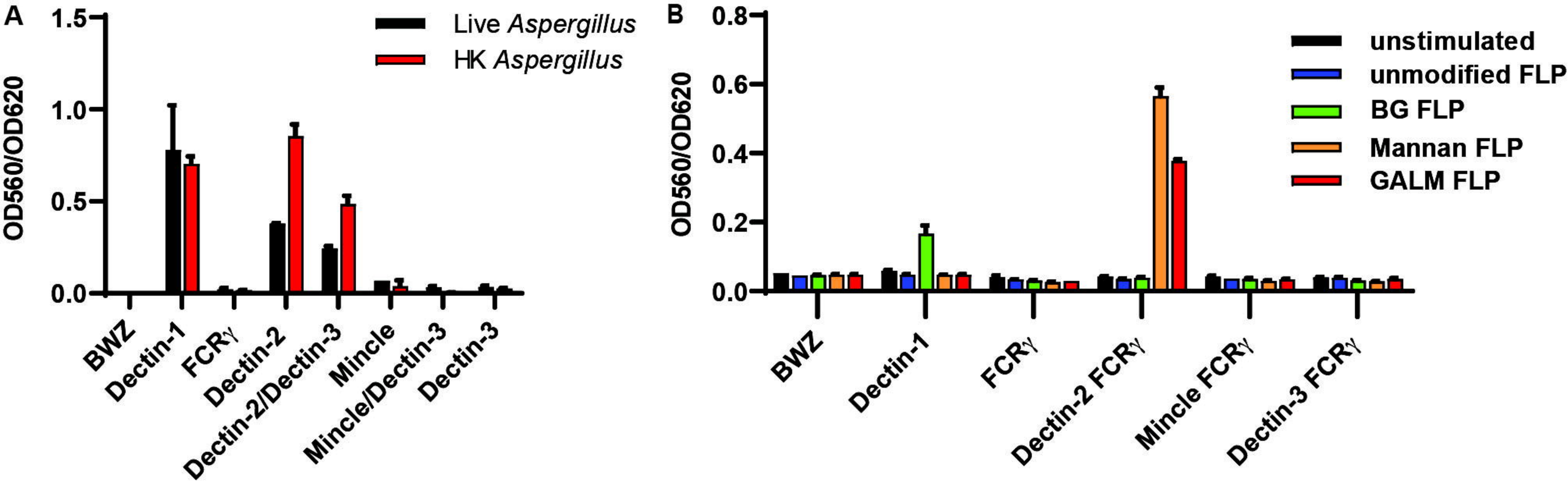

A
1.5
1.0
0.5
0.0
OD560/OD620
Live Aspergillus
HK Aspergillus
BWZ
Dectin-1
FCRγ
Dectin-2
Dectin-2/Dectin-3
Mincle
Mincle/Dectin-3
Dectin-3
B
0.8
0.6
0.4
0.2
0.0
OD560/OD620
unstimulated
unmodified FLP
BG FLP
Mannan FLP
GALM FLP
BWZ
Dectin-1
FCRγ
Dectin-2 FCRγ
Mincle FCRγ
Dectin-3 FCRγ

Figure 1

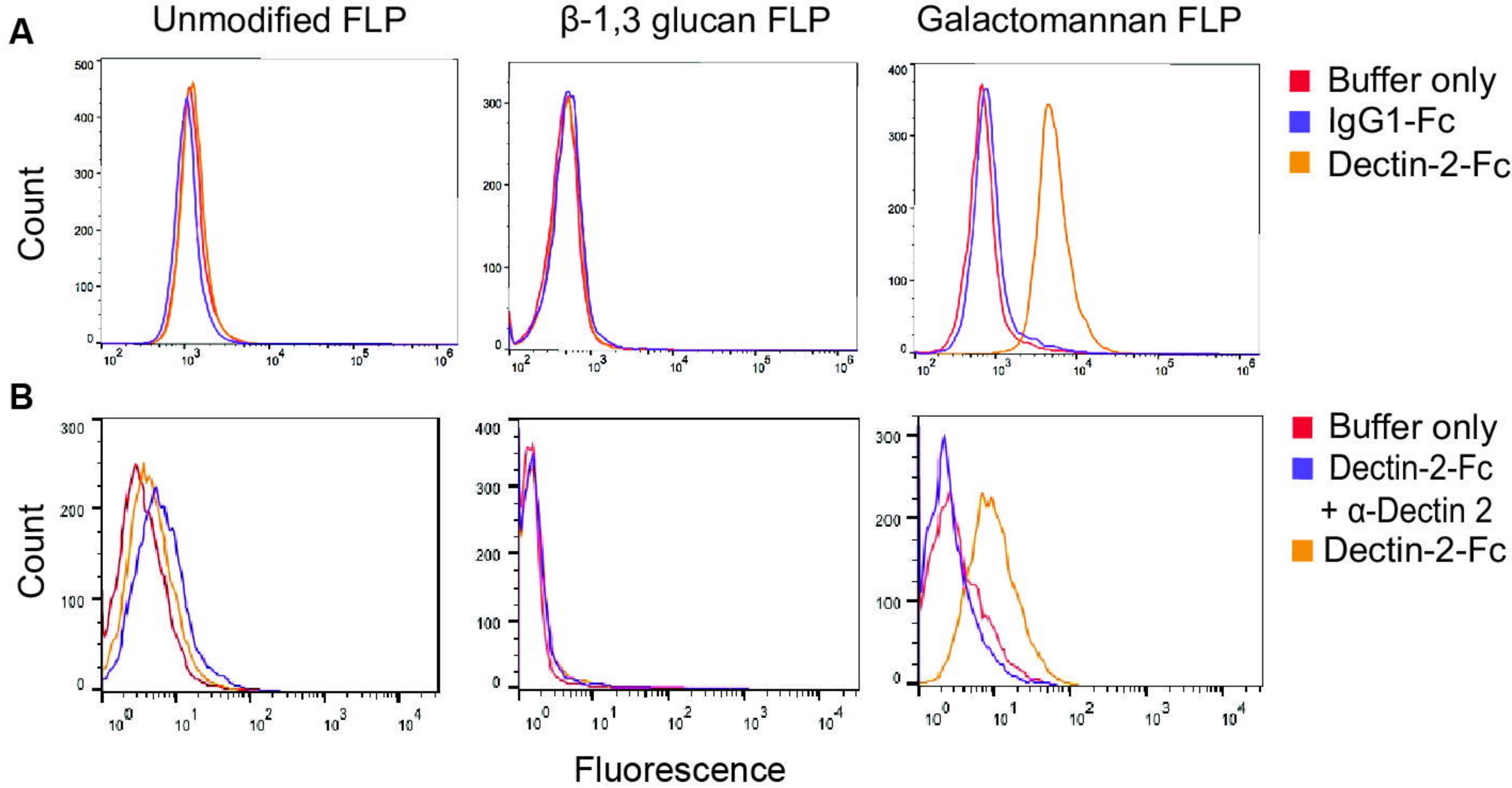
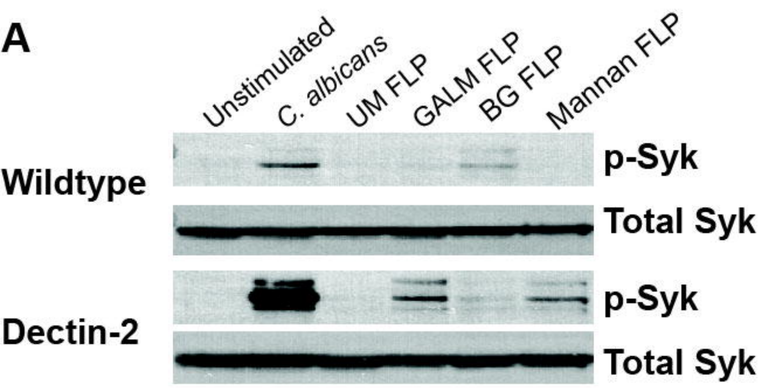
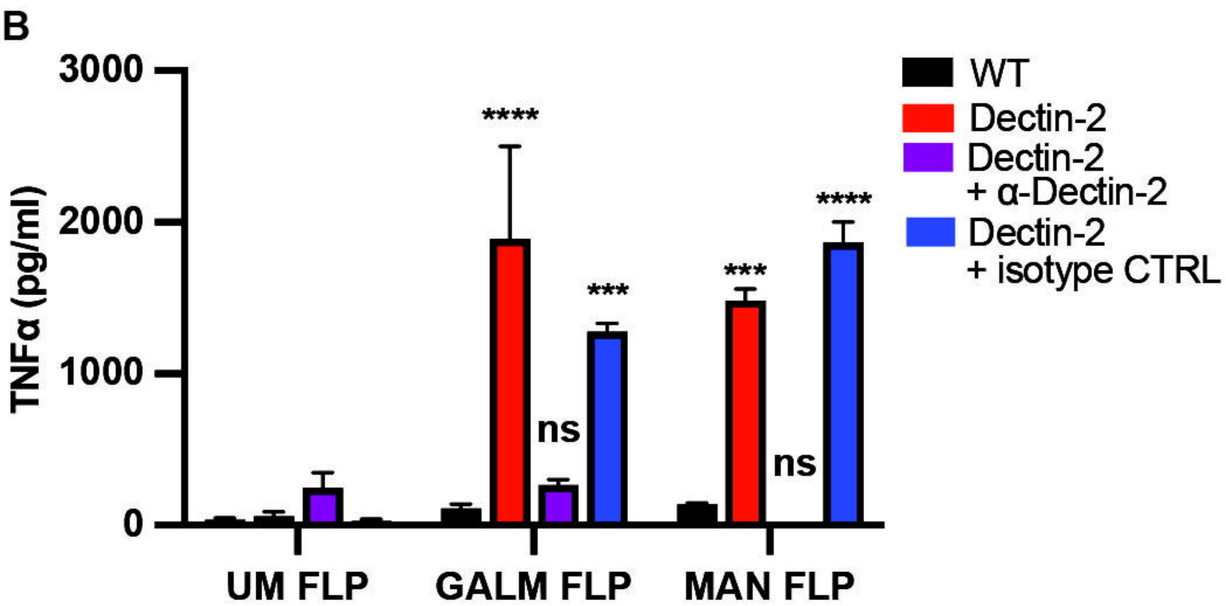

A
Unmodified FLP
β-1,3 glucan FLP
Galactomannan FLP
Count
Buffer only
IgG1-Fc
Dectin-2-Fc
B
Count
Buffer only
Dectin-2-Fc
+ α-Dectin 2
Dectin-2-Fc
Fluorescence

**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)
3000
2000
1000
0
****
***
ns
***
****
ns
UM FLP
GALM FLP
MAN FLP
WT
Dectin-2
Dectin-2
+ α-Dectin-2
Dectin-2
+ isotype CTRL

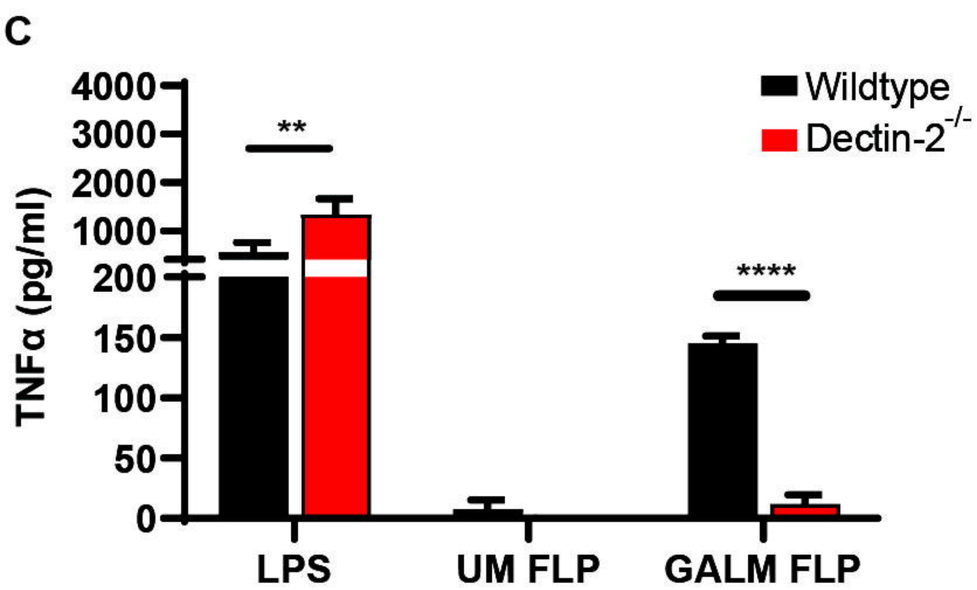

C
TNFα (pg/ml)
4000
3000
2000
1000
200
150
100
50
0
**
****
LPS
UM FLP
GALM FLP
Wildtype
Dectin-2-/-

Figure 3

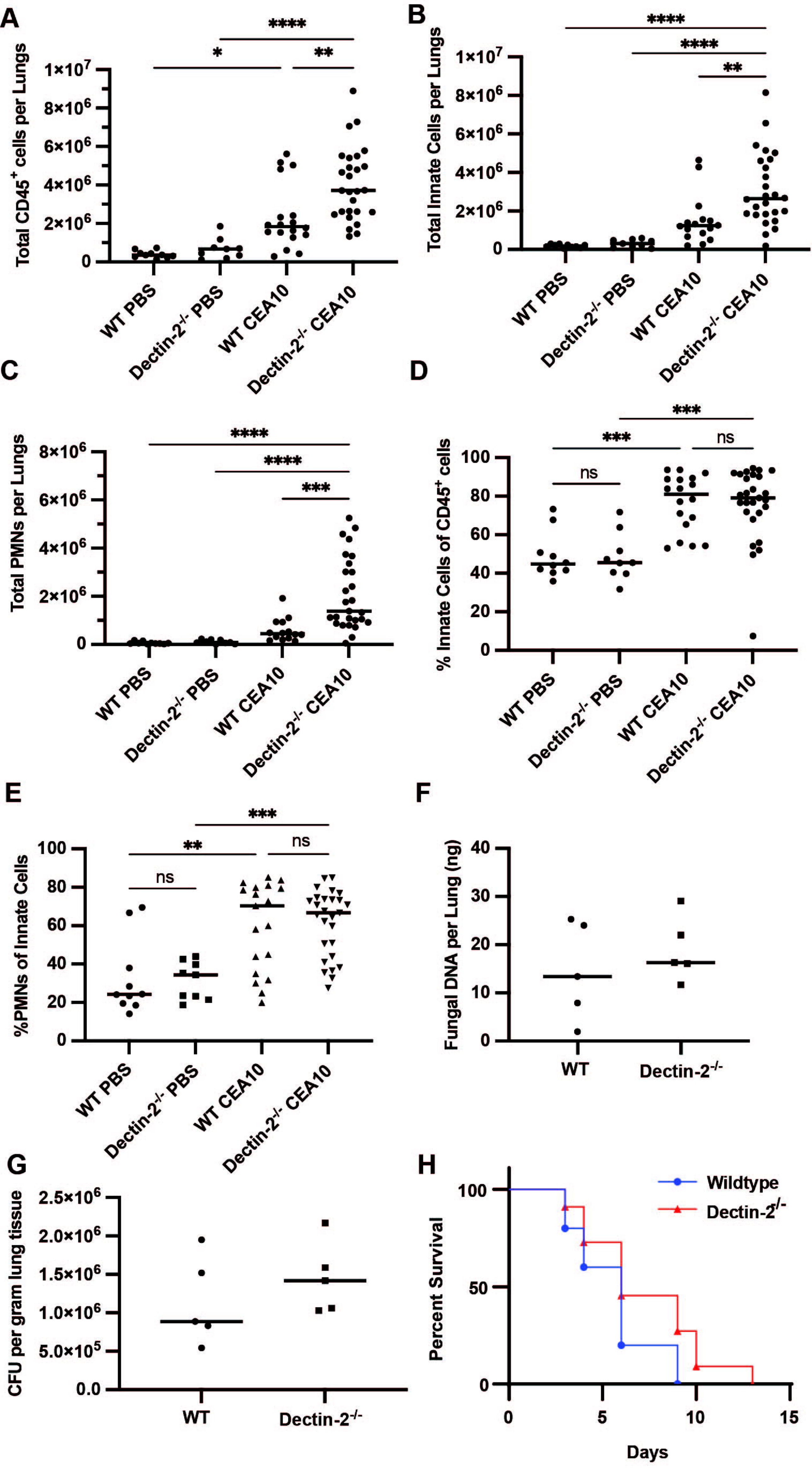

A
Total CD45+ cells per Lungs
1×10^7
8×10^6
6×10^6
4×10^6
2×10^6
0
*
**
****
WT PBS
Dectin-2-/- PBS
WT CEA10
Dectin-2-/- CEA10
B
Total Innate Cells per Lungs
1×10^7
8×10^6
6×10^6
4×10^6
2×10^6
0
****
****
**
WT PBS
Dectin-2-/- PBS
WT CEA10
Dectin-2-/- CEA10
C
Total PMNs per Lungs
8×10^6
6×10^6
4×10^6
2×10^6
0
****
****
***
WT PBS
Dectin-2-/- PBS
WT CEA10
Dectin-2-/- CEA10
D
% Innate Cells of CD45+ cells
100
80
60
40
20
0
***
***
ns
ns
WT PBS
Dectin-2-/- PBS
WT CEA10
Dectin-2-/- CEA10
E
%PMNs of Innate Cells
100
80
60
40
20
0
***
**
ns
ns
WT PBS
Dectin-2-/- PBS
WT CEA10
Dectin-2-/- CEA10
F
Fungal DNA per Lung (ng)
40
30
20
10
0
WT
Dectin-2-/-
G
CFU per gram lung tissue
2.5×10^6
2.0×10^6
1.5×10^6
1.0×10^6
5.0×10^5
0.0
WT
Dectin-2-/-
H
Percent Survival
100
50
0
0
5
10
15
Days
Wildtype
Dectin-2-/-

Figure 4