Dectin-1 Is Required for Resistance to Coccidioidomycosis in Mice

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ABSTRACT **We assessed the role of Dectin-1 in the immune response to the pathogenic fungus *Coccidioides*, both *in vitro* and *in vivo*, using mice with a targeted mutation in *Clec7a*. Elicited peritoneal macrophages responded to formalin-killed spherules (FKS) and alkali-treated FKS by secreting proinflammatory cytokines in a Dectin-1- and β-glucan-dependent manner. The responses of bone marrow-derived dendritic cells (BMDC) to the same stimulants were more complex; interleukin 1β (IL-1β) and tumor necrosis factor alpha (TNF-α) secretion was independent of Dectin-1, while IL-6, IL-10, and granulocyte-macrophage colony-stimulating factor (GM-CSF) were largely but not entirely dependent on Dectin-1. After intranasal infection, Dectin-1$^{-/-}$ mice had lower concentrations of IL-12p70, gamma interferon (IFN-γ), IL-1β, and the Th17 cytokines IL-22, IL-23, and 17A in the lung lavage fluid, which may explain why they were significantly more susceptible to pulmonary coccidioidomycosis two weeks after infection. The Dectin-1 mutation was even more deleterious in (B6 × DBA/2)F_2 mice, which are more resistant to coccidioidomycosis than B6 mice by virtue of protective genes from DBA/2, a genetically resistant strain. We also found that two susceptible strains of mice (B6 and BALB/c) expressed much less Dectin-1 in their lungs than did resistant DBA/2 mice. We conclude that Dectin-1 is necessary for resistance to *Coccidioides immitis*, that Dectin-1 promotes both Th1 and Th17 protective immune responses to this infection, and that there is a correlation between expression of Dectin-1 by the inflammatory infiltrate and resistance to coccidioidomycosis.**

IMPORTANCE **Coccidioidomycosis is a fungal infection endemic in the southwestern United States and neighboring Mexico, causing ~150,000 lung infections in people and resulting in ~17,000 hospitalizations annually in California alone. Very little is known about innate immunity to this fungus. This paper shows that Dectin-1, the primary β-glucan receptor on myeloid cells, is required for resistance to this pathogen. Dectin-1 is part of the innate immune system, and it is needed to direct the acquired immune response toward into a pathway that will lead to macrophage activation. Lungs from infected mice lacking Dectin-1 had lower concentrations of Th1 and Th17 cytokines, two cytokine pathways that are very important for acquired T cell immunity to *Coccidioides* spp. This is the first demonstration that Dectin-1 is required for host resistance to a dimorphic, primary pathogenic fungus.**

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Coccidioidomycosis (San Joaquin Valley Fever) is one of the endemic fungal mycoses in the Western Hemisphere, occurring in the desert regions of the southwestern United States and in countries in Central and South America that have ecosystems similar to that of the lower Sonoran life zone (1). Coccidioidomycosis can be caused by either of two closely related species with different geographic distributions, *Coccidioides immitis* and *Coccidioides posadasii* (2). It is estimated that they cause 150,000 new infections annually in the United States (3). Infection with these soil-dwelling molds is initiated by inhalation of airborne fungal spores (arthroconidia) that, under the influence of temperature and partial pressure of CO_2, undergo a profound metamorphosis to become spherules, the pathognomonic structure for this infection. Spherules enlarge and undergo internal segmentation until they are as large as 150 μm in diameter. The internal segments develop into endospores. Hundreds of endospores are released when a spherule ruptures, and endospores in turn develop into spherules (4). Phagocytes can ingest endospores, developing spherules, and arthroconidia, but mature spherules are too large to be ingested by individual phagocytes.

A striking clinical feature of coccidioidomycosis is that the vast majority of infections are mild and self-limited, usually leaving no more residual damage than a pulmonary granuloma (5). Patients who spontaneously resolve their infections produce only low titers of complement-fixing antibodies that are no longer detectable two years after infection in most patients, but they develop long-lasting immunity, manifested by a positive delayed hypersensitivity skin test (DTH) response to antigens made from the organism (6). In contrast, a small percentage of infections do not resolve spontaneously, and these result in either chronic cavitary pulmonary infections or extrapulmonary foci of infection (dissemination). Patients with disseminated infections make high titers of

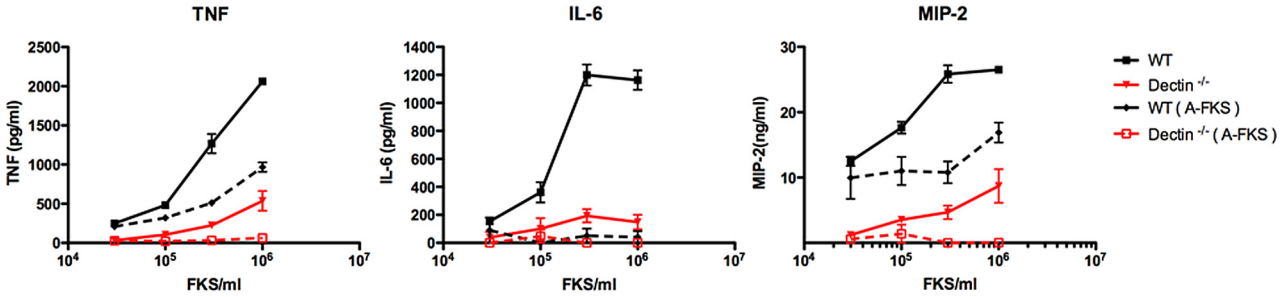

FIG 1 Cytokine responses of elicited peritoneal macrophages to FKS (solid lines) or A-FKS (dashed lines). Macrophages were elicited as described in Materials and Methods and allowd to adhere to 48-well plates at 2×10^5/well for 2 h before the wells were washed to remove nonadherent cells and organisms were added. Values are means for triplicate wells ± 1 standard error of the mean (SEM).

antibody to fungal antigens but do not develop DTH. The higher the antibody titers are, the worse the prognosis is for the patient. Although various innate and acquired cell immunodeficiencies predispose individuals to disseminated infections (7, 8), most cases of disseminated coccidioidomycosis occur in otherwise healthy individuals.

Those observations suggest that patients who suffer disseminated coccidioidomycosis mount Th2 immune responses and that a Th2 immune response cannot control the infection. In mice the protective immune response to this infection is based on development of antigen-specific $CD4^+$ T cells (9). From mouse experiments and observations in humans, we know that certain cytokines are necessary for effective immune responses, including gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), and an as-yet-uncharacterized member of the interleukin 17 (IL-17) family (10–13), suggesting that activation of an M1 macrophage phenotype is protective, but we know very little about how the fungus is killed by the host.

Compared to Caucasians, African Americans and Filipinos have a 5- to 10-fold-greater risk of developing disseminated coccidioidomycosis (14–16), which suggests that there is a genetic basis for susceptibility to sever coccidioidomycosis (17). Inbred mice also vary greatly in their susceptibility to coccidioidomycosis; DBA/2 (D2) mice are 1,000-fold more resistant than C57BL/6 (B6) and BALB/c mice, as determined by 50% lethal dose (LD_{50}) measurements (18, 19). In mice, resistance is the dominant phenotype, and it is a polygenic trait. We mapped resistance loci in recombinant inbred strains of BXD mice and found that one of the resistance loci maps to mouse chromosome 6, not far from the *Clec* cluster of genes, which includes *Clec7a*, encoding Dectin-1, the principal β-1,3-glucan receptor that is expressed on myeloid cells (20). *Clec7a* is alternatively spliced by mice and humans so that they express both a full-length and a truncated version of the Dectin-1 gene (missing exon 3) (21). The functional importance of splicing is not well understood. DBA/2 mice are unusual in that they do not splice out exon 3, and we found a significant association between resistance and expression of the full-length Dectin-1 by recombinant inbred BXD mice (22).

Most studies of the immune response to fungi have used the B6 mouse strain, an inbred strain that is highly susceptible to coccidioidomycosis. When we compared the cytokines made by macrophages and bone marrow-derived dendritic cells (BMDC) from B6 and DBA/2 mice stimulated with formalin-killed spherules (FKS), we found that B6 BMDC make more IL-10 and less IL-12p70 and IL-23 and that blocking Dectin-1 eliminated most of the difference between the two strains but did not completely block cytokine production. Furthermore, RAW macrophages that express a full-length Dectin-1 make more TNF-α and MIP-2 than RAW cells expressing the truncated version of Dectin-1 when stimulated with FKS (22). This shows that there is variation among inbred strains in how their cells respond to spherules *in vitro*, and part of that difference is due to Dectin-1 expression (22).

Dectin-1 clearly is not solely responsible for activating myeloid cells to respond to spherules. Toll-like receptors (TLRs) and Dectin-1 cooperate in some way to generate maximal amounts of proinflammatory cytokines (23). We found that TLR2 and Dectin-1 also interact to produce proinflammatory cytokines from elicited peritoneal macrophages stimulated by FKS (24). Fungal cell walls are composed largely of carbohydrate structures, such as chitin, β-1,3-glucan, phospholipomannans, and mannoproteins (25). The exact composition of most fungal cell walls, including that of *Coccidioides* spp., is not known, but the spherule cell wall does contain chitin, β-glucan, 3-*O*-methyl-mannose, and mannans (26–28). Spherules also have a lipid-rich outer membrane that includes an antigenic glycoprotein (29). Several TLRs have been shown to interact with different surface structures and with fungal DNA and RNA, and some but not all of the corresponding TLR deficiencies have been shown to exacerbate *Candida* infections in mice (30).

In this study, we analyzed the role of Dectin-1 in the *in vitro* and *in vivo* responses to *Coccidioides*, measuring cytokines produced by macrophages and BMDC *in vitro* in response to FKS and in infected lungs. We also compared the resistance of *Clec7a* mutant mice to that of B6 controls. We found that Dectin-1 plays a nonredundant role in resistance to pulmonary *C. immitis* infection in mice.

RESULTS

Since macrophages are both effectors and antigen-presenting cells, and they are abundant in the granulomas that form in response to infection, we determined the effect of a targeted mutation in *Clec7a* on macrophage cytokine responses to formalin-killed spherules (FKS) or hot-alkali-treated FKS (A-FKS). Harsh alkaline hydrolysis of the fungus removes mannoproteins but not β-glucan or chitin. Figure 1 shows dose-response curves for elicited peritoneal macrophages from B6 and Dectin-$1^{-/-}$ mice stimulated with FKS. B6 macrophages made significantly more TNF-α, MIP-2, and IL-6. Dectin-$1^{-/-}$ macrophages were unre-

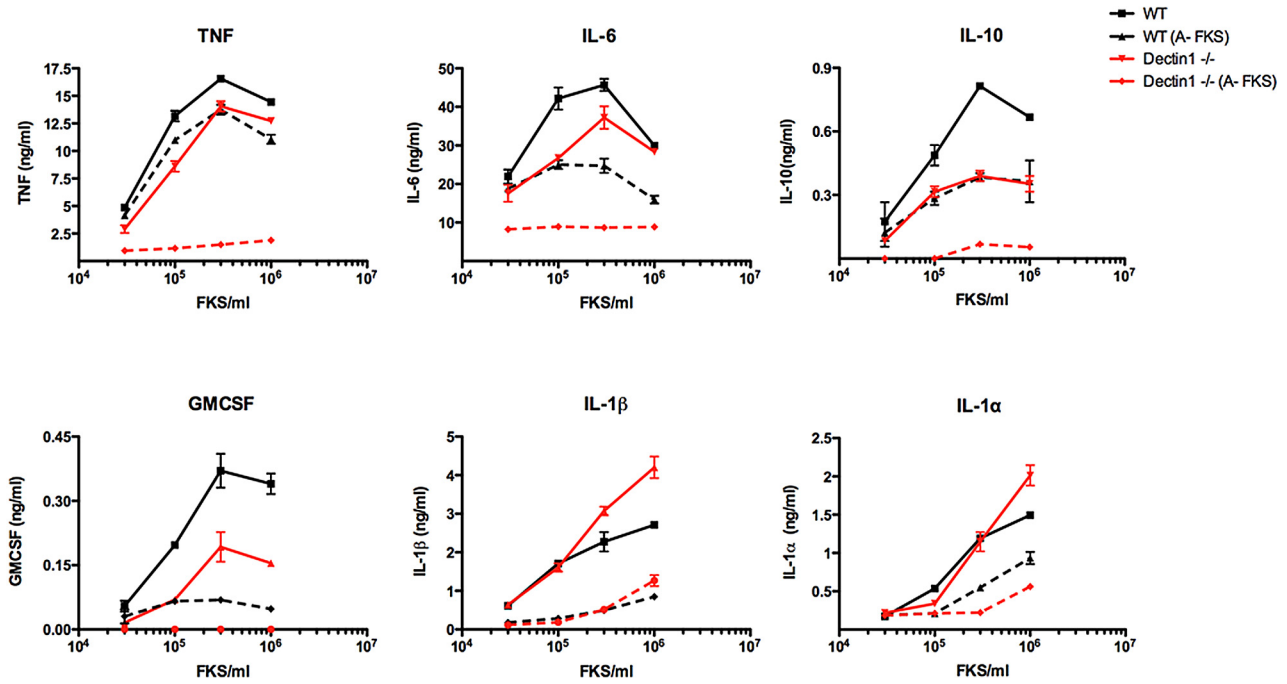

FIG 2 Cytokine responses of B6 (WT) and Dectin-1$^{-/-}$ BMDC stimulated with FKS (solid lines) or A-FKS (dashed lines). Each point is the mean for triplicate assays ± SEM. The results are representative of three separate experiments.

sponsive to A-FKS, while B6 macrophages made significantly more TNF-α, MIP-2, and IL-6 when stimulated with FKS than when A-FKS was the stimulus, suggesting that alkaline hydrolysis remove structures that activated other pattern recognition receptors (PRR) to generate maximal amounts of those cytokines. IL-6 signaling was completely dependent on Dectin-1, but was not induced by A-FKS, so secretion of this cytokine required a collaborative interaction with a PRR that recognized a structure that was removed by hot alkali treatment of FKS. These results are consistent with our previous finding that showed a cooperative interaction between Dectin-1 and TLR2 in RAW cells and peritoneal macrophages from TLR2-deficient mice (24). We did not detect IL-1β, transforming growth factor β (TGF-β), or IL-10 from FKS-stimulated macrophages. To exclude lipopolysaccharide (LPS) contamination of FKS as a confounder in these experiments, we added polymyxin B to FKS and to the LPS control, and only the latter was neutralized by polymyxin (data not shown).

We also compared responses of bone marrow-derived dendritic cells (BMDC) from Dectin$^{-/-}$ and B6 mice to FKS or A-FKS (Fig. 2), and the patterns of response varied for the different cytokines, suggesting involvement of different signaling pathways in these responses. As expected, Dectin-1$^{-/-}$ BMDC did not make any cytokines when stimulated with A-FKS. Dectin-1 was not required for secretion of TNF-α, and B6 BMDC made equivalent amounts of TNF-α whether stimulated with FKS or A-FKS. Since Dectin-1$^{-/-}$ and B6 BMDC made nearly the same amounts of TNF-α in response to FKS, this suggests that FKS activates TNF-α production by more than one pathway and that Dectin-1 is not absolutely required for induction of TNF-α. A-FKS was a less potent inducer of all the other cytokines we measured. IL-1β secretion was not dependent on Dectin-1 and was not induced by A-FKS, suggesting that the stimulus to IL-1β was not β-glucan. FKS induced only low levels of IL-10 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (an order of magnitude less than the other cytokines), and those two responses were partially dependent on Dectin-1, as B6 responses were twice those of Dectin-1$^{-/-}$ BMDC, and B6 BMDC stimulated with A-FKS made about half as much as cells stimulated with FKS. Saijo et al. (31) recently reported that Dectin-2 mediates IL-1β and IL-10 produced by BMDC stimulated with *C. albicans*.

FKS and A-FKS are killed fungi that do not secrete soluble activators, so direct contact between those fungal particles and host cells should be a requirement for stimulation to occur. Therefore, we measured the percentage of elicited peritoneal macrophages and BMDC that were attached to or had ingested fungi under conditions similar to those used in the previous experiments. After an overnight incubation, the slides were washed, stained, and then examined using a 50× objective. There were clumps or chains of organisms with multiple adherent cells (Fig. 3A and B), complicating the measurement, but the slides were read blind by one observer (J.F.) who applied the same criteria to all the samples. There were no significant differences between adherence of FKS and A-FKS to either macrophages or BMDCs (Fig. 3C). A-FKS adhered less well to Dectin-1$^{-/-}$ macrophages, but A-FKS were still adherent to 24% of Dectin-1$^{-/-}$ macrophages, suggesting that they have another receptor for β-glucan, such as complement receptor 3 (CR3) (32). Most of the spherules were too large to be ingested, so we were assessing primarily adherence, not ingestion. However, 5% of Dectin-1$^{-/-}$ BMDC were adherent to FKS, and only 2% were adherent to A-FKS, so in those DCs, not only was Dectin-1 the only receptor for fungal β-glucan, but also β-glucan on intact spherules (FKS) was the primary structure recognized by BMDC. Since FKS adhered to BMDC exclusively via Dectin-1, that implied that β-1,3-glucan is on the sur-

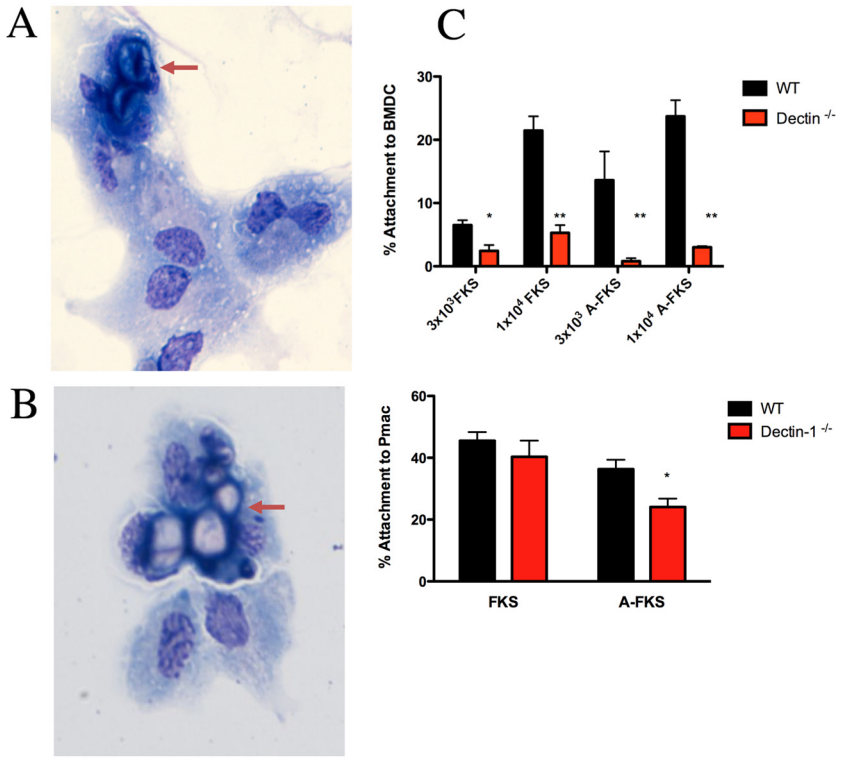

FIG 3 Comparison of adherence of FKS and A-FKS to B6 (WT) and Dectin-1$^{-/-}$ BMDC. (A) Wright-Giemsa-stained B6 BMDC with two adherent FKS (arrow). (B) Clump of A-FKS adherent to BMDC (arrow); note the paler staining of the A-FKS. Magnification (A and B), ×500. (C) Percentages of BMDC and peritoneal macrophages from B6 and Dectin-1$^{-/-}$ mice with adherent fungi. Each condition was tested in triplicate wells, and 4-500 cells were counted in each well. The means ± SEM are shown. *, $P = 0.05$ compared to B6; **, $P < 0.01$ (unpaired t test).

faces of spherules, which we confirmed by incubating the fungus with a soluble Dectin-1–Fc fusion protein; it attached uniformly to the fungal cell wall (Fig. 4). All stages of the fungus except immature hyphae had β-glucan exposed on the surface.

We then infected B6 mice and determined the percentage of CD45$^+$ cells in bronchoalveolar lavage fluid (BALF) 14 days after intranasal (i.n.) infection that expressed Dectin-1 on their surfaces. As shown in Fig. 5A, 11% of viable CD45$^+$ cells expressed Dectin-1. In comparison, 54% of the CD45$^+$ cells from lungs of genetically resistant DBA/2 were Dectin-1$^+$. Furthermore, the mean level of expression Dectin-1 was 25% higher on DBA/2 cells. To establish whether the difference between the two strains was at the transcriptional level, we isolated RNA from the CD45$^+$ cells and, using RT-PCR, determined that the expression level was increased 20-fold in DBA/2 lungs and increased less than 2-fold in B6 lungs (Fig. 5B). There was no difference in the baseline expression of *Clec7a* mRNA in uninfected lungs between the different mouse strains (data not shown).

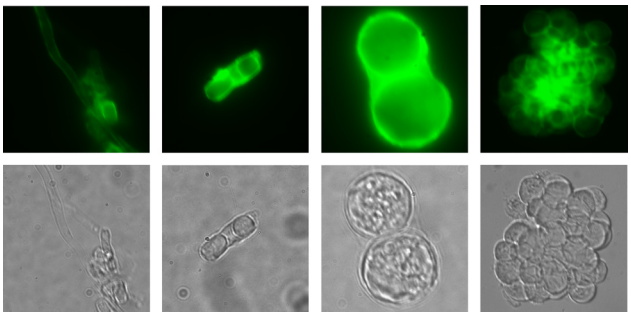

FIG 4 Detection of surface β-glucan using a soluble Dectin-Fc fusion protein. *C. immitis* was grown as a mold on Sabouraud's agar. Spherules were grown *in vitro* in Converse medium. sDectin-1-Fc fusion was made as described in Materials and Methods. The fungi were not fixed before they were exposed to sDectin-1-Fc. The bottom panel shows phase-contrast photos, and the top panel shows the same fields viewed with a fluorescence microscope. From left to right, the panels show immature hyphae, arthroconidia, mature spherules, and a ruptured spherule with a conglomerate of endospores.

We compared the concentrations of various cytokines in the BALF from infected B6 and Dectin-1$^{-/-}$ lungs 14 days after i.n. infection (Fig. 6). Dectin-1$^{-/-}$ BALF had significantly lower concentrations of the Th17 cytokines IL-17a, IL-22, and IL-23 and significantly lower concentrations of the Th1 cytokines IL-12p70 and IFN-γ. The concentration of IL-10 was also twice as high in B6 as in Dectin-1$^{-/-}$ BALF. In contrast, the IL-6 concentration was significantly higher in Dectin-1$^{-/-}$ mice, and the concentration of MIP-2 was 10-fold higher in Dectin-1$^{-/-}$ BALF.

Dectin-1$^{-/-}$ mice were more susceptible to *C. immitis*, as determined by the number of CFU of the fungus recovered from their lungs and spleens 14 days after i.n. infection. As shown in Fig. 7, on average, Dectin-1$^{-/-}$ mice had 10-fold more organisms in their lungs and 100-fold more CFU in their spleens. Because the *Clec7a* mutation was in a B6 genetic background, and B6 mice are genetically susceptible to coccidioidomycosis (19), we were concerned as to whether this result is generally applicable, as most

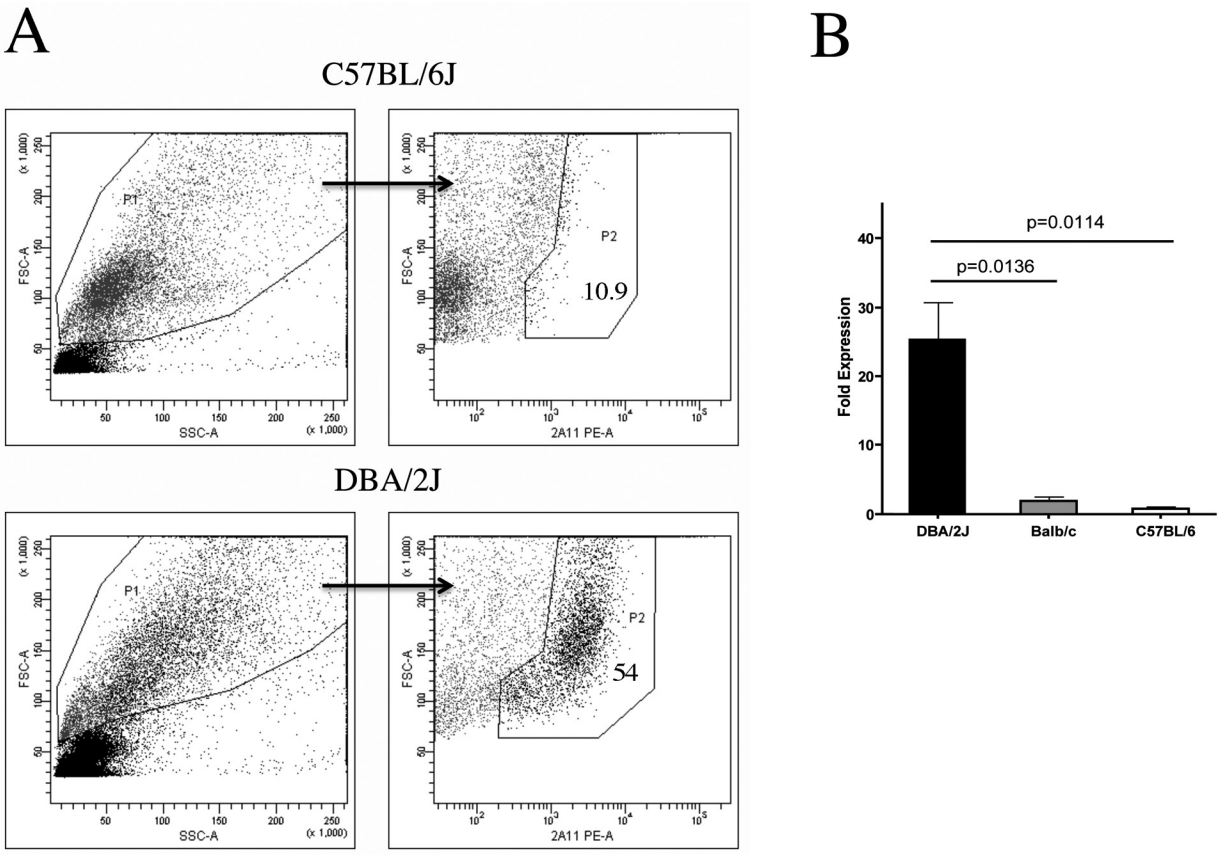

FIG 5 Comparison of Dectin-1 expression on BALF cells from C75BL/6 and DBA/2 mice 14 days after infection. (A) $CD45^+$ cells were positively selected with antibody-coated beads. Based on size and density, live myeloid cells (P1) were selected, and the Fc receptors were blocked with CD16/CD32 for 30 min at 4°C and then labeled with 2A11, a rat monoclonal anti-Dectin-1 antibody, followed by a goat anti-rat IgG-PE. The percentages of anti-rat Ig–PE^+ cells are shown (P2). The mean intensity of staining was 4,600 for DBA/2 cells and 3,681 for B6 cells. Cell acquisition was performed with a dual-laser flow cytometer (FACSCalibur, BD Biosciences, Mountain View, CA). The data were analyzed using Cell Quest Software (BD Biosciences). (B) Change in Dectin-1 mRNA in $CD45^+$ cells in the infected lungs compared to uninfected lungs. mRNA was transcribed to cDNA and measured by quantitative PCR (qPCR) using primers that recognized the conserved region of exon 6. BALB/c mice were included in this experiment as a second susceptible mouse strain.

people recover spontaneously from this infection. We wanted to assess the role of Dectin-1 in a more resistant genetic background, so we took advantage of the fact that $(DBA/2 \times B6)F_1$ mice are resistant to coccidioidomycosis, indicating that the DBA/2 resistance genes are dominant (19). We bred $(B6Clec7a^{tm} \times DBA/2)F_2$ mice and screened the female offspring by PCR to select those that were homozygous for the *Clec7a* mutation and littermates that were homozygous for the wild-type (WT) *Clec7a* gene. The *Clec7a* mutation in this genetic background was even more deleterious, in large part because the F_2 mice that carried two functional *Clec7a* genes were more resistant than B6 mice (Fig. 7B).

DISCUSSION

People regularly inhale *Aspergillus* sp. spores, and *Candida* spp. are essentially part of the normal mucosal flora, but those fungi do not cause invasive infections in immunocompetent hosts. From this we can conclude that people have highly functional immune systems that prevent invasive infections by these ubiquitous fungi. It is likely that we accomplish this with a highly modulated inflammatory response to avoid unnecessary and potentially damaging inflammation (33), a phenomenon that Romani and Puccetti call "protective tolerance to fungi" (34). In contrast, endemic dimor-

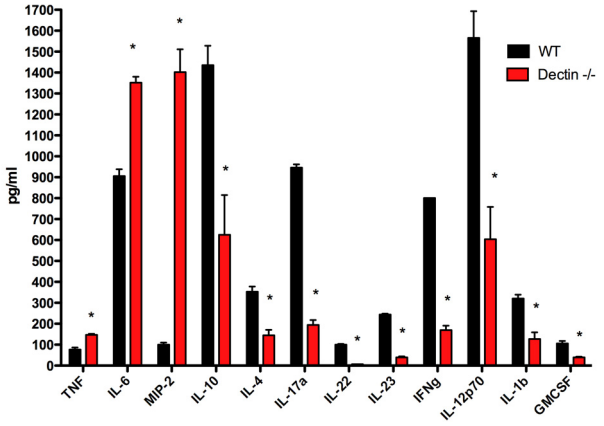

FIG 6 Cytokine concentrations in BALF from mice 14 days after i.n. infection. Data are means ± SEM from 4 mice. WT, wild-type B6 mice. *, $P < 0.05$ compared to B6 BALF concentrations. Note that TNF, IL-6, and MIP-2 concentrations were higher in BALF from Dectin-$1^{-/-}$ mice, whereas all the other measured cytokine concentrations were higher in BALF from B6 mice. We did not detect TGF-β or IL-1α in BALF.

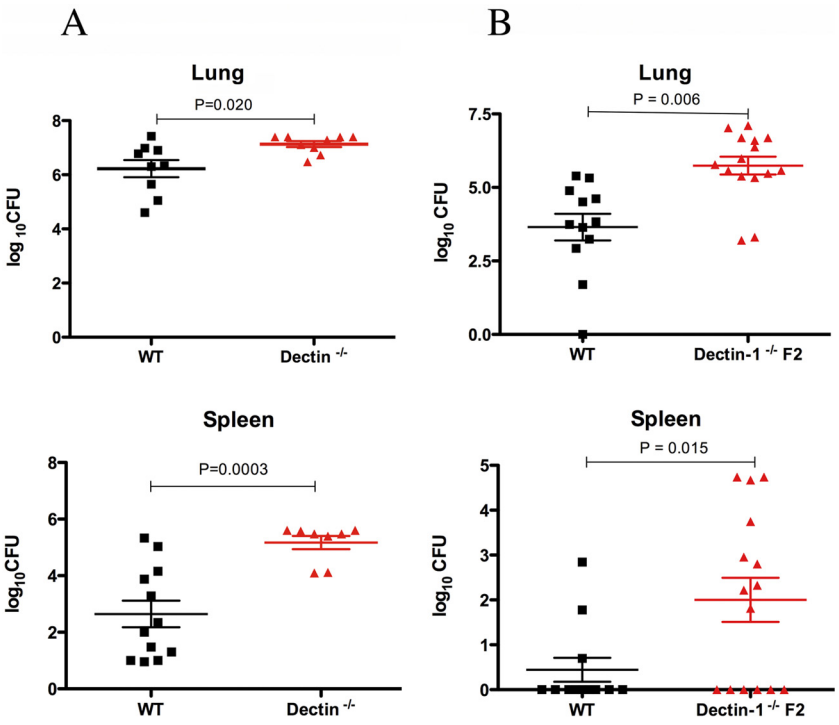

FIG 7 Colony counts of *C. immitis* from lungs and spleens of mice infected i.n. 14 days earlier. (A) B6 (WT) compared to Dectin-1$^{-/-}$ mice in a B6 genetic background. (B) (B6*Clec7a*^{tm} × DBA/2) mice homozygous for *Clec7a* (WT) compared to F_2 mice homozygous for the mutant *Clec7a* gene (Dectin-1$^{-/-}$ F2). Each symbol represents an individual mouse. The horizontal bars are the geometric means ± SEM. Groups were compared with unpaired *t* tests (Prism5; GraphPad).

phic fungi, such as *C. immitis*, are able to cause pneumonia in otherwise healthy individuals, but most patients mount an immune response to the organism that resolves the infection, sometimes at the risk of developing necrotizing granulomas that can damage host tissues. Inbred strains of mice are susceptible to coccidioidomycosis to various degrees, and strains that respond with "tolerance" to *Candida* and *Aspergillus* are rapidly killed by *C. immitis* (35).

Dectin-1 is an important component of the innate immune response to environmental fungi, a response that does not involve formation of granulomas (30). Dectin-1 is a C-type lectin receptor that recognizes β-1,3-glucans in a Ca^{2+}-independent manner (36, 37). Binding of β-glucan to Dectin-1 leads to recruitment of Syk and CARD9 to the intracellular domain of the receptor and then activation of Bcl-10 and MALT1 (38). Cytokine production by β-glucan-stimulated macrophages and DCs results from activation of the transcription factor NF-κB (39). β-Glucans are major structural components of fungal cell walls, so Dectin-1 is an innate immune recognition receptor for defense against fungal infections (40). Dectin-1 stimulates DCs to secrete proinflammatory cytokines and chemokines that shape the acquired immune response (25). In particular, Dectin-1 activation biases the immune response toward Th17, a response that is protective against mucocutaneous fungal infections (41).

The singular importance of Dectin-1 in defense against certain opportunistic fungal infections was established using Dectin-1$^{-/-}$ mice; they are more susceptible to hematogenous *C. albicans* infection (42), *Aspergillus fumigatus* infection (43, 44), and *Pneumocystis carinii* pneumonia (45). The relevance of Dectin-1 to human health was demonstrated by the discovery of a people who were homozygous for an inactivating mutation in *Clec7a* (46). That mutation predisposes to a mild form of mucosal candidiasis but not invasive candidiasis (47). Interestingly, an inactivating mutation in CARD9 predisposes individuals to a more severe form of mucocutaneous candidiasis, which is consistent with CARD9's being an intermediate in signaling pathways for at least two other C-type lectins that also recognize *C. albicans* (48).

In contrast to its role in defense against opportunistic fungi, Dectin-1 has not been shown to be required for resistance to pathogenic fungi (organisms that can cause disease in otherwise healthy individuals with no known immunological deficiencies). Dectin-1 is not required for resistance to *Blastomyces dermatitidis* (13), *Histoplasma capsulatum* (49), or *Cryptococcus neoformans* (50). The yeast form of *Paracoccidioides brasilensis* also has an external layer of α-glucan that obscures the underlying structural β-1,3-glucan (51), and *C. neoformans* has a thick capsule composed of galactoxylomannan and glucuronoxylomannan that serves the same purpose (52). In contrast, we found that *C. immitis* had surface-exposed β-glucan in all phases of its life cycle except immature hyphae (Fig. 4). Therefore, the pathogenesis of coccidioidomycosis must be fundamentally different from that of the other two endemic dimorphic pathogenic fungi, in that *Coccidioides* must deal successfully with the consequences of activating Dectin-1. How it does that is not yet known.

In this paper, we show that Dectin-1 deficiency made mice more susceptible to pulmonary infection with *C. immitis*. This was true whether the *Clec7a* mutation was in the B6 genetic background or in the (B6 × DBA/2)F_2 background (Fig. 7). B6 mice are genetically susceptible to *C. immitis*, but the F_2 mice were more resistant to coccidioidomycosis by virtue of an admixture of genes from the resistant DBA/2 strain (19). Carvalho et al. recently showed that consequences of inactivation of *Clec7a* are greatly affected by the genetic background of the host; BALB/c mice are more resistant to gastric candidiasis than B6 mice, and infection was not exacerbated by the *Clec7a* mutation in BALB/c mice (53). This supports our concern regarding generalizations about the role of Dectin-1 from the B6 experiment. However, since we found that both deficient genetically susceptible and genetically resistant mice had significantly more fungi in their lungs and spleens 14 days after i.n. infection, we believe the finding is generally applicable. Even though the resistant mice we infected were not inbred, the Dectin-1 deficiency overwhelmed any variation in innate resistance in the second generation.

To learn how Dectin-1 deficiency contributed to susceptibility, we determined how Dectin-1 deficiency changed cytokine production *in vitro* and *in vivo*. Since many different cell types are attracted to the infected lungs, and several cytokines and chemokines can be made by many different cell types, including epithe-

lial cells and NK cells, we chose to measure the concentration of cytokines in infected lungs rather than to enumerate the numbers of cytokine-secreting $CD4^+$ T cells. In interpreting our results, we need to be cognizant that the B6 responses are inadequate to direct a protective immune response against coccidioidomycosis, so we were looking for different degrees of inadequate immune responses. Genetically resistant DBA/2 mice make more vigorous Th1 and Th17 responses to this infection than do B6 mice (54, 55). Nevertheless, Dectin-1-deficient B6 mice had even lower levels of the cytokines that are known to be required for immunity to coccidioidomycosis in humans and mice, including TNF-α, the Th1 cytokines IL-12p70 and IFN-γ, and the Th17-related cytokines IL-1β, IL-23, IL-17a, and IL-22 (Fig. 6). IL-6, which also plays a role in Th17 production, was more abundant in the BALF from Dectin-1-deficient mice, perhaps as an acute-phase response to the severe infection. TGF-β works in concert with IL-6 to generate Th17 differentiation *in vitro*, but there was no measurable TGF-β in the BALF of either strain, which could explain why B6 mice do not make a vigorous Th17 response to *C. immitis* (54). In the absence of TGF-β, dually functional T cells that express both IFN-γ and IL-17 can develop. IL-10 also can affect the development of T cell lineages, and Dectin-$1^{-/-}$ mice had only about half the concentration of IL-10 found in WT mice, which could be because β-glucans stimulate IL-10 secretion via Dectin-1 (56). IL-10 was still present at a concentration of 600 pg/ml in Dectin-$1^{-/-}$ BALF, so there must be other pathways that lead to IL-10 production, perhaps by regulatory T cells.

B6 mice have a functional Dectin-1 on DCs and macrophages, and it would be expected to trigger a vigorous innate immune response to the infection and an adaptive immune response biased toward Th17 T cells, but it does not in fact do so (41, 57). One possible explanation is that the short isoform of Dectin-1 is less functional in this infection (22). Another possible explanation is that expression of Dectin-1 on infiltrating hematopoietic cells in the lungs of infected B6 mice is far from maximal; we found that the level of Dectin-1 expression was only 20% of the level in DBA/2 lungs (Fig. 5). *In vitro*, Dectin-1 expression is upregulated by GM-CSF, IL-3, and IL-13 and transcriptionally down-regulated by IL-10 and dexamethasone (58). Since B6 mice make more IL-10 in response to this infection than do DBA/2 mice, this may be one reason why the latter are more resistant to this infection (55). Despite being down-regulated in B6 lungs, Dectin-1 clearly was still important, as Dectin-$1^{-/-}$ mice were more susceptible to infection.

Cytokine levels after *in vitro* stimulation of peritoneal macrophages and BMDC (Fig. 1 and 2) could not be directly correlated with the differences in cytokines found in infected lungs. For example, we could not detect IL-23p19 or IL-12 p70 in BMDC supernatants, but we detected IL-23p19 and IL-12p70 in the B6 BALF. *In vitro*, IL-1β was secreted by WT and Dectin-$1^{-/-}$ BMDC but not by elicited peritoneal macrophages, while *in vivo*, the IL-1β concentration was 50% lower in Dectin-$1^{-/-}$ than WT BALF. Regulation of IL-1β production by macrophages is complex; NF-κB upregulates transcription of pro-IL-1β, which needs to be proteolytically processed by caspase 1 in the inflammasome to form biologically active IL-1β, which is secreted (59). Recently, the β-glucan curdlan was shown to induce both transcription of pro-IL-1β and its processing to IL-1β by human DC in a Dectin-1-dependent process that uses caspase 8, not caspase 1 (60). However, compared to WT DC, nearly as much IL-1β is made by Dectin-1-deficient DC stimulated with *C. albicans*, so apparently there are other pathways to IL-1β, as we found in this study. The receptors and signaling pathways that are activated by spherules to induce IL-1β and TNF-α independently of Dectin-1 are still unknown and will be the subject of future investigations.

There are several ways in which the *Clec7a* mutation could have compromised the immune response to *C. immitis*. FKS and A-FKS adhered less well to BMDC lacking Dectin-$1^{-/-}$ than to B6 BMDC, showing that Dectin-1 was the major opsonic receptor on BMDC for this fungus, although the spherules we used were too large to be ingested by BMDC. The small amount of residual binding of A-FKS to Dectin-$1^{-/-}$ BMDCs and peritoneal macrophages may have been through an alternate β-glucan receptor such as complement receptor 3 (CR3) or one of the scavenger receptors CD5, CL-P1, SCARF1, and CD36 (61). This could also explain the very modest effect of Dectin-1 deficiency on adhesion to macrophages. Because of the need for FKS to attach to BMDC in order to activate those cells, some of the decreases in cytokine production by BMDC that we observed *in vitro* may have resulted in part from decreased contact between the fungal particles and the Dectin-$1^{-/-}$ BMDC. However, since TNF-α and IL-1α and IL-1β secretion by BMDC was not diminished by Dectin-1 deficiency, we do not think that decreased contact can be the explanation for the differences in cytokine levels.

As Romani recently emphasized, fungi can be recognized by many different receptors on macrophages and dendritic cells, and those cells may respond differently to different fungi and even to different forms of the same fungus, depending on which combination of receptors is engaged (62). In addition to C-type lectin receptors, TLR on macrophages and BMDC are also involved in responding to *Candida* yeast cells (63), and this is also true for spherules (24). How these two pathways interact to mediate NF-κB activation is not completely understood, but they can act synergistically to secrete cytokines (64) and to eliminate fungal infections. For instance, the fungus *Fonsecaea pedrosoi*, which causes skin and subcutaneous infections, was recently shown to bind to the C-type lectin receptors Dectin-1 and macrophage-inducible C-type lectin (Mincle) but not to any TLR, and as a consequence, the fungus is unable to trigger proinflammatory cytokine secretion by macrophages and BMDC (65). When a TLR7 agonist is applied to the infected skin, it activates cytokine responses that control the infection. *Coccidioides* spherules behave more like *C. albicans* than *F. pedrosoi* in that FKS interact directly with TLR2, thus providing both signals necessary to activate macrophages to secrete TNF-α, MIP-2, and IL-6 (24).

How activated macrophages kill spherules is not known. Binding of β-1,3-glucan to Dectin-1 on macrophages stimulates the respiratory burst (66), but $gp91^{phox}$ mutant mice are not more susceptible to coccidioidomycosis than their wild-type counterparts (67). Inhibition of NO production does modestly increase susceptibility to this infection (68).

In summary, Dectin-1 is required for resistance to the dimorphic pathogen *C. immitis* in B6 mice. Despite stimulating host mononuclear phagocytes via Dectin-1 and TLR2, *Coccidioides* organisms are capable of infecting normal mice and people and causing a severe granulomatous pneumonia. We propose that Dectin-1 activation is central to directing the adaptive immune response toward Th1 and Th17 pathways, thereby leading to resolution of infection in most normal hosts, by as-yet-unknown mechanisms. Polymorphisms in the Dectin-1 pathway may ex-

plain why some people do not mount an adaptive immune response that leads to cure of the infection.

MATERIALS AND METHODS

Mice. *Clec7a* knockout mice in a B6 background were originally created by Gordon D. Brown (42) and were kindly provided by Stuart Levitz (University of Massachusetts Medical School, MA). BALB/c, B6, and DBA/2 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). (B6*Clec7a*^{tm} × DBA/2)F_2 mice were generated in house, and all female offspring were screened by PCR to detect homozygous *Clec7a* mutant and wild-type progeny. All experiments were carried out by procedures authorized by the Institutional Animal Care and Use Committee of the VA San Diego Healthcare System.

Fungus. *Coccidioides immitis* strain RS was used for all *in vivo* infections. The infection was performed as described previously (68). We used formalin-killed spherules (FKS) that were harvested 96 h after culturing of arthroconidia in Converse medium (a generous gift from John Galgiani, University of Arizona) for *in vitro* activation of cells. To remove mannose residues without destroying β-glucan, we boiled the FKS in 10 N NaOH for 30 min to remove neutral sugars other than β-1,3- and β-1,6-glucan and then washed the alkali-treated spherules 5 times with phosphate-buffered saline (PBS) (69).

Infection. Mice, infected intranasally (i.n.) with ~50 arthroconidia (68), were sacrificed 14 days later to do quantitative cultures of the lungs and spleen and to obtain bronchoalveolar lavage fluid (BALF) as described below.

Genotyping of *Clec7a* mutants. Mouse tail DNA was extracted and PCR amplified using an Extract-N-Amp tissue PCR kit (Sigma). If DNA was further purified with phenol-chloroform extraction, the PCR was carried out using PCR ready mix (Sigma). The PCR products were analyzed by agarose gel electrophoresis. To identify Dectin-1$^{-/-}$ mice, three primers were mixed: GCCAATGCTGCCGACTCCAG (forward) and GC TGTAACTTCTGAAGAAAAC (reverse) to produce a 300-bp product from the wild-type allele and GCGCGCCCCTCGAGCTAGAG (reverse) to produce a 150-bp product from the mutated gene.

Quantification of Dectin-1 expression in mouse lungs. Dectin-1 mRNA was quantified using Sybr green real-time PCR (Bio-Rad, CA) with the ABI Prism 7000 SDS v1.1 detection system (Applied Biosystems, Foster City, CA), and the fold increase in expression of Dectin-1 mRNA was normalized against the expression of glyceraldehyde-3-phosphate dehydrogenase (GADPH) mRNA. The following primers were used: for exon 6 of Dectin-1 gene: ATCAGCATTCTTCCCCAACTCG (forward) and CAGTTCCTTCTCACAGATACTGTATGA (reverse). The GADPH primers used in the real-time PCRs were TGCAGTGGCAAAGTGGA GATT (forward) and TGGAACATGTAGACCATGTAGTTGAG (reverse).

Mouse peritoneal macrophage activation. Peritoneal macrophages were elicited with sodium periodate and harvested 4 days after injection (70). We cultured 1 × 10^6 cells/ml in high-glucose Dulbecco's modified Eagle medium (DMEM) with 10% fetal calf serum (FCS) in a 48-well tissue culture plate and activated them at as described previously (24). We used the synthetic TLR2 agonist Pam3CysSerLys4 (Pam3CSK4) as the control for activation.

Mouse bone marrow-derived dendritic cells (BMDC). We cultured bone marrow cells obtained from mouse femurs and tibias in RPMI with 10% fetal calf serum in the presence of 5 ng/ml recombinant murine GM-CSF (BD Bioscience) as described by Lutz et al. (71). New medium was added on day 3, and the nonadherent cells were harvested on day 6. DCs were positively selected for using anti-CD11c magnetic beads (Miltenyi) according to the manufacturer's protocol. The BMDC were plated at a density of 1 × 10^6 cells/ml and activated in RPMI with 10% FCS.

Mouse bronchoalveolar lavage. Mice were euthanized by asphyxia in a CO_2 environment. The trachea was exposed and intubated with a 16-gauge blunt-end needle. Four successive installations into the trachea of 0.2 ml of sterile saline were aspirated as completely as possible using a 1.0-ml syringe. Cells in the aspirates were sedimented by centrifugation at 400 × *g* for 10 min at 4°C. The lung lavage supernatant was then centrifuged through a 0.45-μm centrifuge tube filter (Spin-X; Costar) to remove any remaining fungal cells. The lavage fluid was assayed immediately for cytokines or stored at −20°C until used.

Cytokine assays. Mouse cytokines in BALF were measured using enzyme-linked immunosorbent assay (ELISA) kits as described in the manufacturer's protocols. The ELISA kits for TNF-α, IL-6, IL-4, IL-10, IL-17, IL-23, IL-12p70, TGF-β, and IFN-γ were purchased from eBioscience (San Diego, CA). The assay kits for IL-1β, IL-12p40, and GM-CSF were from BD Biosciences; MIP-2 and IL-22 assay kits were from R&D. The FlowCytomix multiplex Th1/Th2/Th17/Th22 13-plex cytokine assay (eBioscience) was used in the experiments with isolated macrophages and BMDCs.

Soluble mouse Fc-tagged Dectin-1 (mDectin-1-Fc). cDNA coding for the truncated form of Dectin-1 (isoform B) were generated from C57BL/6 macrophage RNA using Superscript VILO (Invitrogen). PCR product was made using TaKaRa LA *Taq* polymerase (TaKaRa Bio USA, Mountain View, CA) with the primers 5′ ACTGAATTCGTTTTGGCGA CACAATTCAGGGAGAAATC 3′ and 5′ ACTAGATCTCAGTTCCTTC TCACAGATACTGTATG 3′. The DNA was cloned into the EcoRI and BglII sites of pFuse-mIgG2A-Fc2 (IL-2ss) plasmid (InvivoGen, San Diego, CA) to generate a DNA construct coding for Dectin-1 protein with IL-2 signal sequence at the N terminus and the Fc portion of mouse IgG at the C terminus. Plasmid DNA was made from transformed *Escherichia coli* DH5α cells using an Endo-Free plasmid maxikit (Qiagen). The recombinant plasmid was transiently transfected into CHO cells using Lipofectamine (Invitrogen). The cells were cultured in medium with IgG-reduced fetal bovine serum. The recombinant protein was purified using protein A affinity chromatography (Thermoscientific Pierce). Control Fc-only protein was made in the same manner using vector alone DNA. To assay for binding of mDectin-1–Fc to *C. immitis*, the live *in vitro*-grown fungi were incubated with 1 μg/ml of mDectin-1-Fc or control Fc fusion protein on an orbital shaker for 30 min at room temperature. The cells were washed with PBS and incubated with goat anti-mouse IgG2a conjugated to FITC (Life Science, Carlsbad, CA) for 30 min. The cells were washed, fixed with 1% formaldehyde, and viewed under a fluorescence microscope.

Attachment of dendritic cells to FKS. CD11c-positive BMDC from C57BL/6 and Dectin-1$^{-/-}$ mice were placed in 16-well glass Lab-Tek tissue culture chamber slides (Thermo, Fisher, Waltham, MA) at 2 × 10^4 cells per well. FKS or A-FKS was added at two ratios in duplicate, the slide was incubated for 18 h at 37°C in a 5% CO_2 atmosphere, and then nonadherent spherules were washed off with three rinses in PBS. Each step was done in duplicate. The slides were stained with Wright-Giemsa stain and then examined using a 50× oil objective by J.F., who was unaware of the well assignments.

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