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FUNGAL AND PARASITIC INFECTIONS

Myeloid Differentiation Factor 88 and Interleukin-1R1 Signaling Contribute to Resistance to *Coccidioides immitis*

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ABSTRACT Rodents are a natural host for the dimorphic pathogenic fungi *Coccidioides immitis* and *Coccidioides posadasii*, and mice are a good model for human infection. Humans and rodents both express Dectin-1 and Toll-like receptor 2 (TLR2) on myeloid cells, and those receptors collaborate to maximize the cytokine/chemokine responses to spherules (the tissue form of the fungi) and to formalin-killed spherules (FKS). We showed that Dectin-1 is necessary for resistance to pulmonary coccidioidomycosis, but the importance of TLR2 *in vivo* is uncertain. Myeloid differentiation factor 88 (MyD88) is the adapter protein for TLR2 and -4, interleukin-1R1 (IL-1R1), and IL-18R1. MyD88/TRIF$^{-/-}$ and MyD88$^{-/-}$ mice were equally susceptible to *C. immitis* infection, in contrast to C57BL/6 (B6) controls. Of the four surface receptors, only IL-1R1 was required for resistance to *C. immitis*, partially explaining the susceptibility of MyD88$^{-/-}$ mice. We also found that FKS stimulated production of IL-1Ra by bone marrow-derived dendritic cells (BMDCs), independent of MyD88 and Dectin-1. There also was a very high concentration of IL-1Ra in the lungs of infected B6 mice, supporting the potential importance of this regulatory IL-1 family protein in the largely ineffective response of B6 mice to coccidioidomycosis. These results suggest that IL-1R1 signaling is important for defense against *C. immitis* infection.

KEYWORDS *Coccidioides*, innate immunity, MyD88, IL-1R1, IL-18, TLR2, TLR4, IL-1Ra, β-glucan

Coccidioidomycosis is a fungal infection endemic to the Southwestern United States and adjacent areas in northern Mexico. It is estimated that 150,000 new infections occur annually, and the incidence is increasing in Arizona and California (the two states where it is a reportable disease) (1, 2). The area of endemicity may also be expanding, as infections have occurred as far north as Washington State (3). The etiological agents are two closely related dimorphic fungi, *Coccidioides immitis* and *Coccidioides posadasii*, both of which are native to the alkaline desert soil that is part of the Lower Sonoran life zone in the Western Hemisphere. The fungi grow as molds in the soil, where they form arthroconidia (spores) that are small enough to be inhaled into the lungs after they are aerosolized. Under conditions of increased CO_2 and body temperature, arthroconidia undergo a complex metamorphosis into spherules, a unique parasitic form that is pathognomonic of coccidioidomycosis. At maturity, spherules are large (>100 μm in diameter), spherical, thick-walled structures that contain hundreds of nucleated endospores. When spherules rupture, they release their endospores, which can mature into spherules by isotropic growth.

In humans and mice, functional CD4$^+$ T cells are required to prevent extrapulmonary dissemination, the most severe complication of infection (4, 5). The importance of the interleukin-12 (IL-12)/gamma interferon (IFN-γ) pathway is confirmed by case

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reports of disseminated infections in people with Mendelian mutations in that pathway (6, 7) and by studies with mutant mice (8). Tumor necrosis factor alpha (TNF-α) is also important for host defense in mice (9), and the use of anti-TNF-α antibodies is a risk factor for severe infections (10). However, the vast majority of people who develop disseminated coccidioidomycosis have no known genetic or acquired immunodeficiencies and do not appear to be unusually susceptible to other infections.

The biggest risk factor for dissemination in otherwise healthy, nonpregnant people is being an African-American or a Pacific Islander, implying that susceptibility is genetically determined (11, 12). It is likely that host genetics determine the nature of the immune response to *Coccidioides*, as the immune response differs in people who have self-limited infections and in those who have disseminated infections (13). Inbred mice also differ greatly in susceptibility to *C. immitis* infection (14–16). C57BL/6 (B6) and BALB/c mice are highly susceptible to that infection, while DBA/2 mice are very resistant. Recombinant inbred BXD strains also vary greatly in susceptibility to *C. immitis*, and resistance is a multigenic trait in BXD mice (15).

Since the innate immune response can profoundly influence the acquired immune response, we have concentrated our studies on the innate immune responses to *C. immitis* in a mouse model of infection. We compared the responses of genetically resistant DBA/2 mice and B6 mice and found that those two strains differentially splice *Clec7a*, the gene that encodes the β-glucan receptor (Dectin-1). Spherules attach better to the full-length form of Dectin-1 that is made by DBA/2 mice than to the truncated version of Dectin-1 made by B6 mice. Furthermore, recombinant inbred BXD strains that make the truncated form of Dectin-1 both are more susceptible to infection and make more IL-10 after infection (15). However, even the truncated form of Dectin-1 plays a nonredundant role in resistance to *C. immitis* infection, as *Clec7a* (Dectin-1)$^{-/-}$ mice on a B6 background are more susceptible to infection than the parental B6 strain (17). This fits with the concept that C-type lectin receptors (CTLRs), which are expressed on myeloid cells, some B cells, and epithelial cells, orchestrate antifungal immunity (18). A null mutation of Dectin-1 predisposes people to mucosal and skin fungal infections (19). Dectin-1 and other C-type lectin receptors signal through Card 9, and people with mutation of that gene are predisposed to mucosal and invasive fungal infections (20), confirming that this pathway is important for defense against fungal infections in humans as well as in mice.

The importance of Toll-like receptor 2 (TLR2) signaling in resistance to coccidioidomycosis is less certain. In this study, we infected mice with targeted mutations in myeloid differentiation factor 88 (MyD88) and 4 receptors that use MyD88 to determine if they were required for resistance to coccidioidomycosis.

RESULTS

Mouse macrophages and dendritic cells require Dectin-1, TLR2, and MyD88 to secrete cytokines and chemokines in response to stimulation with formalin-killed spherules (FKS) (21), so we anticipated that elicited peritoneal macrophages with mutations in both MyD88 and TRIF (TIR-domain-containing adapter-inducing IFN-β) would be less responsive to FKS, and they were (Fig. 1). We then stimulated bone marrow-derived dendritic cells (BMDCs) from MyD88$^{-/-}$, MyD88/TRIF$^{-/-}$, and control B6 mice with FKS. In contrast to elicited macrophages, MyD88/TRIF$^{-/-}$ and MyD88$^{-/-}$ secreted more TNF-α, IL-10, and IL-6 than did control BMDCs (Fig. 2). We repeated both experiments, with essentially the same results. We were unable to detect IL-1β, IL-12p70, granulocyte-macrophage colony-stimulating factor (GM-CSF), or IL-23 (p19) secreted by either B6 or the mutant BMDCs or macrophages.

Because FKS expresses β-glucan on its surface that is recognized by soluble Dectin-1 (17), we asked whether highly purified particulate β-glucan and FKS are equivalent stimuli for B6 BMDCs. FKS was approximately 10 times more potent as a stimulus for TNF-α and IL-6 than β-glucan, and β-glucan did not elicit an IL-10 response (Fig. 3). This suggests that FKS engages other receptors to amplify signaling, which are unlikely to be TLRs given the results shown in Fig. 2. One caveat in interpreting this comparison

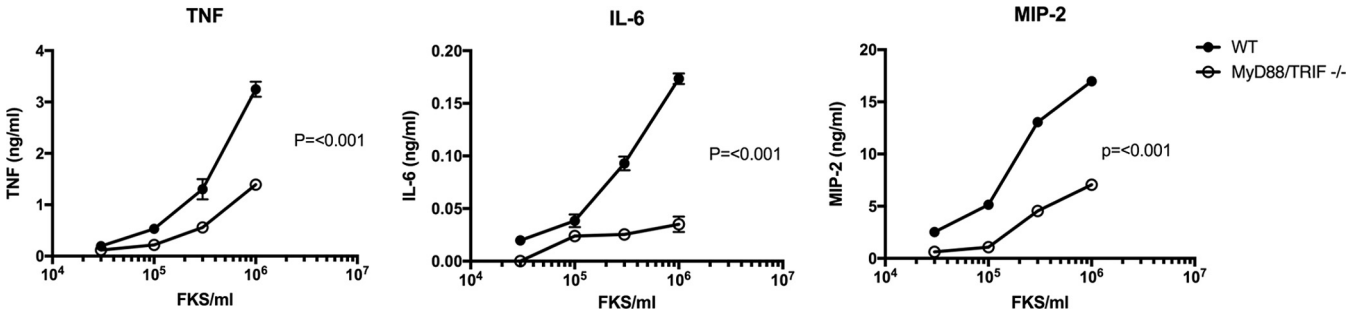

FIG 1 FKS stimulation of cytokine and chemokine production by MyD88/TRIF$^{-/-}$ mouse elicited peritoneal macrophages is impaired. Each point is the average of duplicate wells. Shown are results of one of two independent experiments with essentially the same results. Note the different scales on the *y* axis for the three graphs. WT, B6 cells. Differences between the dose-response curves of B6 and mutant cells were compared by calculating area under the curve and applying Welch's *t* test.

is that FKS and β-glucan are both particulate (22), so the quantities of the two stimuli may not have been comparable, even though we used amounts of particulate β-glucan that have been used by others in cell activation assays, and the highest concentration of β-glucan that we used was turbid. Furthermore, not all preparations of β-glucan have the same potency, and larger particles that cannot be ingested are more potent stimuli for cytokine secretion (22, 23).

Since there is evidence that Dectin-1 and TLR2 act synergistically to enhance cytokine secretion by FKS-stimulated myeloid cells (21), we asked whether TLR signaling was required for resistance to infection. The adapter protein for TLR3 is TRIF. TLR4 uses either TRIF or MyD88, and the adapter for all other TLRs is MyD88. Therefore, we assessed the susceptibility of MyD88, MyD88/TRIF, TLR4, and TLR2 mutants to *C. immitis* infection (Fig. 4). Both MyD88$^{-/-}$ and MyD88/TRIF$^{-/-}$ mice were more susceptible than B6 mice, as manifested by increased dissemination of the fungi from lungs to spleen. MyD88$^{-/-}$ mice also had significantly more organisms in their lungs; the difference in lung CFU recovered from B6 and MyD88/TRIF$^{-/-}$ mice did not quite reach

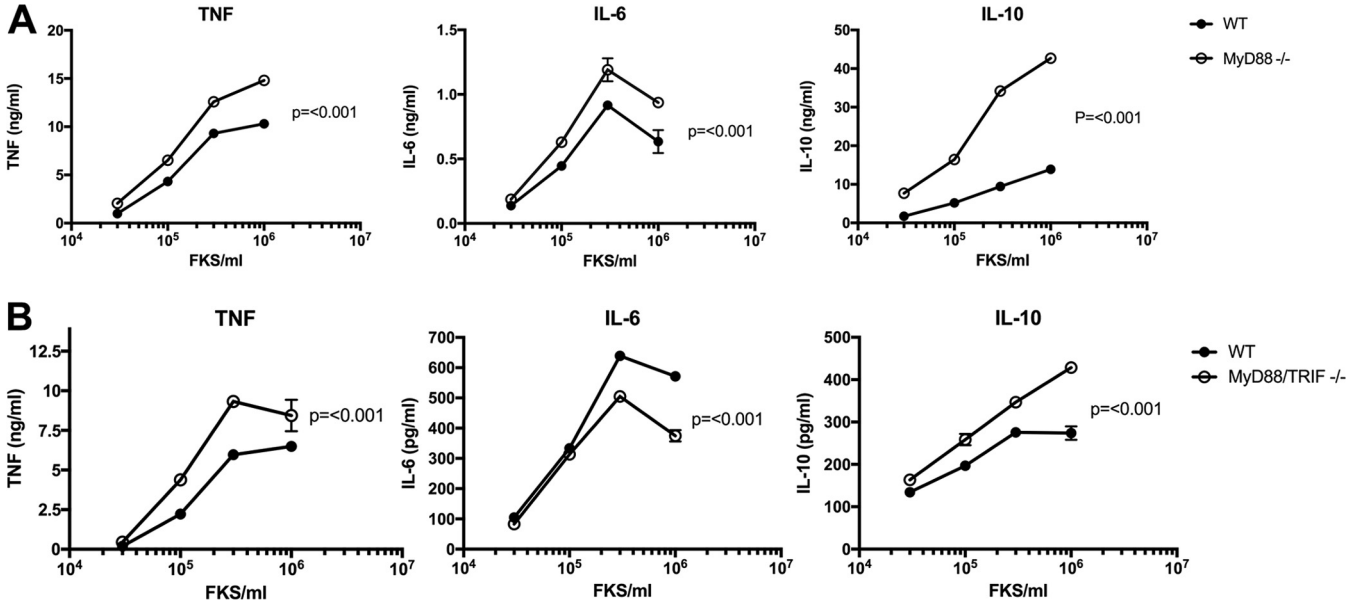

FIG 2 Cytokine responses to FKS by MyD88$^{-/-}$ and MyD88/TRIF$^{-/-}$ BMDCs are not impaired. Error bars show SEM based on triplicate measurements (if there are no error bars, the SEM was smaller than the symbol). (A) MyD88$^{-/-}$ cells made more TNF-α, IL-6, and IL-10 in response to FKS. (B) MyD88/TRIF$^{-/-}$ BMDCs made significantly more TNF-α and IL-10 than did B6 cells. Differences between the dose-response curves of B6 and mutant cells were compared by calculating area under the curve and applying Welch's *t* test.

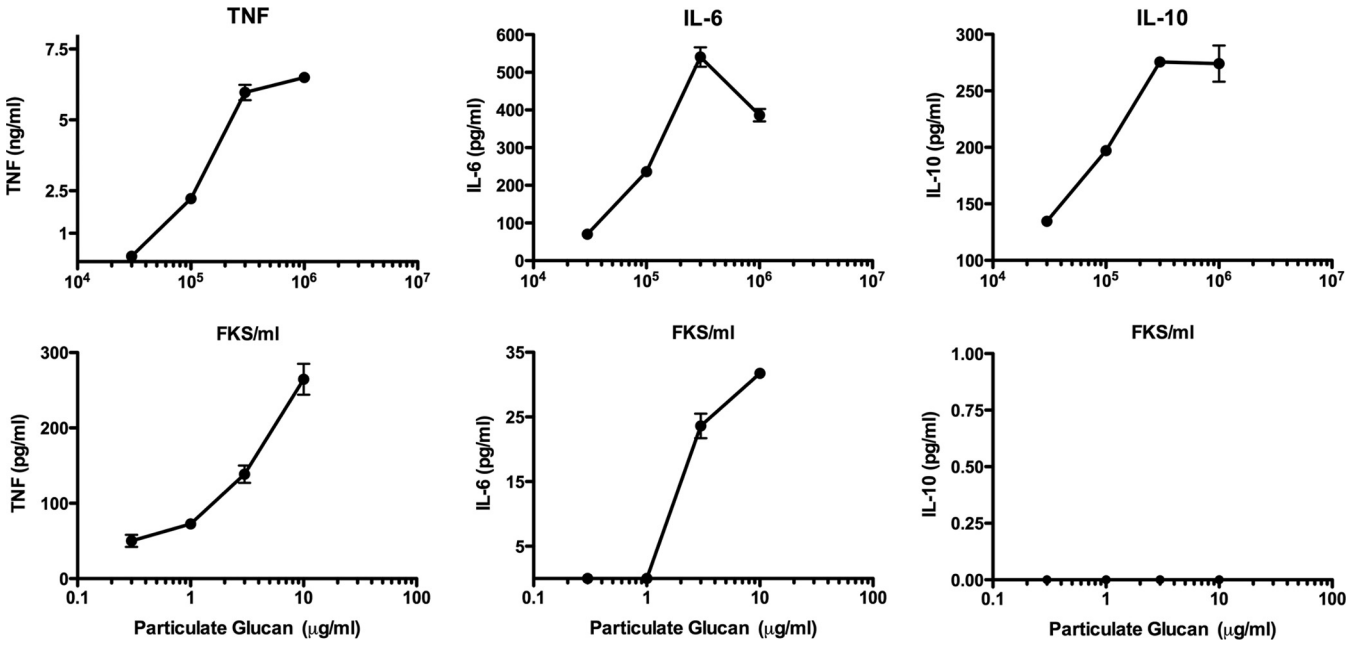

FIG 3 FKS is a more potent activator of BMDC than purified particulate curdlan. B6 BMDCs at a concentration of 1×10^6/ml were incubated overnight with increasing numbers of FKS or increasing concentrations of highly purified β-D-glucan (curdlan). Both particles stimulated secretion of TNF-α and IL-6, although FKS was significantly more potent. Purified β-D-glucan did not stimulate IL-10 production. Note differences in the scales.

statistical significance (Fig. 4A). We concluded that TRIF deficiency did not add to the susceptibility produced by MyD88 deficiency.

In contrast to the MyD88 results, neither TLR2 (Fig. 4A) nor TLR4 (Fig. 4B) was required for resistance to coccidioidomycosis. The latter result confirms the report of Awasthi that TLR4-deficient C3H/HeJ mice are not more susceptible than a control C3H strain (24) We also found that TLR4$^{-/-}$ mice on the B6 genetic background were not

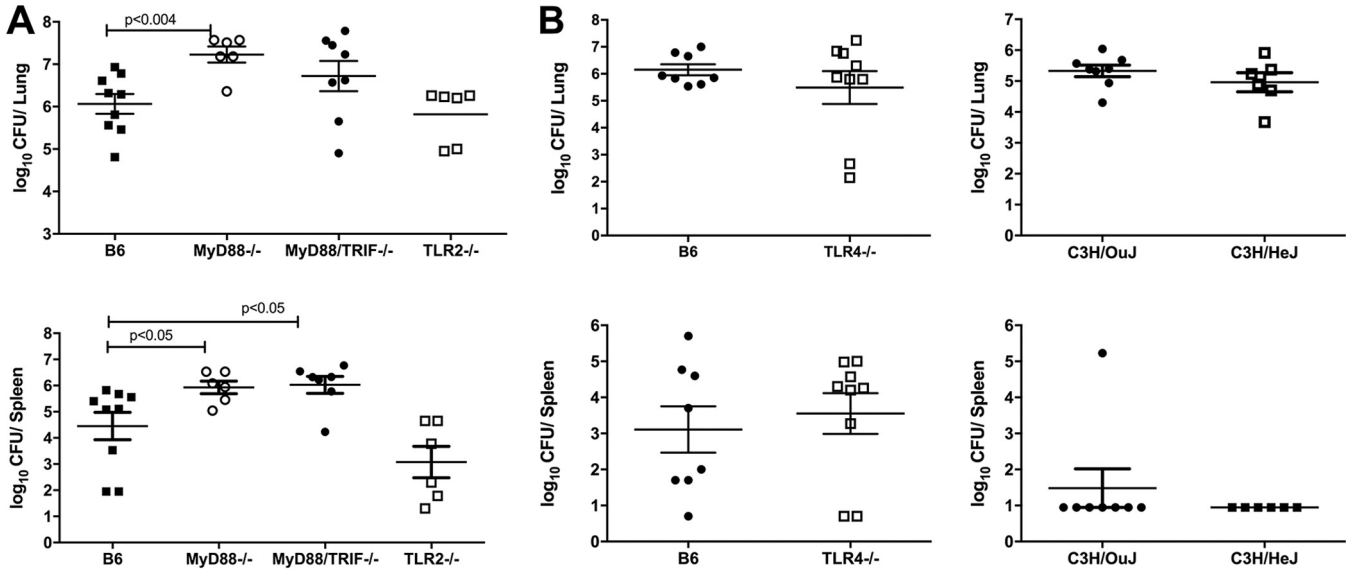

FIG 4 MyD88 is necessary for resistance to *C. immitis* infection but TLR2 and TLR4 are not. CFU in the lungs and spleens were measured 14 days after infection. (A) MyD88$^{-/-}$, MyD88/TRIF$^{-/-}$, and TLR2$^{-/-}$ mice were compared to B6 mice. Each symbol represents an individual mouse, and the horizontal bar shows the geometric mean. MyD88$^{-/-}$ mice had significantly more organisms in their lungs and spleens than did B6 mice. There was no significant difference between MyD88$^{-/-}$ and MyD88/TRIF$^{-/-}$ double-KO mice. TLR2$^{-/-}$ mice had lower median numbers of CFU in lungs and spleens than control B6, but those differences did not reach statistical significance. (B) We infected both B6 mice with a targeted mutation in TLR4 and C3H/HeJ mice, which have a spontaneous inactivating mutation in TLR4, and appropriate control strains. There were no significant differences between the numbers of organisms recovered from TLR4 mutants and controls.

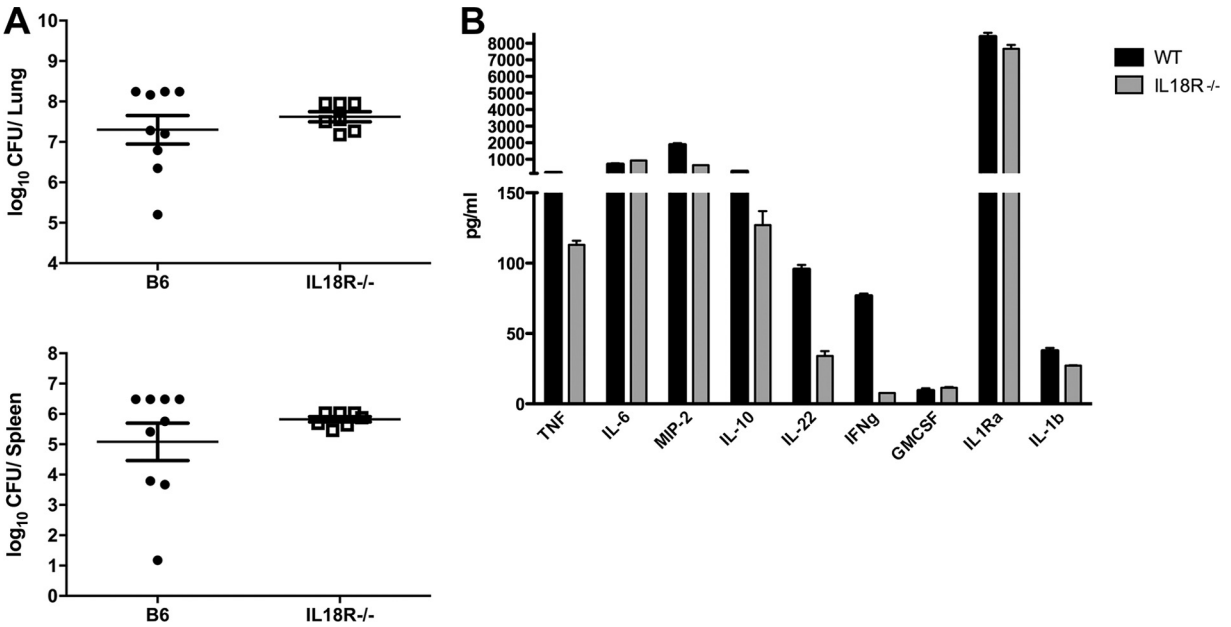

FIG 5 IL-18 signaling in B6 mice is not required for resistance to *C. immitis* infection. We infected IL-18R1$^{-/-}$ mice and B6 controls. In this experiment, mice were not sacrificed until 16 days postinfection to allow time for an acquired TH1 immune response to develop. (A) Comparison of CFU in lungs and spleens showing no significant differences in the geometric means. (B) Cytokine levels (mean of two replicate measurements) in BALF from four B6 mice (black bars) and four IL-18R1$^{-/-}$ mice (gray bars). B6 mice made significantly more TNF-α, IL-10, IL-22, and IFN-γ. However, the concentrations of IL-22 and IFN-γ were very low, even in B6 controls. Note the very high and nearly identical concentrations of IL-1Ra in both groups.

more susceptible than controls. Thus, TLR4 was not necessary for resistance to *C. immitis* in mice on two different genetic backgrounds. After we did this experiment, Hung et al. published findings showing that TLR2-deficient B6 mice are not more susceptible to coccidioidomycosis (25), so we also confirmed their result. Of note, in this pulmonary infection model, C3H mice were more resistant to pulmonary infection than B6 mice, as reflected both in lower lung counts and in lack of dissemination to the spleen; we previously showed that C3H mice are about 10-fold more resistant to *C. immitis* peritonitis than B6 mice (14).

Since MyD88$^{-/-}$ mice were more susceptible to *C. immitis* infection, but neither TLR2$^{-/-}$ nor TLR4$^{-/-}$ mice were more susceptible, we infected mice with mutated IL-1R1 or IL-18R, since those receptors also use MyD88 as their adapter protein. IL-18 induces IFN-γ production (26) and IFN-γ is required for acquired resistance to coccidioidomycosis in mice and people (27) (28), so we expected IL-18R1$^{-/-}$ mice to be more susceptible to *C. immitis*, but they were not (Fig. 5A). The only statistically significant differences in cytokine concentrations in bronchoalveolar lavage fluid (BALF) between IL-18R$^{-/-}$ mice and B6 were slightly higher levels of IL-22 and IFN-γ in the controls, but the absolute amounts of those cytokines were quite low ($<$100 pg/ml). A new finding in this animal model was that both B6 mice and IL-18R$^{-/-}$ mice had very high concentrations of IL-1Ra in the BALF, exceeding the concentrations of all other measured cytokines (Fig. 5B).

We then infected IL-1R1$^{-/-}$ mice, and they were more susceptible than B6 mice, as measured by the amount of fungal dissemination to the spleen, but there was no significant difference in numbers of CFU in their lungs (Fig. 6A). Although IL-1 signaling was required for host defense against extrapulmonary dissemination, caspase 1 was not (Fig. 6B), indicating that canonical NLRP activation was not needed for processing of pre-IL-1β.

The discovery of high levels of IL-1Ra in BALF prompted us to examine the *in vitro* production of that IL-1-regulatory cytokine. We found that BMDCs secreted IL-1Ra in response to FKS in a dose-dependent manner, and this did not require MyD88 or

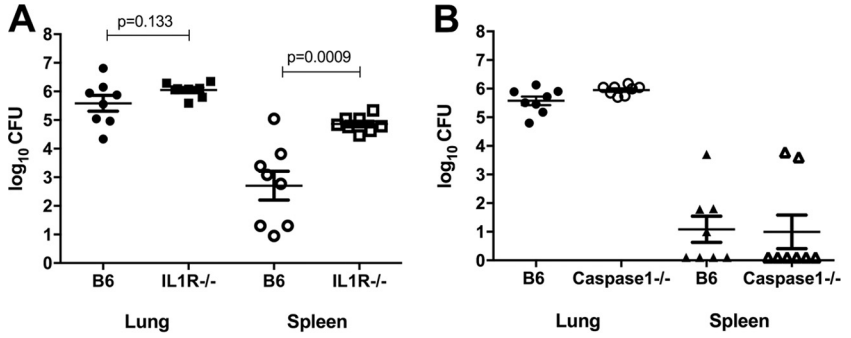

FIG 6 IL-1R1$^{-/-}$ but not caspase 1$^{-/-}$ mice are more susceptible to *C. immitis*. Mice were infected intranasally and sacrificed 14 days later to do quantitative mycology. Each symbol represents 1 mouse, and the horizontal bars show the geometric means. (A) There was no significant difference in lung CFU between the two strains, but the geometric mean CFU were >1 log higher in the spleens of IL-1R1$^{-/-}$ mice, which was highly significant (Student *t* test). (B) There was no significant difference in numbers of CFU in the lungs or the spleens of caspase 1$^{-/-}$ and control B6 mice.

Dectin-1 (Fig. 7). Dectin-1$^{-/-}$ BMDCs made even more IL-1Ra in response to FKS than control cells, whereas the Dectin-1$^{-/-}$ cells were unresponsive to purified β-glucan (Fig. 7) and to alkali-treated FKS (A-FKS) (data not shown). To test whether the MyD88 pathways and Dectin-1 were redundant, we added a monoclonal anti-Dectin-1 antibody to MyD88$^{-/-}$ BMDCs; this did not affect the amount of FKS-induced IL-1Ra in the supernatant. The antibody was functional because it reduced the production of IL-1Ra by A-FKS-stimulated MyD88$^{-/-}$ cells (Fig. 7D). Thus, FKS-stimulated IL-1Ra secretion by BMDCs was not mediated by either a TLR or Dectin-1, even though β-glucan stimulation was entirely dependent on Dectin-1.

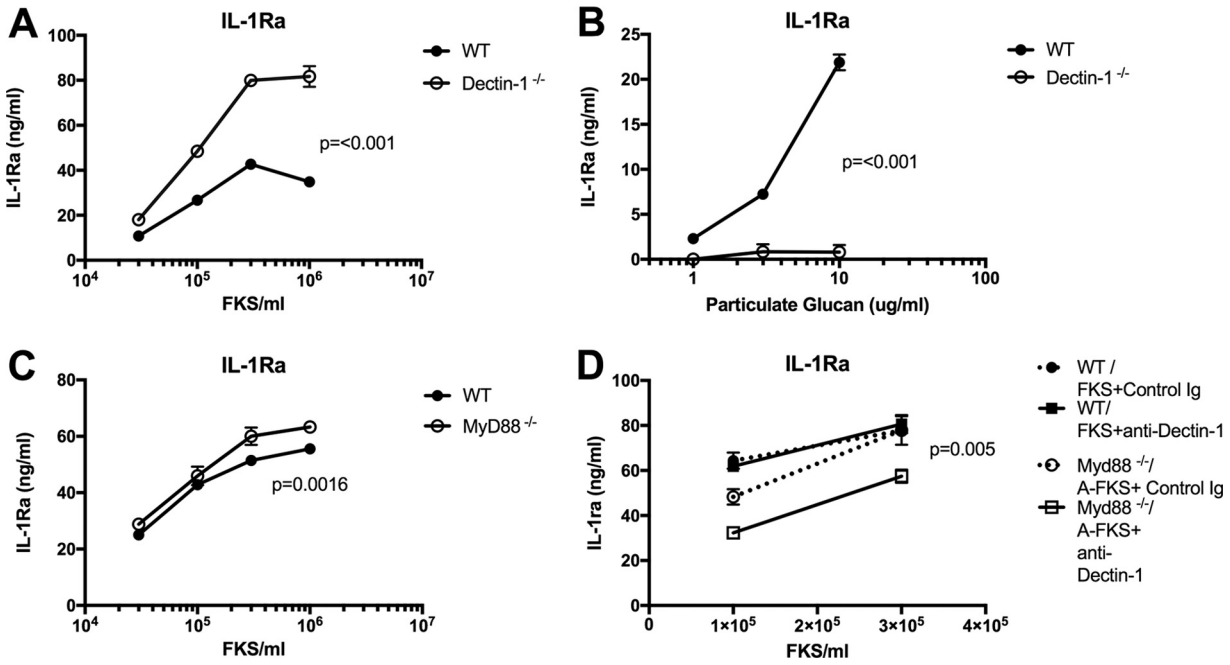

FIG 7 Roles of Dectin-1 and MyD88 in IL-1Ra secretion by WT (B6) BMDCs. IL-1Ra concentrations were measured in the supernatants after overnight incubation, and each dose of FKS and β-glucan was tested in duplicate. Means and SEM are plotted. (A) Dectin-1$^{-/-}$ BMDCs secreted more IL-Ra than WT (B6) cells when stimulated by FKS. (B) Dectin-1 is required for IL-1Ra secretion when the stimulus is purified β-glucan. However, β-glucan is a less potent stimulant than FKS. (C) MyD88 was not required for IL-Ra production as MyD88$^{-/-}$ cells responded more vigorously than B6 cells to FKS. (D) Anti-Dectin-1 (2A11) did not affect the response of MyD88$^{-/-}$ cells to FKS but significantly inhibited the response to A-FKS, confirming that it was a blocking antibody. The *P* value is for the effect of 2A11 on A-FKS-stimulated secretion.

DISCUSSION

Resistance to *C. immitis* and other pathogenic dimorphic fungi requires TH1 $CD4^+$ T cells that make IFN-γ, and probably TH17 cytokines (29, 30). In mice, the kind of CD4 immune response made to fungi depends in large part on the cytokines that are produced by antigen-presenting cells as the result of interactions between fungal structures and signaling receptors. Attachment of the fungus to signaling receptors triggers complex and intersecting signaling pathways that lead to transcription of many cytokine and chemokine genes involved in host defense. The known fungal pattern recognition receptors on myeloid cells include the C3b receptor (CR3), several C-type lectin receptors, and several Toll-like receptors (TLRs). The role of CR3 in responding to spherules has not been established, though CR3 does physically associate with Dectin-1 on lipid rafts, and the two receptors collaborate to promote macrophage TNF and IL-6 responses to *Histoplasma capsulatum* (31). The C-type lectin receptor Dectin-1 is necessary but not sufficient for spherules to stimulate cytokine production by macrophages (21). We have previously shown that both the mannose receptor (MR) and Dectin-2 are also required for cytokine production by elicited macrophages and that the MR is also needed on BMDCs, so other receptors play a role either in the attachment of FKS to cells or in initiation of signaling responses. However, neither is needed *in vivo* to protect against infection (32).

In this paper we show that elicited peritoneal macrophages from mice deficient in both MyD88 and TRIF, like elicited peritoneal MyD88 knockout (KO) macrophages, did not respond to stimulation by FKS, while unexpectedly, those adapter proteins were not required for BMDCs to respond to FKS. The explanation for that discrepancy between macrophage and DC cell responses to FKS is not known, but Goodridge et al. have previously shown that GM-CSF, which we used to grow BMDCs, changes the way cells respond to β-glucans (33). They attributed this primarily to differences in recruitment of caspase recruitment domain 9 (CARD9) to the phagosome, as CARD9 is required for NF-κB activation by zymosan (34). Another important finding in their paper was that different sources of macrophages responded differently to β-glucan stimulation, implying that it may be difficult to predict from *in vitro* assays how macrophages will behave during infection. They also showed that macrophages from different sites responded differently to β-glucan. This of course raises the question of how inflammatory macrophages and naturally derived DCs will respond *in vivo* during infection. As a way of determining if the results of *in vitro* assays with purified cell types were predictive of the behavior *in vivo*, we infected mutant mice. We have previously shown that Dectin-1 deficiency impaired FKS-stimulated cytokine secretion by the same kinds of myeloid cells used in these experiments, and it made mice more susceptible to *C. immitis* infection (17). Thus, there was a congruence of *in vitro* and *in vivo* results. In this study, we found a discrepancy between the need for *in vivo* and *in vitro* results for MyD88, because *in vitro* it was not needed for cytokine secretion by BMDCs, while $MyD88^{-/-}$ mice were more susceptible to infection ($MyD88/TRIF^{-/-}$ mice were also more susceptible but not more than the $MyD88^{-/-}$ mice, so we concluded TRIF is not required for resistance to *C. immitis*). The increased susceptibility of $MyD88^{-/-}$ mice manifested as increased colony counts in the lung and spleen compared to those in B6 mice, which are themselves genetically susceptible (14).

MyD88 is the adapter protein for all TLRs except TLR3, and for IL-1R1 and IL-18R, so we tested the susceptibility of TLR2 and TLR4 to *C. immitis* because both of those TLRs have been shown to respond to collaborate with Dectin-1 for induction of cytokine production by either FKS or other pathogenic fungi to promote cytokine secretion (21, 35). However, we did not find that TLR2, TLR4, or the IL-18R was needed for resistance. In contrast, $IL\text{-}1R1^{-/-}$ mice were more susceptible, as measured by increased dissemination to the spleen, without an increase in lung counts. We speculate that the reason that IL-1R1 signaling did not appear to be needed in the lungs is that the lungs of the control B6 mice were rendered functionally IL-1 deficient by an abundance of IL-1Ra, amounting to a $>$300-fold excess of IL-1Ra over IL-1β in the BALF (Fig. 5B). Others have

shown that a high ratio of IL-1Ra to IL-1β neutralizes the proinflammatory effects of IL-1β (36, 37). A high ratio has been shown also to be functionally important in the humans, as high levels of circulating IL-1Ra are correlated with a higher mortality rate for pneumococcal pneumonia (38). In addition, patients treated for rheumatoid with anakinra, a synthetic IL-1Ra, have a rate of severe infection of more than 10% (39), although there are no reports of an excess of fungal infections in anakinra recipients (40). Transgenic mice overexpressing IL-1Ra are more susceptible to *Listeria monocytogenes* infections (41).

IL-1Ra is considered an anti-inflammatory cytokine, but it can affect all IL-1-mediated immune functions. IL-1β has many effects on the innate and adaptive immune system, including sustaining a TH17 immune response. This could be one reason why *C. immitis*-infected B6 mice had undetectable amounts of IL-17A in their BALF (Fig. 5B). Relatively little is known about the regulation of IL-1Ra expression, although synthesis is stimulated by proinflammatory substances such as lipopolysaccharide (LPS) and TNF-α, and it is secreted by many different cell types (42). Tilg et al. found that infusions of IL-6 increased serum levels of IL-1Ra and stimulated human macrophages to secrete IL-1Ra (43). *Coccidioides* induces IL-6 production by myeloid cells *in vitro* and is abundant in the BALF of infected B6 mice (Fig. 5B). We found that IL-1Ra was made by FKS-stimulated BMDCs and that this did not require MyD88 or Dectin-1 (Fig. 7). Smeekens et al. showed that *Candida albicans* stimulates human monocytes to secrete IL-1Ra (44). Similar to our results, they found that β-glucan stimulates IL-1Ra secretion, but it is much less potent than *C. albicans* yeast, and the response to yeast cells does not require Dectin-1, TLR2, or CR3. They postulated that there was another receptor for *C. albicans* β-glucan that acted through Akt and phosphatidylinositol 3 (PI3) kinases. Dectin-1 has previously been identified as a mediator of IL-1Ra secretion using antibody to cross-link Dectin-1 (45). We confirmed that β-glucan stimulation of IL-1Ra secretion by BMDCs requires Dectin-1, but neither Dectin-1 nor MyD88 was required if the stimulus was FKS. Since A-FKS stimulus also requires Dectin-1, the non-Dectin-1 ligands were removed by hot alkali treatment; most likely they were surface mannans.

B6 mice are inherently susceptible to *Coccidioides* (14). In these experiments we gained additional insights into why B6 mice are so susceptible to this infection, including that they had very low levels of IL-18, IL-23, and IL-β in their BALF. If the BALF cytokine levels accurately reflect what is made in the lung and draining nodes after infection, the absence or paucity of those cytokines would result in impaired TH1 and TH17 responses. We have previously shown infected B6 mice make much more IL-10 in their lungs than do resistant DBA/2 mice and that IL-10-deficient B6 mice are more resistant to infection than B6 mice, suggesting that IL-10 may play a role in inhibiting the cytokine responses noted above. Although we did not measure IL-10 in the BALF of MyD88$^{-/-}$ mice, we did find that MyD88$^{-/-}$ BMDCs made more IL-10 in response to FKS. This needs further investigation.

The importance of MyD88 in other fungal infections is variable. Bello-Irizarry et al. showed that MyD88 signaling is harmful in mice infected with *Pneumocystis*, as the mutant mice had more intense inflammation in their lungs, suggesting an anti-inflammatory role for MyD88 (46). Dubourdeau et al. found that MyD88 deficiency does not make mice more susceptible to *Aspergillus fumigatus* (47), although Bellocchio et al. found that cyclophosphamide-pretreated TLR4- and MyD88-deficient mice are more susceptible to pulmonary aspergillosis than similarly treated B6 mice (48). Jhingran et al. found that MyD88 is needed for epithelial cells to secrete CXC chemokines that attracted polymorphonuclear leukocytes (PMN) early after *A. fumigatus* infection (49), which may be critical in neutropenic animals. MyD88 (50) but not TLR2 (51) is required for resistance to the primary pathogen *Paracoccidioides brasiliensis*. MyD88$^{-/-}$ mice are also more susceptible to the primary pathogen *H. capsulatum*, and they do not make a TH1 immune response to that fungus (52). *Blastomyces dermatitidis* is another primary pathogen that uses MyD88 both for innate and acquired immune responses (29). There is no information about whether the MyD88 pathway is required for protection against opportunistic or primary pathogenic fungi in humans (53). About 60 people with

MyD88 or IRAK-4 deficiencies have been reported, and while they are more susceptible to a variety of bacterial infections, they were not plagued by infections due to opportunistic fungal pathogens such as *Pneumocystis*, *Candida*, or *Aspergillus* (54). It is not clear from that report whether any of those patients lived in areas of the Western hemisphere where they might have been exposed to endemic dimorphic fungi. In general, the mouse data suggest that MyD88 is required for resistance to the small number of primary pathogenic endemic fungi (now including *Coccidioides*), but not for resistance to opportunistic fungal pathogens that are often controlled by the innate immune response.

IL-18R signaling did not contribute to the susceptibility of MyD88$^{-/-}$ mice (Fig. 5A). This was an unexpected result since IL-18 is important for the induction of IFN-γ from NKT cells and IL-2 and IL-12 from CD4^{+} T cells (55), and IFN-γ is known to be important for resistance to this infection. One possible explanation for this result is that control B6 mice made so little IL-18 that it was not detected in their BALF (Fig. 5B). IL-18R1$^{-/-}$ mice also had no detectable IL-18 in their BALF, excluding binding of IL-18 to its cellular receptor as the explanation for the paucity of IL-18 in the BALF. As further confirmation that susceptible mice do not make IL-18, infection with *C. immitis* did not increase IL-18 mRNA expression in BALB/c mice (J. Fierer, unpublished data). In this study, we found that B6 mice also had undetectable amounts of IL-12 in the BALF (Fig. 5). Since IL-12 induces the synthesis of the IL-18Rβ chain, which is required for a functional IL-18 receptor, it is perhaps not surprising that IL-18R1$^{-/-}$ mice were not more susceptible to this infection. We have previously shown that BMDCs from resistant DBA/2 mice make much more IL-12p70 than do FKS-stimulated BMDCs (15) and that infected DBA/2 mice expressed 4 times more Stat1 and 16 times more IFN-γ than B6 mice in their lungs (56). Thus, the B6 genetic background of the mutants that we studied clearly affected the result of the experiment, and our results should not be interpreted as evidence that the IFN-γ pathway plays no role in immunity to this pathogen.

Our results are largely, but not completely, in agreement with those of Hung et al. (25). For instance, they did not find nonimmune IL-1R1$^{-/-}$ or MyD88$^{-/-}$ mice to be more susceptible than B6 mice to infection with *C. posadasii*, but neither mutant was fully protected by the live vaccine. Furthermore, MyD88$^{-/-}$ mice were unable to clear the avirulent vaccine strain from subcutaneous tissues after vaccination, clearly establishing the need for a MyD88-mediated response to defend against this pathogen. A possible explanation for the difference in our results with nonimmune mice is that Hung et al. induced a more severe pulmonary infection in order to test their vaccine; in our experiments the median lung CFU in B6 mice were in the 10^6 range, whereas in their experiments the median lung CFU were 10- to 100-fold higher. The increased severity of the infection in their experiments may have obscured the difference between the results for mutant and control mice.

MATERIALS AND METHODS

Mice. We purchased C57BL/6J, IL-1R1$^{-/-}$, IL-18R$^{-/-}$, C3H/HeJ, and C3H/OuJ mice from Jackson Laboratory (Bar Harbor, ME). All TLR-deficient and MyD88/TRIF$^{-/-}$ mice on a B6 background were originally a gift from Shizuo Akira. We bred MyD88$^{-/-}$ mice by crossing MyD88/TRIF$^{-/-}$ to B6 mice and then intercrossing the progeny for 3 generations. At each generation we used PCR analysis to select for breeding those mice that were homozygous MyD88$^{-/-}$ and had retained at least one TRIF wild-type (WT) allele. After three intercrosses we had 2 breeding pairs that were homozygous for the mutant MyD88 and the WT TRIF genes. All experiments were carried out using procedures authorized by the Institutional Animal Care and Use Committee of the VA San Diego Healthcare System.

Fungus. *C. immitis* strain RS was used for all *in vivo* infections. For *in vitro* cell stimulation assays, we used formalin-killed spherules (FKS) that were made from spherules that were harvested after 96 h of growth in Converse medium (a generous gift from John Galgiani, University of Arizona). Alkali-treated FKS (A-FKS) was made by boiling FKS in 10 N NaOH (17).

Infection. Intranasal infections were performed as described previously (57). We infected 6- to 12-week-old female mice with 50 to 100 arthroconidia. Mice were sacrificed 14 days later to do quantitative cultures of the lungs and spleen and, in some cases, to obtain bronchoalveolar lavage fluid (BALF) as described below. We infected C3H male mice.

Mouse peritoneal macrophage activation. Peritoneal macrophages were elicited with sodium periodate and harvested 4 days later (28). We cultured 1×10^6 adherent cells/ml in Dulbecco modified Eagle medium (DMEM) with high glucose and 10% fetal calf serum (FCS) in a 48-well tissue culture plate

and activated them as described previously (21). We used the synthetic TLR2 agonist $Pam_3CysSerLys4$ (Pam_3CSK4), purified LPS (InvivoGen, San Diego), or poly I·C (Amersham) as the control for TLR activation.

Mouse BMDCs. We cultured bone marrow-derived dendritic cells (BMDCs) obtained from mouse femurs and tibias in RPMI medium with 10% fetal calf serum in the presence of 5 ng/ml of recombinant murine GM-CSF (BD Bioscience) on tissue culture plates (Cellstar; Greiner Bio-One), as described by Lutz et al. (58). 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 BMDCs were plated at a density of 1×10^6/ml in RPMI medium with 10% low-endotoxin fetal calf serum.

Mouse bronchoalveolar lavage. Lung lavage was done as described previously (17), with some modification. Mice were euthanized by asphyxia in a CO_2 environment. The trachea was exposed and intubated with a 16G blunt-end needle. Four successive installations of 0.2 ml of sterile saline into the trachea were aspirated out of the lungs as completely as possible, using a 1.0-ml syringe. Cells in the aspirates were sedimented by centrifugation at 1,800 rpm (Sorvall) 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 extracellular fungal cells. The lavage fluid was assayed immediately for cytokines or stored at −20°C until used.

Reagents. Mouse cytokines in BALF were measured using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's protocols. The ELISAs for TNF-α, IL-6, IL-4, IL-10, IL-17, IL-23, IL-12p70, transforming growth factor β (TGF-β), IL-1Ra, and IFN-γ were purchased from eBioscience (San Diego, CA). The ELISAs for IL-1β, IL-12p40, and GM-CSF were from BD Biosciences, and MIP-2 and IL-22 assay kits were from R&D. Purified particulate β-glucan from *Saccharomyces cerevisiae* was prepared as previously described (59). Curdlan was purchased from Wako Pure Chemicals (VA). β-Glucan was purified from spherules as previously described (21). We purchased ultrapure LPS from *Escherichia coli* O111:B4 and anti-Dectin-1 blocking antibody (2A11) from InvivoGen (San Diego, CA), and the antibody was added at a final concentration of 10 μg/ml.

Statistical analysis. Geometric means and standard errors of the means (SEM) were determined using GraphPad Prism 7 (San Diego, CA) software and were compared using unpaired *t* test. If there were 3 or more groups, we used Dunnett's test for one-way analysis of variance (ANOVA). Dose-response curves were compared by calculating the areas under the curves using GraphPad Prism 7 and comparing them using Welch's *t* test.

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