Interleukin-17A (IL-17A)-producing CD4+ T helper (Th17) cells have been shown to be essential for defense against pulmonary infection with *Coccidioides* species. However, we have just begun to identify the required pattern recognition receptors and understand the signal pathways that lead to Th17 cell activation after fungal infection. We previously reported that Card9−/− mice vaccinated with formalin-killed spherules failed to acquire resistance to *Coccidioides* infection. Here, we report that both MyD88−/− and Card9−/− mice immunized with a live, attenuated vaccine also fail to acquire protective immunity to this respiratory disease. Like Card9−/− mice, vaccinated MyD88−/− mice revealed a significant reduction in numbers of both Th17 and Th1 cells in their lungs after *Coccidioides* infection. Both Toll-like receptor 2 (TLR2) and IL-1 receptor type 1 (IL-1r1) upstream of MyD88 have been implicated in Th17 cell differentiation. Surprisingly, vaccinated TLR2−/− and wild-type (WT) mice showed similar outcomes after pulmonary infection with *Coccidioides*, while vaccinated IL-1r1−/− mice revealed a significant reduction in the number of Th17 cells in their infected lungs compared to WT mice. Thus, activation of both IL-1r1/MyD88- and Card9-mediated Th17 immunity is essential for protection against *Coccidioides* infection. Our data also reveal that the numbers of Th17 cells were reduced in IL-1r1−/− mice to a lesser extent than in MyD88−/− mice, raising the possibility that other TLRs are involved in MyD88-dependent Th17 immunity to coccidioidomycosis. An antimicrobial action of Th17 cells is to promote early recruitment of neutrophils to infection sites. Our data revealed that neutrophils are required for vaccine immunity to this respiratory disease.

*Coccidioides* species are etiological agents of coccidioidomycosis, which is also known as San Joaquin Valley fever, a potentially life-threatening respiratory mycosis that is endemic to the southwestern United States and arid regions of Mexico and Central and South America (1). The incidence of symptomatic coccidioidomycosis in the United States increased from 2,265 reported cases in 1998 to 22,401 cases in 2011, based on data from the National Notifiable Diseases Surveillance System (NNDSS) (2). This database likely underestimates the actual burden of disease, since reporting cases of coccidioidomycosis is not mandated in every state within the known regions of endemicity. A vaccine against coccidioidomycosis would promote the well-being of at-risk populations in the United States, in addition to people who reside in arid areas of Latin America (3). Both clinical data and results of experimental animal studies have shown that T-cell immunity is essential for protection against coccidioidomycosis, and mammalian hosts with a deficiency of CD4+ T cells are at elevated risk of contracting this respiratory disease (3). Many studies of coccidioidomycosis have reported gamma interferon (IFN-γ) production as a correlate of vaccine-induced protection in mice (4, 5). Although there is controversy about the beneficial or harmful roles of interleukin-17 (IL-17) and Th17 cells in fungal infections, we have shown that murine IL-17 receptors and Th17 cells are essential for vaccine immunity to *Coccidioides* infection (6, 7).

Antifungal responses are initiated through host recognition of fungal pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) in innate immune cells. Host recognition of fungal invasion triggers cascades of signaling events to activate secretion of proinflammatory cytokines and induction of T-cell differentiation. Among these signaling molecules, caspase adaptor recruitment domain family member 9 (Card9) and myeloid differentiation factor 88 (MyD88) are two cytosolic adaptors that transduce signals from C-type lectin receptors (CLRs) and the Toll/IL-1 receptor (TIR) superfamily, respectively (8–10). In humans, deep dermatophytosis appears to be an important clinical manifestation of Card9 mutations (11). A mutation in Card9 results in significantly reduced numbers of Th17 cells in patients with chronic mucocutaneous candidiasis (12). In mice, Card9 has been reported to be essential for the development of Th17 cells in response to primary *Candida albicans* infection (13). Card9−/− mice are unable to control a subcutaneous (s.c.) injection of the live, attenuated vaccine strain of *Blastomyces dermatitidis* and succumb to widespread disseminated disease (14). To circumvent the potential susceptibility of Card9−/− mice to live, attenuated vaccine strains of the evaluated fungal pathogens, we have immunized them with heat-killed yeast of the species *Blastomyces dermatitidis* and *Histoplasma capsulatum* or formalin-killed spherules (FKS) of *Coccidioides posadasii*. Our data revealed...
that the Card9 axes of the signal pathway are required for the development of antigen-specific Th17 cells for these three dimorphic fungal pathogens (14).

Coccidioides spp. are dimorphic fungi characterized by a parasitic cycle that is unique among the medically important fungi (15). The saprobic phase of Coccidioides grows as a filamentous (hyphal) form and produces small, dry spores (arthroconidia) that are released into the air upon disturbance of the soil. In vivo, the spores grow isotropically and develop into large, multinucleate parasitic cells (spherules; >80 μm in diameter). The latter undergo a process of segmentation of their cytoplasm, followed by differentiation of a multitude of endospores (2 to 10 μm in diameter) that are released when they enlarge and cause the spherule wall to rupture. The FKS vaccine is made of a formalin-killed mixture of spherules and endospores. Immunization of susceptible mice with the FKS vaccine has been reported to protect against a potentially lethal respiratory challenge with this pathogen (14, 16). However, we have observed an unacceptable level of inflammatory response at the injection site in mice following subcutaneous vaccination of an optimal, protective dose (3.0 mg) of the FKS vaccine (16). A genetically defined, live attenuated strain of C. posadasii (ΔT) has been generated that lost its ability to endosporeulate in vivo but is able to elicit protective immunity to coccidiodomycosis in disease-susceptible mice (16). In contrast to FKS, this live vaccine shows only a minimal inflammatory response at sites of immunization in the C57BL/6 mouse model. In the present study, we investigated whether immunocompromised animals, including Card9−/−, MyD88−/−, TLR2−/−, and IL-1R1−/− mice, could control subcutaneous inoculation of the live, attenuated ΔT strain.

Dependence of MyD88 in antifungal immunity is inconsistent in murine models of fungal diseases. MyD88 knockout mice are not more susceptible to invasive aspergillosis and blood-borne disseminated paracoccidioidomycosis than are wild-type mice (17, 18). In contrast, MyD88 is essential for adaptive and innate immunity to Blastomyces, Paracoccidioides, and Pneumocystis infections (7, 9, 19). The best-characterized PRRs upstream of MyD88 are Toll-like receptors (TLRs) that have leucine-rich repeats and share a cytoplasmic TIR domain with IL-1, IL-18, and IL-1r1 and reported for individual mice of each group of 10 to 12 animals as otherwise stated. The strains included C57BL/6 (stock number 000664), B6.129P2(SJL)-MyD88tm1.1Defr/J (MyD88+/− mice; stock number 090088), and B6.129S7-IL1r1tm1Imx/J (IL-1r1−/− mice; stock number 003245). Breeding pairs of Card9−/− mice were provided by Xin Lin at the MD Anderson Cancer Center. Breeding pairs of TLR2−/− mice were a gift from Bernard Arulandam at the University of Texas at San Antonio (UTSA). Mice were housed in a specific-pathogen-free (SPF) animal facility at UTSA and handled according to guidelines approved by the University Institutional Animal Care and Use Committee. Mice were relocalized prior to vaccination and infection to an animal biosafety level 3 (ABSL3) laboratory.

Vaccination protocol and evaluation of protection. All strains of mice were gender matched and were 8 to 12 weeks old when used in this study. Primary immunization of C57BL/6 and selected knockout mice with FKS or spores isolated from the live, attenuated strain (ΔT) was performed in the abdominal region by the s.c. route as reported previously (16). The vaccine doses were 10⁹ FKS and 5 × 10⁹ CFU of the ΔT vaccine in 100 μl phosphate-buffered saline (PBS). This initial immunization step was followed 14 days later with a boost of 10⁸ FKS or 2.5 × 10⁴ CFU of the ΔT vaccine. Control mice were immunized with PBS following the same vaccination protocol as above. Mice were challenged 4 weeks after completion of the vaccination protocol by intranasal (i.n.) instillation with approximately 80 viable spores of the virulent isolate of C. posadasii (C735) suspended in 35 μl PBS as previously reported (16). Fungal burden in the lungs and spleen was determined at 14 days postchallenge (dpc) by plating serial dilutions of separate lung and spleen homogenates on GYE agar containing 50 μg/ml chloramphenicol or 75 μg/ml hygromycin as reported elsewhere (16). The ΔT vaccine strain carrying a resistance marker to hygromycin can grow on GYE agar containing 50 μg/ml chloramphenicol or 75 μg/ml hygromycin as reported elsewhere (16). The ΔT vaccine strain carrying a resistance marker to hygromycin can grow on GYE agar containing this antibiotic. The number of CFU of Coccidioides was expressed on a log scale and reported for individual mice of each group of 10 to 12 animals as previously described (16). Survival studies of vaccinated versus nonvaccinated mice were conducted over 60 days postchallenge as previously reported (16).

Intracellular cytokine staining. Pulmonary leukocytes were isolated from lungs of both vaccinated and nonvaccinated mice at 8 and 12 days postchallenge as previously reported (6, 24). Aliquots of pulmonary leukocytes were stimulated with anti-CD3 and -CD28 in the presence of GolgiStop in 10% fetal bovine serum-complemented RPMI 1640 for 4 h at 37°C. Permeabilized cells were stained with selected fluorochrome-conjugated monoclonal antibodies (MAbs) specific for CD4, CD8, IFN-γ, or IL-17A to determine absolute numbers of the specific cytokine-producing CD4⁺ or CD8⁺ T cells, as previously described (6, 25). The leukocytes were gated for CD4⁺ or CD8⁺ T cells, and their levels of cytokine expression were determined. The absolute numbers of the specific cytokine-producing CD4⁺ or CD8⁺ T cells relative to the total lung-infiltrated leukocytes per lung homogenate at 8 and 12 days postchallenge were calculated by multiplying the percentage of each gated population by the total number of viable pulmonary leukocytes determined by hemocytometer counts as previously reported (6).

Depletion of neutrophils. The monoclonal antibody against Ly6G (clone IA8) was obtained from Bio-X-Cell (West Lebanon, NH). This rat immunoglobulin G2a (IgG2a) reacts with the Ly6G antigen expressed by murine neutrophils but not with other cell populations (26). Two groups of vaccinated and nonvaccinated C57BL/6 mice were injected intraperitoneally (i.p.) with 200 μg MAb 24 h before i.n. challenge with C. posadasii spores. This treatment was repeated every 2 days after infection until mice were sacrificed at 12 days postchallenge. Each mouse received a total of 7 injections. Control mice received equivalent amounts of normal rat IgG.
MyD88 elicited a moderate inflammatory response in WT C57BL/6 and attenuated strains of mice at 14 dpc. The mean CFU in lungs of nonvaccinated and vaccinated mice measured as CFU, as reported previously (16). Survival data were examined by using the Kaplan-Meier test via log rank analysis to compare survival plots, as reported previously (16). A P value of <0.05 was considered statistically significant.

RESULTS
Vaccine immunity to Coccidioides infection requires both MyD88 and Card9 adaptors. We previously reported that Th17 cells are essential for vaccine-induced protection against Coccidioides infection (6, 7). Here, we compared the relative contributions of MyD88 and Card9 in activation of vaccine immunity to pulmonary infection with Coccidioides. First, we tested whether the live, attenuated ΔT vaccine could be used to protect MyD88Δ−/− and Card9Δ−/− mice. Vaccination with live ΔT spores elicited a moderate inflammatory response in WT C57BL/6 and MyD88Δ−/− mice, as revealed by slight swelling at sites of immunization and a lower concentration of neutrophils observed in paraffin sections of skin biopsies, while the ΔT-vaccinated Card9Δ−/− mice developed severe inflammation at the injection sites, comparable to those caused by the FKS vaccine (16). We examined cultured homogenates of skin biopsies, lungs, and spleen from the mice that were immunized with a total of 7.5 × 10^6 spores of the ΔT strain at 4 weeks after the last vaccination. The results indicated viability of the vaccine strain at sites of immunization in the skin of mice but an absence of the hygromycin-resistant ΔT strain in the lungs and spleen. We detected a range of CFU (10^1.2 to 10^4.2) in skin biopsies of 70%, 50%, and 30% of the vaccinated Card9Δ−/−, MyD88Δ−/−, and wild-type mice, respectively, but none of these mice showed symptoms of coccidioidomycosis, loss of body mass, or reduced mobility. Thus, the live ΔT vaccine can be used to explore vaccine immunity to Coccidioides infection in these examined strains of knockout mice.

MyD88Δ−/−, Card9Δ−/−, and WT mice were vaccinated with the live ΔT vaccine and intranasally challenged with approximately 80 Coccidioides spores, a potentially lethal dose. The relative efficacy of protection against coccidioidomycosis was compared by measuring numbers of CFU in the lungs and spleen of these three strains of mice at 14 dpc. The mean CFU in lungs of nonvaccinated MyD88Δ−/− and Card9Δ−/− mice (7.7 ± 0.4 and 7.1 ± 1.1 log_{10}, respectively [means ± standard errors of the means]) were comparable to those of wild-type mice (7.4 ± 0.8 log_{10}) (Fig. 1A). Similarly, nonvaccinated MyD88Δ−/− and Card9Δ−/− mice did not show significant differences in CFU in their spleens (4.8 ± 0.6; 4.3 ± 1.2 log_{10}) compared to reactions in nonvaccinated WT mice (4.6 ± 0.5 log_{10}) (Fig. 1B). Both vaccinated MyD88Δ−/− and Card9Δ−/− mice had elevated numbers of CFU that were 2.5 logs higher than the response in vaccinated WT mice (Fig. 1A and B). Lung and spleen CFU of the ΔT-vaccinated Card9Δ−/− mice were comparable to the results for FKS-vaccinated mice, as previously reported (14). Thus, Card9 is also required for vaccine immunity elicited by the live, attenuated vaccine. All three strains of nonvaccinated mice (controls) approached a moribund state at 12 to 24 dpc, whereas vaccinated WT mice survived for over 60 days (Fig. 1C), as previously reported (6, 16). In contrast, comparative studies revealed that the vaccinated MyD88Δ−/− and Card9Δ−/− mice showed 30% and 20% survival, respectively, and these rates were significantly reduced compared to those of vaccinated WT mice (P < 0.05) (Fig. 1C). These results indicated that both MyD88 and Card9 are essential for vaccine-induced resistance to Coccidioides infection.

MyD88 and Card9 are required for acquisition of both Th17 and Th1 cells in Coccidioides-infected lungs. Next, we determined the numbers of activated CD4^+ T cells, including Th17 and Th1 cells that had infiltrated the lungs of vaccinated and nonvaccinated MyD88Δ−/− and Card9Δ−/− mice at 8 and 12 dpc, and we compared the results to those of WT mice (Fig. 2A to D). Expression of CD44 on the surface of T cells is a marker of activation (6). Vaccinated MyD88Δ−/− and Card9Δ−/− mice had significantly reduced numbers of CD44^+ CD4^+ T cells in their lungs at both 8 and 12 dpc compared to vaccinated WT controls (Fig. 2A). Phenotypic analysis of activated pulmonary CD4^+ T cells revealed that significantly reduced percentages and numbers of Th17 (CD4^+ IL-17A^+) cells were present in lungs of vaccinated MyD88Δ−/− and Card9Δ−/− mice than in vaccinated WT mice at both 8 and 12 dpc (Fig. 2B and C). Interestingly, marked reductions in numbers of Th1 (CD4^+ IFN-γ^+) cells were also observed in vaccinated MyD88Δ−/− and Card9Δ−/− mice compared to WT mice (Fig. 2D). Thus, both MyD88 and Card9 adaptors are required for the vaccine-induced acquisition of Th17 and Th1 cells in the lungs of Coccidioides-infected mice.
TLR2 is dispensable for vaccine immunity to Coccidioides infection. Since MyD88 and upstream TLR2 have been shown to be required for macrophages to sense Coccidioides spherules (21), we postulated that TLR2 might be essential for recognition of the live, attenuated vaccine in eliciting vaccine immunity. To test this hypothesis, we compared vaccine-induced resistance and acquisition of subtypes of CD4+ T cells in lungs and spleens of TLR2−/− and WT mice during the first 14 dpc. To our surprise, vaccinated TLR2−/− mice presented with similarly reduced levels of CFU in lungs and spleen as in vaccinated WT mice (Fig. 3A and B). In parallel, vaccinated TLR2−/− and WT mice showed 100% survival for a period of 60 days postchallenge (Fig. 3C and 1C). Phenotypic analysis of pulmonary CD4+ T cells also revealed that numbers of Th17 and Th1 cells of the vaccinated TLR2−/− mice were comparable to those of WT mice (data not shown). These results indicated that TLR2 is dispensable for pulmonary acquisition of Th17 and Th1 cells and vaccine-induced resistance to Coccidioides infection.

The IL-1 receptor is essential for acquisition of Th17 cells and vaccine-induced resistance to Coccidioides infection. Since IL-1r1 can also transduce a signal through MyD88 to activate Th17 differentiation, we asked whether IL-1r1 contributes to vaccine-induced resistance to Coccidioides infection and acquisition of Th17 cells in the lungs. Vaccinated IL-1r1−/− mice presented...
with intermediate numbers of CFU in lungs and spleen compared
to the numbers of CFU in vaccinated MyD88<sup>−/−</sup> and WT mice,
whereas all strains of nonvaccinated mice had comparable, high
numbers of CFU in lungs and spleen (Fig. 4A and B). We further
measured the numbers of activated CD4<sup>+</sup> CD4<sup>+</sup> T cells, Th17,
and Th1 cells in lungs of IL-1r<sup>−/−</sup> mice and compared these
results to those of MyD88<sup>−/−</sup> and WT mice at 8 and 12 dpc (Fig.
5A to D). Our data revealed that IL-1r<sup>−/−</sup> mice had reduced
numbers of activated CD4<sup>+</sup> CD4<sup>+</sup> T cells at 8 and 12 dpc com-
pared to WT mice (Fig. 5 A). Like MyD88<sup>−/−</sup> mice, vaccinated
IL-1r<sup>1−/−</sup> mice revealed significantly reduced percentages and
numbers of Th17 cells in their lungs at both 8 and 12 dpc com-
pared to vaccinated WT mice (Fig. 5B and C). Interestingly, num-
bers of Th1 cells in lungs of vaccinated IL-1r<sup>1−/−</sup> mice were com-
parable to those of wild-type mice (Fig. 5D). These data suggest
that IL-1r is essential for induction of Th17 cells and resistance to
Coccidioides infection.

Vaccine-induced resistance to Coccidioides infection is de-
pendent on neutrophils. Th17 immunity can promote early in-
filtration and activation of phagocytic cells, especially neutrophils,
and Th1 cells, Th17, and WT mice, respectively, at sites of infection.
We previously reported that vaccinated WT mice had elevated numbers of neutrophils in lungs during the early stage (before 7 dpc) of Coccidioides infection compared to the response in nonvaccinated mice (6). We asked whether neu-
trrophils are required for vaccine immunity to Coccidioides infec-
tion. C57BL/6 mice were treated with a monoclonal antibody (IA8
MAb) to selectively deplete Ly6G<sup>+</sup> neutrophils during the effector
phase of Coccidioides infection. Mice intraperitoneally injected
with rat IgG served as controls. We confirmed an efficient deple-
tion (95% ± 3%) of CD11b<sup>+</sup> LFA1<sup>+</sup> neutrophils in the lungs of
both vaccinated and nonvaccinated mice at 12 dpc. Mice were
subjected to fungal burden assays at this time point. Vaccinated
mice that were depleted of neutrophils had 38- and 48-fold more
CFU in lungs and spleen than controls given rat IgG (P < 0.05)
(Fig. 6A and B), respectively. Nonvaccinated mice depleted of
neutrophils showed a trend of elevated numbers of CFU in
lungs and spleen compared to control littermates that received
rat IgG; however, the increase was not statistically significant
(Fig. 6A and B).

**FIG 4** IL-1r1 is essential for vaccine-induced resistance to Coccidioides infec-
tion. The CFU of Coccidioides detected in dilution plate cultures of lungs (A) and
spleen (B) homogenates at 14 dpc of WT, IL-1r<sup>1−/−</sup>, and MyD88<sup>−/−</sup> mice
(n = 10 per group) vaccinated with the ΔT vaccine (+) or immunized with
PBS (−) as controls are reported. Asterisks indicate statistically significant
differences (P < 0.05) between vaccinated and nonvaccinated mice of the same
strain, while daggers indicate significant differences between the vaccinated
IL-1r<sup>1−/−</sup> and MyD88<sup>−/−</sup> mice compared to the WT mice. Dollar signs indi-
cate significant differences between the MyD88<sup>−/−</sup> and IL-1r<sup>1−/−</sup> mice. The
reported results are representative of two independent experiments.

**FIG 5** IL-1r1 is required for acquisition of Th17 cells to Coccidioides-infected lungs. Fluorescence-activated cell sorting analysis results for activated CD4<sup>+</sup> T cells
and Th17 and Th1 cells are shown for lungs homogenates derived from vaccinated compared to nonvaccinated WT, IL-1r<sup>1−/−</sup>, and MyD88<sup>−/−</sup> mice. (A) The
numbers of CD4<sup>+</sup> CD4<sup>+</sup> T cells at 8 and 12 dpc; (B) percentages of gated CD4<sup>+</sup> T cells positive for IL-17A at 12 dpc; (C) and D) the numbers of IL-17A- and
IFN-γ-producing Th17 and Th1 cells, respectively, are shown for the two strains of knockout mice compared to the WT mice. Asterisks indicate significantly
higher absolute numbers of the responsive T-cell phenotypes in lungs of vaccinated compared to nonvaccinated mice of the same strain, while daggers represent
significant differences in the IL-1r<sup>1−/−</sup> and MyD88<sup>−/−</sup> mice compared to WT mice. Dollar signs indicate significant differences between the MyD88<sup>−/−</sup> and
IL-1r<sup>1−/−</sup> mice. Data are mean values ± standard errors of the means of 4 mice per group. The reported results are representative of two independent
experiments.
DISCUSSION

The development of robust and durable vaccines requires a fundamental understanding of how protective immune responses are induced. We applied a live, attenuated vaccine (\(\Delta T\)) to explore the nature of vaccine immunity in mice during the initial 2-week period after intranasal challenge with a potentially lethal dose of \textit{Coccidioides} spores. The numbers of pulmonary Th1 and Th17 cells showed a progressive increase in vaccinated mice and corresponded with a reduction of fungal burden (6). Profiles of cytokines detected in lung homogenates of \(\Delta T\)-vaccinated mice were indicative of a mixed Th1, Th2, and Th17 immune response. While mice lacking an IFN-\(\gamma\) receptor (IFN-\(\gamma R\)) or IL-4 receptor (IL-4R) can develop comparable vaccine immunity without loss of \(\Delta T\) vaccine-induced resistance, deficiency of the IL-17 receptor (IL-17RA) results in a significant increase in susceptibility to \textit{Coccidioides} infection (6). Although vaccinated \(\Delta T^{+/−}\) mice can survive for a period of 45 days postchallenge, immune-deficient mice present with significantly elevated numbers of CFU in lungs and spleen at 14 days postchallenge compared to vaccinated wild-type mice (6, 7). Furthermore, we have created a strain of TCR transgenic mice (Bd 1807) whose CD4\(^+\) T cells respond to a shared epitope among Blastomyces dermatitidis, Histoplasma capsulatum, and \textit{Coccidioides posadasii}. Adoptive transfer of CD4\(^+\) T cells prepared from Bd 1807 mice confers significant reductions of fungal burden in the lungs and spleen of the recipient mice vaccinated with FKS and challenged with a potentially lethal dose of \textit{Coccidioides} spores (29). On adoptive transfer into the vaccinated mice, Bd 1807 cells were activated and differentiated into Th1 cells after trafficking to the lungs upon challenge with one of these three dimorphic fungi (29). Later, we found that Bd 1807 cells could also differentiate into Th17 effector cells in lungs of the recipient mice (7). Our data suggest that vaccine-induced Th17 cells are indispensable for protective immunity to infection with \textit{Coccidioides posadasii}, \textit{Blastomyces dermatitidis}, and \textit{Histoplasma capsulatum} (7).

We found that Card9 is an essential signal adaptor governing the activation of protective immunity elicited by both the live, attenuated vaccine (\(\Delta T\)) and FKS against pulmonary \textit{Coccidioides} infection (14). Dectin-1, Dectin-2, and Mincle are the best-characterized CLR families upstream of the spleen tyrosine kinase (Syk)-coupled Card9 axis of the signal cascade (30). Dectin-1 contains an immunoreceptor tyrosine-based activation motif (ITAM), whereas Dectin-2 and Mincle transduce signals through association with the ITAM-containing Fc receptor \(\gamma\) chain (Fc\(\gamma R\)). Dectin-1 has been shown to be involved in innate recognition of FKS by peritoneal macrophages to produce inflammatory cytokines (21). Dectin-1 is also required for resistance to primary \textit{Coccidioides} infection (31, 32). Recently, we showed that the development of both Th17 and Th1 cells was impaired in FKS-vaccinated \(\Delta T^{+/−},\) Dectin-2\(^{−/−}\), and Fc\(\gamma R\)\(^{−/−}\) mice compared to WT and Mincle\(^{−/−}\) mice (14). In this study, we found that the live \(\Delta T\) vaccine, like FKS, also activates the Card9-mediated signal pathway to elicit protective immunity to \textit{Coccidioides} infection.

Our data revealed that MyD88, like Card9, is also required for vaccine-induced antifungal Th17 and Th1 responses and resistance to \textit{Coccidioides} infection. Both \(\Delta T^{+/−}\) and \(\Delta T^{−/−}\) knockout mice were impacted by the acquisition of Th17 cells, which are essential for vaccine immunity to \textit{Coccidioides} infection (6). Similarly, antifungal Th17 and Th1 responses to pulmonary infections with \textit{Blastomyces dermatitidis} and \textit{Paracoccidioides brasiliensis} are also dependent on MyD88 (7, 9). Unexpectedly, our results revealed that TLR2 is not essential for vaccine immunity to \textit{Coccidioides} infection, although it is required for peritoneal macrophages to secrete proinflammatory cytokines upon incubation with \textit{Coccidioides} spherules in \textit{vitro} (21). Comparable results were also reported for systemic \textit{Aspergillus} infection and pulmonary \textit{Blastomyces dermatitidis} infection, suggesting that TLR2 is dispensable during early differentiation and recruitment of CD4\(^+\) T cells in the airway (7, 33, 34). In addition to TLR2, other TLRs, including TLR4, TLR1, TLR6, and TLR9, have been implicated in innate recognition of fungal infections (35) and may compensate for the loss of TLR2 upon \textit{Coccidioides} infection.

Besides serving as an immune adaptor for TLRs, MyD88 also mediates signal transduction via IL-1, IL-18, and IL-33 receptors (20, 36, 37). Both IL-1\(\alpha\) and IL-1\(\beta\) interact with IL-1r1, while IL-18 and IL-33 bind to IL-18r and IL-33r, respectively. IL-1r1 is expressed on both antigen-presenting cells and T cells. Activation of IL-1r1/MyD88 can provide both extrinsic and intrinsic signals for CD4\(^+\) T cell development (37–39). IL-1\(\alpha\) and IL-1\(\beta\) induce mRNA expression of hundreds of genes in multiple cell types, such as monocytes and macrophages (40). IL-1\(\alpha\) and IL-1\(\beta\) induce expression of their own genes, which serves as a positive feedback loop that amplifies the IL-1 response in an autocrine or paracrine manner (38). Selective expression of receptors for an IL-1 family member has been shown in CD4\(^+\) T cells primed in the presence of Th17, Th1, or Th2 cells. IL-1r1 is required for the upregulation of IRF4 and RORC (two fundamental Th17 transcription factors) during early development and differentiation of Th17 cells (39). Activation of IL-1r1/MyD88 enhances antigen-driven expansion of Th17 cells both \textit{in vitro} and \textit{in vivo} (37, 41). IL-18r shows heightened expression on Th1 cells and IL-33r on Th2 cells (37). Our current model of coccidioidomycosis could not distinguish the relative contribution of the extrinsic and intrinsic roles of IL-1r1 in the development of a CD4\(^+\) T-cell response to \textit{Coccidioides} infection. However, the \(\Delta T\)-vaccinated IL-1r1\(^{−/−}\) mice specifically reduced acquisition of Th17 cells, suggesting that the IL-1r1/MyD88 axis of immunity is required for vaccine-induced Th17 immunity and resistance to pulmonary...
Coccidioides infection. Of note, our data revealed that the Th17 response was reduced in IL-1r1−/− mice to a lesser extent than in MyD88−/− mice, raising the possibility that members of the TLR family, other than TLR2, may also be involved in induction of Th17 immunity to Coccidioides infection.

Interestingly, vaccinated IL-1r1−/− mice, but not MyD88−/− mice, showed sustained acquisition of Th1 cells during the first 12 days post-Coccidioides infection. Presumably, in the absence of IL-1r1, TLRs, IL-18r, and IL-33r contribute to these MyD88-dependent CD4+ T-cell responses to Coccidioides infection. IL-18 is known to play an important role in Th1 polarization, but it also promotes production of Th2-type cytokines (e.g., IL-4, IL-5, IL-9, and IL-13) from T cells, NK cells, basophils, and mast cells (39, 42). IL-33 and IL-33r drive polarized Th2 cells to produce IL-4, IL-5, and IL-13 in mice. Future investigations of IL-18r- and IL-33-mediated signals will elucidate their roles in vaccine immunity to Coccidioides infection.

Th17 immunity promotes infiltration and activation of neutrophils as a mode of action in mediating vaccine immunity against fungal infection and inflammatory diseases (7, 43). Although the functional mechanisms of neutrophils have not yet been studied, our data suggest that neutrophils are essential for vaccine-induced resistance to Coccidioides infection. Human neutrophils have been shown to kill arthroconidia (spores) of Coccidioides (44). As the spores germinate and transform into spherules, the fungal cells progressively become larger and more resistant to the inhibitory effects of human neutrophils (45). Rupture of mature spherules and release of endospores trigger an influx of neutrophils (46). Ingestion of young spherules (spherule initials) and endospores elicits an oxidative burst, albeit to a lesser extent than that induced by spores (47). Several studies have shown that neutrophils can kill spherule initials, while mature spherules are resistant to phagocytic killing (45, 46). Neutrophils can be polarized toward proinflammatory or anti-inflammatory cells (N1 versus N2) in response to environmental signals (48). Neutrophils can also produce IL-10, a global anti-inflammatory cytokine in lung homogenates during mice for the first 14 days after Coccidioides infection (49). Thus, neutrophils have emerged as components of the effector and regulatory circuits of the innate and adaptive immune systems (50). Vaccinated and challenged C57BL/6 mice reveal early infiltration of neutrophils into lungs at 5 to 7 dpc that is sustained in moderate numbers during the first 14 dpc of Coccidioides infection (6). No severe tissue damage due to uncontrolled recruitment of neutrophils has been noted based on histopathological examination of lung tissue of such vaccinated mice. In contrast, nonvaccinated mice do not recruit polymorphonuclear lymphocytes into the lungs until 8 to 9 dpc, when Coccidioides spherules rapidly propagate and start to disseminate to extrapulmonary tissue and organs (6, 25, 28). The sharp influx of neutrophils into lungs of nonvaccinated mice that fail to develop Th17 cells after 8 days postchallenge is probably due to activation of the complement systems. Although nonvaccinated mice accumulate large numbers of neutrophils in their lungs before reaching a moribund state, partly because of their delayed recruitment, the mice are unable to inhibit reproduction of Coccidioides spherules.

Both ligands of Toll/TLRs and CLRs are of growing interest as adjuvants to enhance vaccine efficacy (51). Withrich and colleagues have also shown that addition of IL-1 to weak antigens can enhance vaccine capacity to induce protection against lethal Blastomyces dermatitidis infection in mice and is far more effective than lipopolysaccharide (52). Our data suggest that an adjuvant system composed of ligands to stimulate both Card9-deficient and MyD88-mediated signals is worthy of investigation. For example, porous glucan particles that consist primarily of β-1,3-d-glucans and allow for carrying pathogen-specific antigens and Toll/TLR ligands, such as CpG oligonucleotides and/or IL-1, have been exploited as receptor-targeted vaccine delivery systems (53). We postulate that this glucan particle-based vaccine platform, which delivers antigens and provides adjuvanticity, can be used to induce robust Th17 and Th1 immunity to combat Coccidioides infection.

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REFERENCES


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