Susceptibility of TLR4-defective C3H/HeJ mice to Coccidioides posadasii infection

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Coccidioides posadasii is one of the two fungal pathogens that cause coccidioidomycosis. The inhalation of air-borne arthroconidia leads to the formation of endospore-forming spherules in the lungs and pulmonary infection. In severe condition, the endospores are disseminated to other non-pulmonary organs in the body. The Toll-like receptors (TLR) expressed by a number of immune and non-immune cells can significantly impact the host defense and susceptibility to C. posadasii infection. In this study, we infected TLR4-defective C3H/HeJ mice with a sublethal dose of C. posadasii and studied fungal dissemination, mortality and humoral response. We also measured IL-12 cytokine secreted by C. posadasii-infected dendritic cells. We found that the C3H/HeJ mice were equally susceptible to C. posadasii as compared to C3H/OuJ mice which have intact TLR4. No significant changes were observed in pulmonary fungal load, survival and humoral response. The blockade of TLR4 did not affect C. posadasii-induced IL-12 secretion. However, the fungal counts were 10 times less in spleens of C3H/HeJ mice as compared to C3H/OuJ mice (P < 0.05). Our results suggest that the TLR4 may not be involved in inducing protective host defense against C. posadasii, but it appears to be critical for fungal dissemination.

Keywords  Toll-like receptor 4, Coccidioides posadasii, infection, innate immunity

Experiments in mice show that the inflammatory responses to Aspergillus are in part mediated by TLR4 [10,11]. Similar to aspergillosis, coccidioidomycosis is an invasive disease, and increased susceptibility in patients and mouse models has been linked to immunocompromised condition and reduced T cell response [12]. In a continuation of our ongoing efforts to study the role of innate immune factors in coccidioidomycosis [13–16], we investigated the role of TLR4 in a mouse model of C. posadasii-induced infections.

Materials and methods

Mouse model
Six-week-old female BALB/c, C3H/HeJ and C3H/OuJ mice were purchased from Jackson Laboratories, ME. The information on genotype, phenotype and differences among mouse strains is provided in Table 1. Inbred mice have been shown to differ in their TLR4 genotype and responses to lipopolysaccharide (LPS, the most potent ligand for TLR4) [17,18]. C3H/HeJ mice express defective TLR4 and remain hyporesponsive to LPS treatment. C3H/OuJ mice with C3H background, expressing intact TLR4 protein were included as control.
Susceptibility of mice to *C. posadasii* infection

Since BALB/c mice are extremely susceptible to *C. posadasii* infection [12], a depressed Th1 immune response has been found responsible for their increased susceptibility to infection. The dendritic cells (DCs) play a critical role in activating pathogen-specific T cell response [19]. Results of our previous study suggest that the reduced responses by BALB/c mice-derived DCs may be partly responsible for their increased susceptibility to *C. posadasii* infection [14]. It is well established that the DCs respond to infectious stimuli through pathogen-pattern recognition receptors (e.g., TLRs) [20]. Thus, in the present study we used BALB/c mice-derived DCs to decipher the role of TLR4 in inducing innate immune response to *C. posadasii* infection.

Mice were housed for at least one week for acclimatization prior to conducting any experiment. All procedures involving animals were approved by Institutional Animal Care and Use Committee. Infected mice were housed in Animal Biosafety Level-3 (ABSL-3) facility at the University of Texas Health Science Center at San Antonio, San Antonio, TX (UTHSCSA).

*Coccidioides posadasii*

*C. posadasii* strain Silveira was cultured on 1% glucose-0.5% yeast extract agar (GYE) and used throughout the study. The arthroconidia were harvested in endotoxin-free 0.15 M NaCl solution (Baxter Health Care Products, IL), and counts were enumerated by hematocytometer. The viability of arthroconidia was confirmed by plating the fungal suspension on GYE agar. All the experiments with live *C. posadasii* were approved by Institutional Biosafety Committee and performed in the Biosafety Level-3 (BSL-3)/Animal Biosafety Level-3 (ABSL-3) facility at UTHSCSA.

*Intranasal infection with C. posadasii arthroconidia*

First, the sublethal dose of *C. posadasii* was determined. The C3H/OuJ mice (*n* = 10 each per infectious dose) were anaesthetized by intramuscular injection of ketamine-xylazine (75 μg/g and 10 μg/g body weight, respectively) and challenged with *C. posadasii* arthroconidia (*n* = 20, 47, 93 viable arthroconidia). Subsequently, a larger number of mice (C3H/OuJ and C3H/HeJ, 10 mice of each, two experiments) were infected with live *C. posadasii* arthroconidia (*n* = 38–45) via the intranasal route [13]. After injection, mice were housed in ABSL-3 facility and given food and water *ad libitum*. The infected mice were sacrificed on the 10th day of infection. At the time of necropsy, blood was collected by cardiac puncture, and organs were harvested.

**Fungal burden assay**

Ten-fold dilutions of the lung and spleen tissue homogenates were inoculated on Mycobiotic agar plates (Difco, Detroit, MI) and incubated at 33°C. The number of mycelial colonies were counted and normalized with gram-wet weight of tissues.

**Antibody response**

Serum was separated from blood samples, filtered through 0.2 μm syringe filter and stored at −80°C for further analysis. *Coccidioides*-specific IgG response was determined as per the published method [21]. Briefly, the 96-well plate was coated with (0.1 μg /well) purified recombinant *Coccidioides*-Ag2/PRA protein (kindly provided by Dr Rebecca Cox, UTHSCSA; now deceased) and incubated with 10-fold diluted serum samples. The antibody IgG isotypes were determined by incubating with alkaline phosphatase-conjugated anti-mouse IgG1, IgG2a, IgG2b and IgG3. The p-nitrophenyl phosphate substrate system was used and OD was read at 410 nm on a microplate reader (Dynatech Inc, Lake Success, NY).

**Clinical symptoms and mortality**

A separate set of infected mice (C3H/HeJ and C3H/OuJ, total of 20 mice each, two experiments) was housed in ABSL-3 facility and given food and water *ad libitum*. The

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**Table 1** TLR4 genotype and phenotype of mouse strains included in the study.

<table>
<thead>
<tr>
<th>Mouse strains</th>
<th>Open reading frame mutations in TLR4 gene</th>
<th>Phenotypic characteristics</th>
<th>MHC II haplotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3H/HeJ</td>
<td>Mutation in exon 3, proline (712) to histidine mutation in cytoplasmic domain of TLR4. This point mutation modifies the protein, creating a dominant inhibitory effect on LPS signaling.</td>
<td>Hyporesponsive to LPS</td>
<td>k</td>
</tr>
<tr>
<td>C3H/OuJ</td>
<td>Same C3H background as C3H/HeJ mice, does not carry the proline (712) to histidine mutation.</td>
<td>Normoresponsive to LPS</td>
<td>k</td>
</tr>
<tr>
<td>BALB/c</td>
<td>Mutations in exon 3, methionine (209) to isoleucine, valine (254) to isoleucine, glutamic acid (593) to aspartic acid in ectodomain of TLR4, arginine (761) to histidine in cytoplasmic domain of TLR4 [17], does not carry the proline (712) to histidine mutation.</td>
<td>Normoresponsive to LPS</td>
<td>d</td>
</tr>
</tbody>
</table>
mice were monitored on a daily basis for clinical symptoms (reduction in body weight and mobility, ruffled fur) and mortality for a period of 40 days post intranasal challenge.

**Culture and isolation of dendritic cells (DCs)**

The DCs were cultured using bone marrow specimens of BALB/c mice as per the method described earlier [14]. Briefly, the bone marrow cell suspension was seeded on 6-well tissue culture plates (Nalge-Nunc International Corp, NY) in RPMI 1640 medium containing 10 mM N-2-Hydroxyethylpiperazine-N’-2-ethanesulfonic acid, 10 μg/ml gentamicin, 100 U/ml penicillin, 100 μg/ml streptomycin, 10% fetal bovine serum, 1% MEM nonessential amino acids, 50 μM β-mercaptoethanol, 10 ng/ml recombinant mouse-GM-CSF and 10 ng/ml IL-4 (both cytokines from Peprotech, NJ). The cells were incubated at 37°C in 5% CO₂ atmosphere. On day 6, the nonadherent cells were collected and DCs were harvested using 14.5% metrizamide (Sigma, MO) solution. The viability, morphology and phenotype of DCs were confirmed by trypan blue dye exclusion, Diff-Quik Wright-Giemsa staining and flow-cytometry, respectively.

**TLR4 blockade and in vitro infection of DCs**

The non-adherent DCs (2.5 × 10⁶ cells per well) were incubated with anti-mouse TLR4 antibody (eBioscience, CA; 1 μg/well diluted in culture medium containing 1% normal mouse serum) for 30 min. The live *C. posadasii* arthroconidia (at the ratio of 1 arthroconidium:10 DCs) and 100 ng/ml *Salmonella minnesota* LPS (Sigma, MO) were then added to the cells. After 24 h of incubation, the cell-free supernatants were collected, filtered through 0.2 μm syringe filters and stored at −80°C for further analysis.

**Measurement of IL-12 levels**

The levels of secreted IL-12 were measured in cell-free supernatants of DCs treated with *C. posadasii* by ELISA [14]. Briefly, microwells were coated with primary antibody, purified anti-mouse IL-12 p40/p70 (BD Pharmingen, CA). Following the blocking of the nonspecific sites, the wells were incubated with diluted cytokine standard solution (recombinant mouse IL-12) or supernatants. The biotinylated anti-mouse IL-12 p40/70 antibody was added followed by streptavidin-peroxidase conjugate and tetramethylbenzidine substrate solution (Sigma, MO). The reaction was stopped by adding 0.2 N H₂SO₄. The optical density was read spectrophotometrically at 450 nm. The limit of detection for IL-12 was 10 pg/ml.

**Statistical analysis**

The results were analyzed by Mann-Whitney t test using Prism software (Graphpad, San Diego, CA). Survival curves were compared by Kaplan-Meier method.

**Results**

**Lethality of C3H/OuJ mice with *C. posadasii***

The C3H/OuJ mice having intact TLR4, were injected with 20 (viable 20), 50 (viable 47) and 100 (viable 93) arthroconidia to determine the lethal and sublethal doses of *C. posadasii*. All the mice challenged with 100 (93 viable) arthroconidia died within 14 days of intranasal challenge. After 35 days post challenge with 50 (47 viable) and 20 live arthroconidia, about 60% and 90% of the mice survived (Fig. 1). Thus, for subsequent infection experiments, we challenged C3H/HeJ (having defective TLR4) and C3H/OuJ (having intact TLR4) mice with a sublethal dose of 38–45 viable *C. posadasii* arthroconidia.

**Role of TLR4 in fungal dissemination**

Within 10 days of intranasal challenge with sublethal dose of arthroconidia (n = 38–45), both C3H/HeJ (having defective TLR4) and C3H/OuJ (having intact TLR4) mice lost approximately 1.9–2.8 g of body weight (about 10% of the pre-infection weight; Fig. 2a). The lungs and spleens of infected C3H/OuJ and C3H/HeJ mice weighed the same and showed visible signs of inflammation (Fig. 2b, c). When fungal burden in lungs of C3H/HeJ was compared to that of C3H/OuJ mice, there was no significant difference (Fig. 2d). However, the fungal load was 1.0 times more in spleens of C3H/OuJ mice as compared to C3H/HeJ mice (P < 0.05, Fig. 2e) suggesting that the
intact TLR4 expressed by C3H/OuJ mice may enable the dissemination of *C. posadasii* to spleen. In another set of mice, mortality was followed for a period of 40 days post infection challenge (Fig. 2f). Both types of mice started to show signs of sickness around day 11–12, with deaths beginning around day 13. There was no significant difference in survival curves or timing of appearance of symptoms in C3H/OuJ and C3H/HeJ mice. The humoral response is an indicator of the active infection and the positive anti-*Coccidioides* antibody titer is considered of prognostic value [12]. The antibody response was measured in pooled serum specimens of *C. posadasii* infected C3H/HeJ and C3H/OuJ mice. We found similar antibody titers for different IgG isotypes in pooled serum samples of C3H/HeJ and C3H/OuJ mice (Fig. 2g).

**TLR4 does not affect the secretion of Th-1 promoting-IL-12 cytokine by *C. posadasii*-infected DCs**

The Th1 response is known to be protective against *C. posadasii* infection [12]. The antigen-presenting cells including DCs play a critical role in this process [19]. The Th-1 stimulating ability of DCs partly depends on its ability to secrete IL-12 cytokine [22]. Thus, we measured the role of TLR4 in IL-12 secretion by DCs. The DCs derived from murine bone marrow specimens were 98% viable and...

![Fig. 2](image-url)

Fig. 2 Fungal burden, mortality and humoral response in *Coccidioides posadasii*-infected, TLR4 defective C3H/HeJ mice. C3H/HeJ and C3H/OuJ mice (10 mice of each type in each experiment, two experiments) were injected intranasally with 38–45 live *C. posadasii* arthroconidia on day 0. Mice were either sacrificed on day 10 (shown in a–c, g) or monitored for clinical symptoms and mortality for a period of 40 days (shown in f). (a–c) The body weight (a) and wet weights of spleen (b) and lung (c) of mice sacrificed on day 10 are shown as mean ± SEM. (d–e) The spleen and lung tissue homogenates were subjected to fungal burden assay. The cfu counts normalized per g lung (d) and spleen (e) are shown as mean ± SEM. (f) The survival curves of *C. posadasii*-infected C3H/HeJ and C3H/OuJ mice (*n* = 20 each) were analyzed by Kaplan Meier method. (g) *C. posadasii* antigen (Ag2/PRA)-specific antibody response was detected in serum samples of infected mice. The serum samples of mice in each experiment were pooled and subjected to IgG isotyping by immunoassay. Results shown in Figs. 1a–e and 1g are from one representative experiment. ns: statistically not significant, *P* < 0.05 as compared to C3H/OuJ control mice.

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showed typical phenotype of immature DCs after 6 days of in vitro culture (data not shown). The antibody-mediated blockade of TLR4 inhibited the LPS-induced IL-12 (P < 0.05), but it did not affect the IL-12 secretion by C. posadasii-infected DCs (Fig. 3).

Discussion

The infection starts by inhalation of C. posadasii arthroconidia, which in the lungs develop into endosporulating spherules that cause pulmonary coccidioidomycosis [23]. In the most severe disseminated coccidioidomycosis, the endospores spread to non-pulmonary organs. We hypothesized that the Coccidioides-arthroconidia are recognized by pathogen pattern recognition receptors expressed in the lung such as surfactant proteins (SP-A and SP-D) and TLR. The TLR-mediated mechanisms may be involved in regulation of the innate immune response and fungal pathogenesis. Earlier we showed that surfactant proteins bind to C. posadasii-derived antigens, and TLR2 may be important in innate immune signaling [14,16]. We also found that there was significant difference in level of TLR4 gene expression between C. posadasii-susceptible BALB/c and C. posadasii-resistant DBA/2 mouse strains [14]. As per the literature reports, the difference in susceptibility pattern of DBA/2 vs. BALB/c mice has also been linked to the difference in the levels of protective Th1 response [12]; however, the role of TLR4 has not been elucidated in host response against coccidioidomycosis. A recent study by Viriyakosol et al. reported that the TLR4 does not affect the macrophage response against C. posadasii in an in vitro cell culture infection model [24]. Since multiple cells, host factors and fungal virulence factors may be involved in the infection process in vivo, we studied the susceptibility of TLR4-defective mice to C. posadasii.

The results of the present study suggest that the TLR4 does not affect the Th1 (related to protective immunity)-inducing-IL-12 secretion, antibody response, progression of clinical symptoms and mortality of C. posadasii infected mice. However, as evident in Fig. 2(e), the CFU counts in spleens of C3H/OuJ mice were significantly increased as compared to the ones in spleens of C3H/HeJ mice. Although the fungal recovery was assessed only in lung and spleen, these results suggest that the intact TLR4 may be involved in dissemination of C. posadasii to non-pulmonary organs, including spleen. At the same time, mortality studies have shown that the TLR4 deficiency was not able to change the late course of infection as no differences were observed in survival curves of C. posadasii-infected C3H/HeJ and C3H/OuJ mice. The TLR4 deficiency is probably compensated by the remaining pathogen-pattern recognition receptors, such as dectin [24,25], surfactant proteins [16] and TLR2 [14,24], which have been shown to participate in innate immunity against C. posadasii. The final outcome is more likely determined by a combined effect of interaction of C. posadasii with multiple pathogen recognition receptors during the full course of infection. A more comprehensive study is needed in this direction to characterize the influence of TLR4 and synergistic or antagonistic effects of the other receptors on the effector and regulatory immune responses against C. posadasii infection.

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References


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