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Viable spores of *Coccidioides posadasii Δcps1* are required for vaccination and provide long lasting immunity

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ABSTRACT

Coccidioidomycosis is a systemic fungal infection for which a vaccine has been sought for over fifty years. The avirulent *Coccidioides posadasii* strain, *Δcps1*, which is missing a 6 kb gene, showed significant protection in mice. These studies explore conditions of protection in mice and elucidate the immune response. Mice were vaccinated with different doses and viability states of *Δcps1* spores, challenged with virulent *C. posadasii*, and sacrificed at various endpoints, dependent on experimental objectives. Tissues from vaccinated mice were harvested for *in vitro* elucidation of immune response. Vaccination with viable *Δcps1* spores was required for protection from lethal challenge. Viable spore vaccination produced durable immunity, lasting at least 6 months, and prolonged survival (≥6 months). The *C. posadasii* vaccine strain also protected mice against *C. immitis* (survival ≥ 6 months). Cytokines from infected lungs of vaccinated mice in the first four days after Cp challenge showed significant increases of IFN-γ, as did stimulated $CD4^+$ spleen cells from vaccinated mice. Transfer of $CD4^+$ cells, but not $CD8^+$ or B cells, reduced fungal burdens following challenge. IFN-γ from $CD4^+$ cells in vaccinated mice indicates a Th1 response, which is critical for host control of coccidioidomycosis.

1. Introduction

Coccidioidomycosis (Valley Fever) is caused by the two fungal species, *Coccidioides immitis* and *C. posadasii. Coccidioides* spp. were thought to be restricted to the southwestern United States and northern Mexico in North America, however, the disease has been reported and the fungus recovered from soil in northeastern Utah and southwestern Washington [1–3]. These cases suggest that the historical boundaries of the fungus are expanding, putting even more humans and animals at risk of endemic exposure. Coccidioidomycosis causes significant morbidity. Approximately 40% of infections result in clinical illness. Hospital-related costs of the disease in California alone between 2000 and 2011 totaled $2.2 billion, and attributable deaths averaged 160–170 per year nationally [4,5]. With a rate and range of disease in dogs similar to humans, Arizonans alone are spending more than $60 million per year caring for dogs with coccidioidomycosis [6,7].

A vaccine to prevent coccidioidomycosis has the potential to save healthcare dollars and prevent morbidity and mortality in both humans and dogs. We have developed an attenuated, live vaccine candidate by deleting the *CPS1* gene in *C. posadasii* to create *Δcps1* strain [8]. We showed that *Δcps1* was avirulent in both wild-type and profoundly immunodeficient mice. Vaccination protected against death and high fungal burdens in two different mouse strains and by three different routes of immunization [8]. In this report, we expand on the initial studies by examining the mechanism of protection and the ability of this vaccine to protect against challenge by both *C. immitis* and *C. posadasii*. We show that the live vaccine produces protracted survival after lethal challenge, and a long duration of immunity. We tested various doses of *Δcps1* to determine the minimal efficacious dose to optimize its use as a vaccine. We examined the *Δcps1*- induced cytokine responses in the lung and showed that the vaccine produces a Th1 skewed response with little detectable IL-17. These experiments allow us

Abbreviations: BSL, biosafety level; GYE, glucose yeast extract; Cp, *Coccidioides posadasii*; Ci, *Coccidioides immitis*; IN, intranasal; SC, subcutaneous; IP, intraperitoneal.

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to define the correlates of protection that will be critical in extending these results clinically to dogs and ultimately humans.

2. Materials and methods

Mice: Six- to eight-week old female BALB/cAnNHsd (BALB/c) and C57BL/6NHsd (B6) mice were purchased from Envigo (Indianapolis, IN). Mice were housed and used according to NIH guidelines under an approved Institutional Animal Care and Use protocol. All procedures utilizing wild-type *Coccidioides* strains were performed at animal biosafety level (ABSL) 3. All other experiments were performed under ABSL2 containment.

Fungal strains: *Coccidioides posadasii*, Silveira strain (Cp), and *Coccidioides immitis*, RS strain (Ci), were grown to maturity on 2X glucose-yeast extract (GYE) agar, and arthroconidia (spores) were harvested as previously described [9]. *Δcps1*, an avirulent strain derived from Cp with the 6 kb *CPS1* gene replaced with the *hphB* cassette (hygromycin resistance marker) [10], was grown and harvested as above with the addition of hygromycin (50 μg/ml) to medium. To verify the mutant strain, colonies were recovered on 2X GYE containing hygromycin, which suppresses growth of wild-type strains, and the mutation was confirmed by PCR. All growth and use of wild-type *Coccidioides* strains was performed at BSL3. *Δcps1* experiments were performed using BSL2 as authorized by the University of Arizona Institutional Biosafety Committee.

Vaccine preparation and vaccination: Spore suspensions were serially diluted and plated to determine viable numbers and then adjusted to the required concentration in 0.9% USP endotoxin-free saline (saline). Doses of spores used for vaccination ranged from 500 to 500,000 given once or twice intranasally (IN) or subcutaneously (SC). Doses and route are described in the individual studies. For the irradiated *Δcps1* preparation, spores were exposed to 900 gray radiation using a GammaCell 40, (Best Theratronics, Ottawa, ON, Canada), resulting in a >99.9% reduction in viability, and administered IN or SC. For the ethanol-killed preparation, *Δcps1* spores were incubated in 70% EtOH for 30 min, then washed twice and resuspended in 0.9% saline. Sterility was verified by culture. Spores were administered IN or SC. Saline injection served as a vaccine control.

Challenge with wild-type *Coccidioides:* Infections with wild-type strains of Cp or Ci (~100 spores/mouse) were administered IN by insufflation of 30 μl of spore suspension in 0.9% saline under ketamine-xylazine anesthesia as previously described [11].

Analysis of lung cytokines: B6 mice were vaccinated IN with *Δcps1* twice and infected four weeks after the booster. The right lungs from these mice were collected on days 1, 2, 4, and 6 post-infection (p.i.). Lungs were processed individually for single cell suspensions as previously described [12] Briefly, lungs were minced and fragments digested with 0.5 mg/ml collagenase I (Worthington Biochemica, Lakewood, NJ) and 0.02 mg/ml DNase I (Sigma Aldrich, St. Louis, MO) in RPMI-1640. 5×10^5 cells/well were placed in a 24-well tissue culture plate with 500 μl of complete RPMI medium with 10% fetal calf serum (RPMI/c) and were incubated for 24 h at 37 °C, 5% CO_2, to allow secretion of cytokines. Supernatants were centrifuged at 300*g* for 5 min, passed through a 0.2 μm filter for sterilization, and frozen at −80 °C until analysis by Luminex using a mouse 31-plex Panel (EMD Millipore, Billerica, MA).

Splenocyte stimulation and flow cytometry: Spleens were collected from mice vaccinated IN or SC with *Δcps1*, or vaccinated then challenged with Cp spores, and processed into single cell suspensions as previously described [12]. Cells were resuspended in RPMI/c medium, stained with trypan blue, and viability and concentration were determined. Cells (5×10^5) were dispensed into 96-well culture plates and incubated with 10 μg/ml of sterile *Coccidioides* spherule lysate at 37 °C, 5% CO_2, for 16 h. Protein Transport Inhibitor Cocktail (eBiosciences, San Diego CA) was added for the final four hours of incubation, per the manufacturer's instructions, to allow accumulation of cytokines for intracellular staining. Cells were then processed for flow cytometric analysis as previously described. Data was collected using a BD- LSR II flow cytometer (BD Bioscience, Mountain View, CA) and analyzed using FlowJo (FlowJo, Ashton, OR) [12].

Statistical analysis: Lung fungal burden data were log-transformed and analyzed by ANOVA with Tukey's correction for multiple comparisons. Cytokine secretion was analyzed by False Discovery Rate (FDR) where the FDR was set to 5%. Normally distributed data were analyzed by *t*-test to detect differences in group means. All calculations were performed using Prism (GraphPad Software, La Jolla, CA).

3. Results

3.1. Viable *Δcps1* spores are required to induce protection in mice

To determine if viable *Δcps1* spores are required to produce protection, B6 mice (N = 6/group) were vaccinated either IN or SC twice, two weeks apart, with viable spores (100,000), irradiated spores (100,000), or spores killed with EtOH (500,000). Saline injection was used as a control. Four weeks after booster, mice were infected IN with 100 spores of Cp. Mice were sacrificed on day 11 p.i. due to moribund animals in the saline control, irradiated spore, and EtOH-killed spore groups. All mice vaccinated with viable spores were clinically well at sacrifice. Fungal burdens from the saline control group, both irradiated spore groups, and both killed spore groups were significantly higher than from mice vaccinated with the live spores ($P < 0.01$ all comparisons) (Fig. 1). In this study, mice vaccinated IN with live spores also had significantly lower lung fungal burdens than those vaccinated SC ($P = 0.02$), but this result is inconsistent with other studies where we have not shown a significant difference in lung fungal burden between IN and SC administration [8]. Spleen cultures from mice given live *Δcps1* spores were negative for fungal growth. There was growth in 5/6 spleens from the mice given irradiated spores, and from all

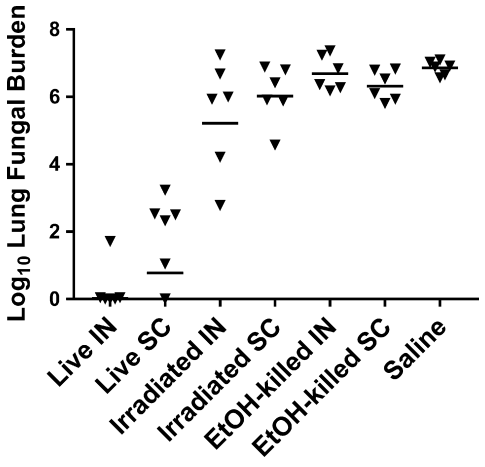

Fig. 1. B6 mice (n = 6/group) were vaccinated twice with live, irradiated (>99.9% killed), or EtOH-killed spores of *Δcps1*and challenged intranasally 4 weeks later with lethal Cp. Culture at 11 days p.i. shows that only the live spores significantly reduced lung fungal burden compared to killed preparations or saline control ($p < 0.001$, all comparisons). The route of immunization IN (intranasal) vs. SC (subcutaneous) was not significant ($p = 0.542$). Statistical analysis was by ANOVA with Tukey's correction for multiple comparisons on log-transformed data. Bar = geometric mean for each group.

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