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Dendritic cell-based immunization induces *Coccidioides* Ag2/PRA-specific immune response ☆

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ABSTRACT

Valley Fever, or coccidioidomycosis, is caused by a soil-borne, highly virulent fungal pathogen, *Coccidioides* spp. Infection with *Coccidioides* can be life-threatening. Since an effective treatment is not available and the T cell-mediated immune response is protective, vaccine development is of interest. In this study, a primary dendritic cell (DC)-vaccine was evaluated for its ability to stimulate *Coccidioides* antigen-specific immune response in an extremely susceptible BALB/c mouse model. The DC-vaccine (Ag2-DC) was prepared by non-virally transfecting the primary bone marrow-derived DCs with a plasmid DNA encoding Ag2/PRA (protective epitope of *Coccidioides*). Mice were intranasally immunized with Ag2-DC on days 2 and 10. Immunized mice were necropsied on days 8, 32, and 44. Major organs and blood samples were harvested. The most common indicators of injury (protein, lactate, and albumin), Ag/PRA-specific cytokine-secreting cells, and IgG and its isotypes were determined by biochemical and immunologic assays, respectively. No signs of sickness were noted. Similarly, no significant changes were observed in the levels of total lung protein, lactate, and albumin, in immunized mice compared with healthy control mice. Interferon (IFN-γ), and interleukin (IL)-4 and IL-17 cytokine-secreting cells were observed in lung and lymph nodes upon Ag2-DC immunization. Our results showed that the levels of serum IgG and its isotypes were increased in Ag2-DC-immunized mice. This report provides evidence of DC immunization-stimulated Ag2/PRA-specific immune responses.

1. Introduction

Coccidioidomycosis is caused by a dimorphic fungal pathogen, *Coccidioides posadasii* or *C. immitis*. It is an endemic infection in areas of the southwestern U.S., south-central Washington, northern Mexico, and parts of Central and South America [1,2]. Isolated cases are regularly reported worldwide, and are related to patients' travel to endemic regions. Severe health issues related to occupational exposure to *Coccidioides* arthroconidia have been reported among military personnel and veterans. The infection is initiated by inhalation of air-borne arthroconidia, which leads to formation of endosporulating spherules in the lungs. Local pulmonary infection is most often self-limited. In high-risk individuals, the systemic spread of endospores in non-pulmonary organs results in disseminated infection, which causes significant morbidity and mortality. Individuals with immunocompromised conditions, and of certain racial and ethnic origins, are at high risk of developing more severe disseminated coccidioidomycosis due to their poor immune status [3]. Treatment strategies include antifungal drug therapy, surgical debridement, or a combination [4]. Current therapies, often required long-term, are expensive and ineffective in preventing relapse or reactivation. Thus, new avenues are needed to improve the treatment and clinical management of coccidioidomycosis.

Abbreviations: Ag2/PRA, Antigen 2/Proline rich antigen; Ag2-DC, Dendritic cells transfected with a plasmid DNA encoding Ag2/PRA; ANOVA, Analysis of variance; 1% BSA-TBST, 1% bovine serum albumin in Tris-buffered saline containing 0.05% Tween 20; DC, Dendritic cells; DMEM, Dulbecco's minimum essential medium; ELISA, Enzyme-linked immunosorbent assay; ELISPOT, Enzyme-linked immunospot assay; EDTA, Ethylenediamine tetraacetic acid; GM-CSF, Granulocyte macrophage colony stimulating factor; HEPES, 2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid; IFN-γ, Interferon gamma; IgG, Immunoglobulin G; IL, Interleukin; MEM, Minimum essential medium; MHC, Major histocompatibility complex; PMA, Phorbol 12-myristate-13-acetate; PMSF, Phenylmethylsulfonyl fluoride; Th, T helper cell; TMB, 3,3′,5,5′ tetramethylbenzidine; V-DC, Dendritic cells transfected with vector plasmid DNA.

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Previous studies have demonstrated that the susceptibility of certain individuals and mouse strains (BALB/c and C57BL/6) to more severe disseminated infection is associated with defective cellular immune responses (reviewed in [5]). The individuals at high risk of developing disseminated coccidioidomycosis are of primary interest to the research studies performed in BALB/c mice described here. Considering the cost of morbidity and potential lifelong disability from disseminated infection, the DC-immunization approach described here could be feasible as a therapeutic vaccine more focused toward reconstitution of those patients with defunct immune systems and suffering from disseminated coccidioidomycosis.

Activation of cell-mediated immune responses depends on an effective antigen-presentation by antigen-presenting cells, such as dendritic cells (DCs). It is evident from studies in patients with disseminated coccidioidomycosis [6,7] and in susceptible mice, such as the BALB/c and C57BL/6 mouse strains [8,9], that suppressed DC responses are associated with defective T cell responses. Replenishment of the DC-mediated antigen-presentation could stimulate T and B cell responses and help control the severity of infection. Our investigations are focused on studying the DC responses in different mouse strains, developing a DC-based immunization approach, and investigating the efficacy of DC-based immunization against coccidioidomycosis [8,10,11]. BALB/c mice are extremely susceptible to coccidioidomycosis, and *Coccidioides*-infected BALB/c mice present immunological features (less interferon gamma, IFN-γ) similar to those observed in patients with disseminated disease [5]. We used genetically transfected DCs (Ag2-DC) that expressed a well-characterized protective epitope of *Coccidioides*, known as Antigen-2 or proline rich antigen (Ag2/PRA) [11,12]. Initial results on preparation of Ag2-DC, characterization, trafficking of Ag2-DC, and efficacy upon prophylactic immunization in infection models are reported elsewhere [11–14].

In this report, we describe the Ag2/PRA-specific T cell and antibody responses induced by DC-based vaccination. The intranasal DC-immunization contributes to significant retention of DCs in lung, primary organ of coccidioidomycosis [11,12]. Thus, some common indicators (lactate, albumin and protein) of lung injury were measured in mice intranasally immunized with Ag2-DC.

2. Materials and methods

2.1. Mice

Six-week-old female BALB/c mice were obtained from Jackson Laboratories, ME. All procedures involving animals were approved by the Institutional Animal Care and Use Committee of the University of Oklahoma Health Sciences Center (OUHSC), Oklahoma City. The animals were given an acclimatization period of at least one week prior to any experiment.

2.2. Murine bone marrow-derived primary DCs

Primary DCs were obtained by culturing the murine bone marrow cells that were harvested from BALB/c mice ($n = 30$ at each time of immunization) according to the method described earlier [8,15]. Briefly, bone marrow from femur and tibia bones was flushed using RPMI-1640 medium (Invitrogen-Gibco, CA). A single-cell suspension was obtained by passing the harvested bone marrow cells through nylon mesh. The cell suspension was then seeded onto 6-well tissue culture plates (Nalge-Nunc International Corp, NY) in RPMI-1640 medium containing 10 mM N-2-Hydroxye thylpiperazine-N'-2-ethanesulfonic acid (HEPES), 10 μg/ml gentamicin (Invitrogen-Gibco, CA), 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen-Gibco, CA), 10% fetal bovine serum (Invitrogen-Gibco, CA), 1% minimum essential medium nonessential amino acids, 50 μM β-mercaptoethanol, 10 ng/ml recombinant mouse granulocyte-macrophage colony stimulating factor (GM-CSF), and 10 ng/ml recombinant mouse interleukin (IL)-4 (both cytokines from Peprotech, NJ). The cells were incubated at 37 °C in a 5% carbon dioxide atmosphere. The DCs were harvested the next day using Optiprep density gradient solution (Accurate Chemicals, NY) [16]. Briefly, nonadherent cells were collected, washed, and subjected to density gradient separation, per the manufacturer's instructions. The DCs were isolated from the top layer (density < 1.065 g/ml) of the Optiprep density gradient. Harvested BALB/c mice bone marrow-derived primary DCs were used for transfection.

2.3. Transfection of primary DCs for immunization

Harvested DCs were washed with serum-free Dulbecco's minimum essential medium (DMEM; Invitrogen-Gibco, CA) before transfection. A non-viral, lipid-based transfection reagent, TransIT-TKO (Mirus Bio, WI), was used. The transfection reagent-DNA complex (4 μl:2 μg of each plasmid DNA) was prepared in DMEM by adding endotoxin-free plasmid DNA (2 μg vector plasmid DNA or 2 μg plasmid DNA encoding Ag2/PRA) to the TransIT-TKO reagent. The cells were incubated with the transfection reagent-DNA complex at 37 °C in a 5% carbon dioxide incubator. After 4 h of incubation, an additional 250 μl of complete medium was added. The percent transfection efficiency was evaluated by visual enumeration of the DCs transfected with a plasmid DNA encoding green fluorescent protein *versus* total number of cells, using a fluorescent microscope with an appropriate filter (Olympus Optical Co. Ltd, Tokyo, Japan). The viability of transfected cells was assessed by flow cytometry after staining the cells with propidium iodide (1 μg/ml). The protein expression of Ag2/PRA was studied in transfected cells by dot-immunoblotting with Ag2/PRA-specific antibody [10]. Recombinant Ag2/PRA protein served as a positive control. Purified recombinant Ag2/PRA protein and Ag2/PRA-specific antibody were obtained from Dr. John Galgiani (University of Arizona, Tucson, AZ).

Transfected DCs were scraped, washed, and suspended in sterile Dulbecco's phosphate buffered saline. Syngeneic BALB/c mice ($n = 10$ per experiment) were anesthetized, held upright, and instilled with DCs transfected with vector plasmid DNA (V-DC) or plasmid DNA encoding Ag2/PRA (Ag2-DC) suspended in 30–40 μl Dulbecco's phosphate buffered saline in each of the nares in alternate fashion. Mice were housed at a 45° angle for 30 min. Mice were then observed routinely for signs of sickness (ruffled fur, diarrhea, eye exudate, lack of reactivity, prostration, abnormal breathing) every subsequent day [17]. Mice were immunized again on day 10 with V-DC or Ag2-DC. Finally, mice were sacrificed on days 8, 32, and 44. Blood was collected using cardiac puncture, and clotted. Serum was separated by centrifugation at room temperature. Serum samples were stored at −80 °C prior to further analysis. Lung, thymus, and peripheral lymph nodes were processed for isolation of cells and assessment of immune function. A tissue piece from randomly selected region of lung was snap-frozen in liquid nitrogen for further analysis.

2.4. Lung lymphocytes

The lymphocytes were isolated from fresh lung tissue using Lympholyte-M solution (Cedarlane, Canada) per the manufacturer's instructions. Briefly, lung tissue pieces were minced between sterile frosted glass slides. A single cell suspension was obtained by passing the minced lung tissue through a nylon filter (BD Biosciences, CA). Five ml of the lung cell suspension was layered over an equivalent volume of Lympholyte M solution and cen-

trifuged for 20 min at 1,000–1,500 × g at room temperature. After centrifugation, the lymphocytes were collected from the interface and washed prior to further analysis.

2.5. Thymocytes and peripheral lymph node cells

Thymus and peripheral lymph nodes were crushed between sterile frosted glass slides. The cell suspensions were washed twice and suspended in RPMI-1640 medium containing 10 mM HEPES, 10 μg/ml gentamicin, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (Invitrogen-Gibco, CA). The viability of cells was determined by standard trypan blue dye exclusion test. The cell morphology and phenotype were determined by microscopy and flow cytometry per previously published methods [12].

2.6. Measurement of total protein, lactate, and albumin levels in lung tissue homogenates

Frozen lung tissue pieces were homogenized in a buffer containing 1% Igepal CA630, 0.1% sodium dodecyl sulfate, 0.5 mg/ml leupeptin, 0.7 mg/ml pepstatin, 0.5 mM disodium ethylenediamine tetraacetic acid (EDTA, pH 8.0), 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 0.5% sodium deoxycholate, 200 μM sodium orthovanadate, and 10 μM sodium fluoride, and were processed as described earlier [18,19]. Total protein concentration was measured in 1:100 diluted lung tissue homogenates with the bicinchoninic acid assay (Pierce, CA).

The lactate levels were measured in diluted lung tissue homogenates with a fluorometric assay kit (Biovision, CA), per the manufacturer's instructions. Briefly, 50 μl of diluted (1:500, 1:1,250 or 1:5,000) lung tissue homogenates or L-lactate standard solutions were mixed with 46 μl of lactate assay buffer, 2 μl of enzyme mix, and 2 μl of probe. The reaction mix was then incubated for 30 min at room temperature in the dark. The fluorescence (excitation/emission wavelengths = 530/590 nm) was read on a Biotek Synergy 2 multimode microplate reader.

Albumin concentration was determined in lung tissue homogenates with an ELISA kit (Bethyl Labs, TX), per the manufacturer's instructions. Briefly, albumin standard solutions or 1:5,000 diluted lung tissue homogenates were added to the antibody pre-coated wells and incubated for 1 h at room temperature. Microwells were washed and incubated with anti-albumin detection antibody and horseradish peroxidase solution. Immune complexes were then detected by adding 3,3′,5,5′ tetramethylbenzidine (TMB) substrate and stop solutions before taking the optical density readings at 450 nm.

Levels of total protein were normalized with lung weight. Concentrations of lactate and albumin were normalized with total protein and lung weight.

2.7. Release of interferon-gamma (IFN-γ), interleukin (IL)-4, and IL-17 cytokines by lung lymphocytes, thymocytes, and lymph node cells in response to Ag2/PRA

For specific time points, we pooled freshly harvested lung lymphocytes, thymocytes, and lymph node cells from all mice in a group (V-DC and Ag2-DC; $n = 4–5$ mice per group). The IFN-γ, IL-4, and IL-17 cytokine-secreting cells were enumerated in response to Ag2/PRA by using an enzyme-linked immunospot (ELISPOT) assay (eBioscience, CA) as per the manufacturer's instructions. Briefly, the microwells of ELISPOT assay plates (Millipore, MA) were coated with capture antibodies for mouse IFN-γ, IL-4, or IL-17, overnight at 4 °C. The lung lymphocytes, thymocytes, and lymph node cells (0.1–1.0 million cells per microwell) were added to the mouse IFN-γ, IL-4, or IL-17-specific antibody-coated wells and incubated with 1 μg/ml Ag2/PRA (obtained from Dr. John Galgiani, University of Arizona, Tucson, AZ) or controls (medium or 2.5 ng/ml phorbol 12-myristate-13-acetate, PMA, and 250 ng/ml ionomycin) for 48 h at 37 °C in a humidified 5% carbon dioxide incubator. The microwells were then incubated with biotinylated detection antibodies for the respective cytokines for 2 h at room temperature, followed by avidin-horseradish peroxidase reagent. After incubation for 45 min at room temperature, 3-amino-9-ethyl carbazole substrate solution was added. The reaction was stopped with deionized water. The microwell plates were air-dried and spots were counted using a dissecting microscope. The microwells with cells incubated with medium alone or PMA and ionomycin served as negative and positive controls, respectively. Antibody-coated microwells incubated with medium alone served as background controls.

2.8. Detection of Ag2/PRA specific antibody in serum samples from immunized mice

Levels of total IgG and IgG isotypes were determined as described earlier [20]. Microwells (Immulon 4HBX, Thermo Fisher Scientific, MA) were coated with 0.1 μg Ag2/PRA protein diluted in 0.1 M sodium bicarbonate buffer, pH 9.6, overnight at 4 °C. The next day, microwells were washed and incubated with 1:100, 1:1000, and 1:10,000 diluted serum samples in 1% bovine serum albumin in Tris-buffered saline containing 0.05% Tween 20 (1% BSA-TBST) at room temperature for 3 h, and then overnight at 4 °C. The microwells were washed and then incubated for 1 h at 30 °C with alkaline phosphatase-conjugated anti-mouse IgG, IgG1 and IgG3 (1:2,000), IgG2a (1:500), and IgG2b (1:1,000) antibodies (Invitrogen-Gibco, CA) diluted in 1% BSA-TBST. The immune complex was then detected with a substrate solution containing 1 mg/ml p-nitrophenylphosphate (Sigma, MO), 1 M diethanolamine, and 0.5 mM magnesium chloride, pH 9.8. After 30 min of incubation with substrate solution, the reaction was stopped with 3 M sodium hydroxide. Optical density was read at 405 nm.

2.9. Statistics

The results were analyzed by *t*-test or Analysis of Variance (ANOVA) for statistical significance using Graphpad Prism software (San Diego, CA). Statistical significance was noted at $p < 0.05$, or as otherwise indicated.

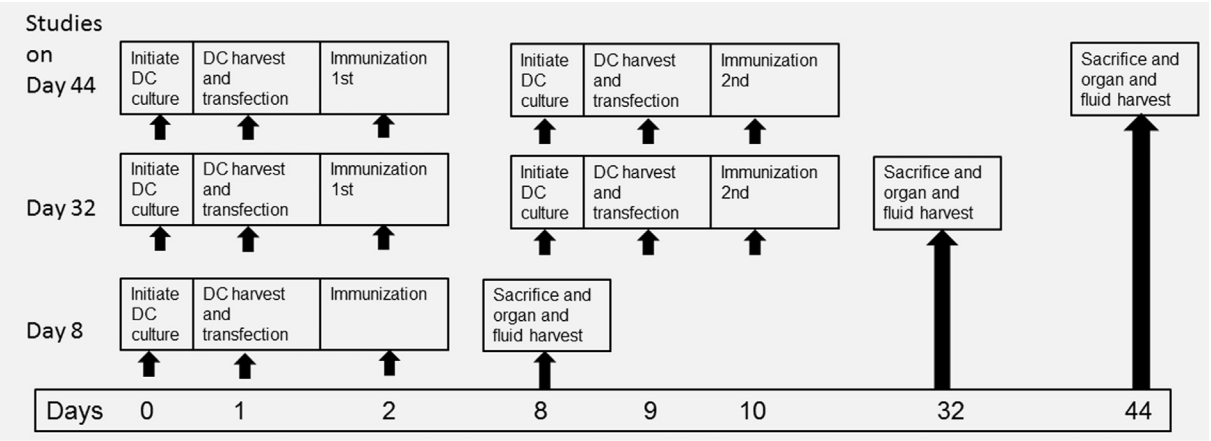
3. Results

3.1. Characteristics of V-DC and Ag2-DC

The BALB/c mice-derived DCs showed characteristics of immature myeloid DCs, and were negative for typical macrophage (CD14), T cell (CD3), and B cell (CD45RA) markers, as reported earlier [8,10,12]. At the time of immunization, about 78–90% of transfected cells remained viable. Mice were immunized with syngeneic primary bone marrow-derived DCs transiently transfected with vector plasmid DNA (V-DC) or plasmid DNA encoding Ag2/PRA (Ag2-DC) on days 2 and 10. The transfection efficiency was about 30–50%, and Ag2-DC expressed Ag2/PRA. The numbers of V-DC and Ag2-DC and the schedule of immunizations and endpoints are shown in Fig. 1.

3.2. Indicators of lung injury post-immunization

All mice immunized with V-DC or Ag2-DC survived for the complete duration. No signs of sickness were observed in the animals. Since significant localization of cells was noted in lungs of DC-vaccinated mice [11,12], selected markers of lung injury were mea-



Studies on days	Immunization	V-DC	Ag2-DC
8	Immunization	0.5 x 10^6 cells	0.5 x 10^6 cells
32	1st immunization	1.0 x 10^6 cells	1.0 x 10^6 cells
	2nd immunization	0.4 x 10^6 cells	0.4 x 10^6 cells
44	1st immunization	0.6 x 10^6 cells	0.6 x 10^6 cells
	2nd immunization	0.2 x 10^6 cells	0.2 x 10^6 cells

Fig. 1. Timeline and dose (number of cells) of immunizations of BALB/c mice with Ag2-DC and V-DC on days 2 and 10. Immunized mice were sacrificed on days 8, 32, or 44. Organs (lung, thymus, and peripheral lymph nodes) and blood were harvested for analyzing the Ag2/PRA-specific immune response and injury parameters.

sured over time. Total protein, lactate, and albumin levels were analyzed in lung tissue homogenates of vaccinated mice as markers of injury to the lung. Our results demonstrate that there was no significant change in levels of protein in V-DC (range 34,390–42,594 μg per g lung) and in Ag2-DC (28,860–40,982 μg per g lung) immunized groups compared with those levels in nonimmunized healthy control mice (24,298 μg per g lung). Similarly, no change was noted in lactate (V-DC 12.2–18.6, Ag2-DC 21.1–25.9, and control 8.54, μmole per g lung) or albumin (V-DC 4,788–6,038, Ag2-DC 4,298.82–5,498.08 and control 2,651.01 μg per g lung) levels between the groups (Fig. 2).

3.3. Ag2/PRA-specific T cell response in immunized mice

The antigen-specific cytokine response was determined by incubating lung lymphocytes, lymph node cells, and thymocytes with Ag2/PRA. Specifically, IFN-γ, IL-4 and IL-17 cytokine-secreting cells were quantified and normalized per million cells using an ELISPOT assay (Fig. 3). As expected, no spot was visible in microwells with medium alone in the absence of cells (background control). The cytokine-secreting T cells were detected upon treatment with PMA and ionomycin, and were present in greater numbers than were observed in unstimulated cell populations. The cytokine-secreting lung lymphocytes were enumerable in immunized mice as early as day 8 of immunization (Fig. 3A-i). The Ag2/PRA-specific cytokine-secreting thymocytes were not detected in sufficient numbers at any time after immunization (Fig. 3A-ii). Few lymph node cells secreted IFN-γ and IL-17 in response to Ag2/PRA. Only subtle differences were noted in the frequency of cytokine-secreting cells in Ag2-DC and V-DC immunized mice in response to Ag2/PRA.

3.4. Systemic humoral immune response was detected in immunized mice

The antibody levels were analyzed against Ag2/PRA in mice immunized with Ag2-DC and V-DC (Fig. 4). The optical density readings were in the range of 0.08–0.09 for blank and with bovine serum albumin. Also, the optical density values in nonimmunized mice were lower or equivalent to those in V-DC immunized mice. An antibody response was observed within 8 days of the first immunization. Increased levels of Ag2/PRA-specific total IgG were observed in Ag2-DC-immunized mice on days 8 and 32 ($p < 0.05$) compared with those in V-DC-immunized mice. Specifically, a significant increase was noted in IgG2a, IgG2b, and IgG3 within 8 and 32 days of immunization (Fig. 4). Although the mean IgG1 antibody level was increased in Ag2-DC-immunized mice on day 44, this increase was not statistically significant compared with that in V-DC-immunized mice (Fig. 4).

4. Discussion

Literature reports, including the one from our laboratory, have demonstrated that DCs derived from susceptible BALB/c and C57BL/6 mouse strains secrete reduced levels of IL-12 in response to *Coccidioides* as compared with the resistant DBA/2 mouse strain [8,9,11,21]. These results led us to focus on using the DC-based immunization approach for replenishment of the DC and T cell responses. Our results demonstrated the efficacy of Ag2-DC (prepared using C57BL/6 mice-derived JAWS II DCs) in reducing fungal burden, in stimulating IFN-γ response, and in improving lung pathology in syngeneic mouse models of coccidioidomycosis induced by intraperitoneal and intranasal challenge with live *Coccidioides* arthroconidia [11]. Our initial results have been encouraging in support of the DC-based immunization approach.

In our previously published studies, the phenotypic characteristics of primary bone marrow-derived DCs and Ag2-DC were investigated over time, and trafficking of intranasally administered Ag2-DC was analyzed in BALB/c mice [8,12–14]. Our results demonstrated that substantial numbers of intranasally administered Ag2-DC are retained in the mucosal organs, mainly in the lung and gut [11,12]. Thus, Ag2-DCs are anticipated to present Ag2/PRA to resident naïve T cells, which can eventually be activated to become effector or memory T cells at mucosal surfaces. This is

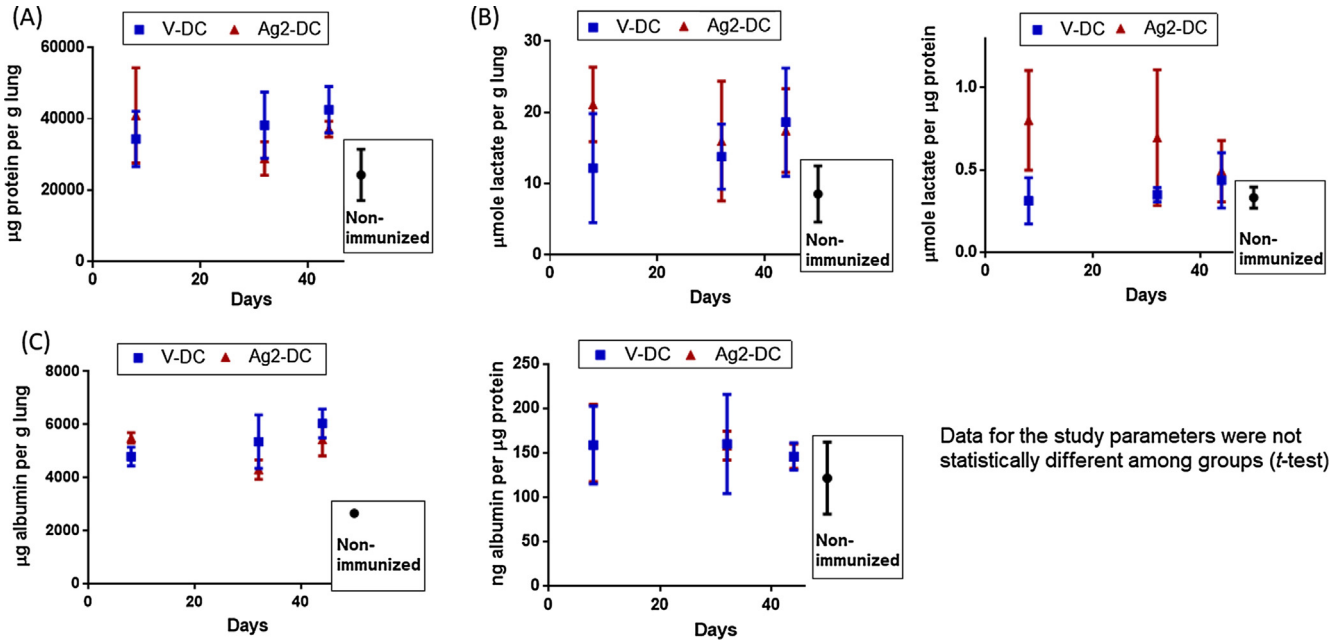


Fig. 2. Levels of total protein, lactate, and albumin in lung tissues harvested from mice immunized with Ag2-DC or V-DC. The concentrations of protein were normalized with grams lung weight (A). The lactate (B) and albumin (C) concentrations were normalized with protein concentration in lung and gram lung weight. Nonimmunized mice were included as control. Results are mean and *SEM* of triplicate measurements from four-to-five mice per group. Small error bars are contained within the symbol. *t*-test was performed for statistical analysis.

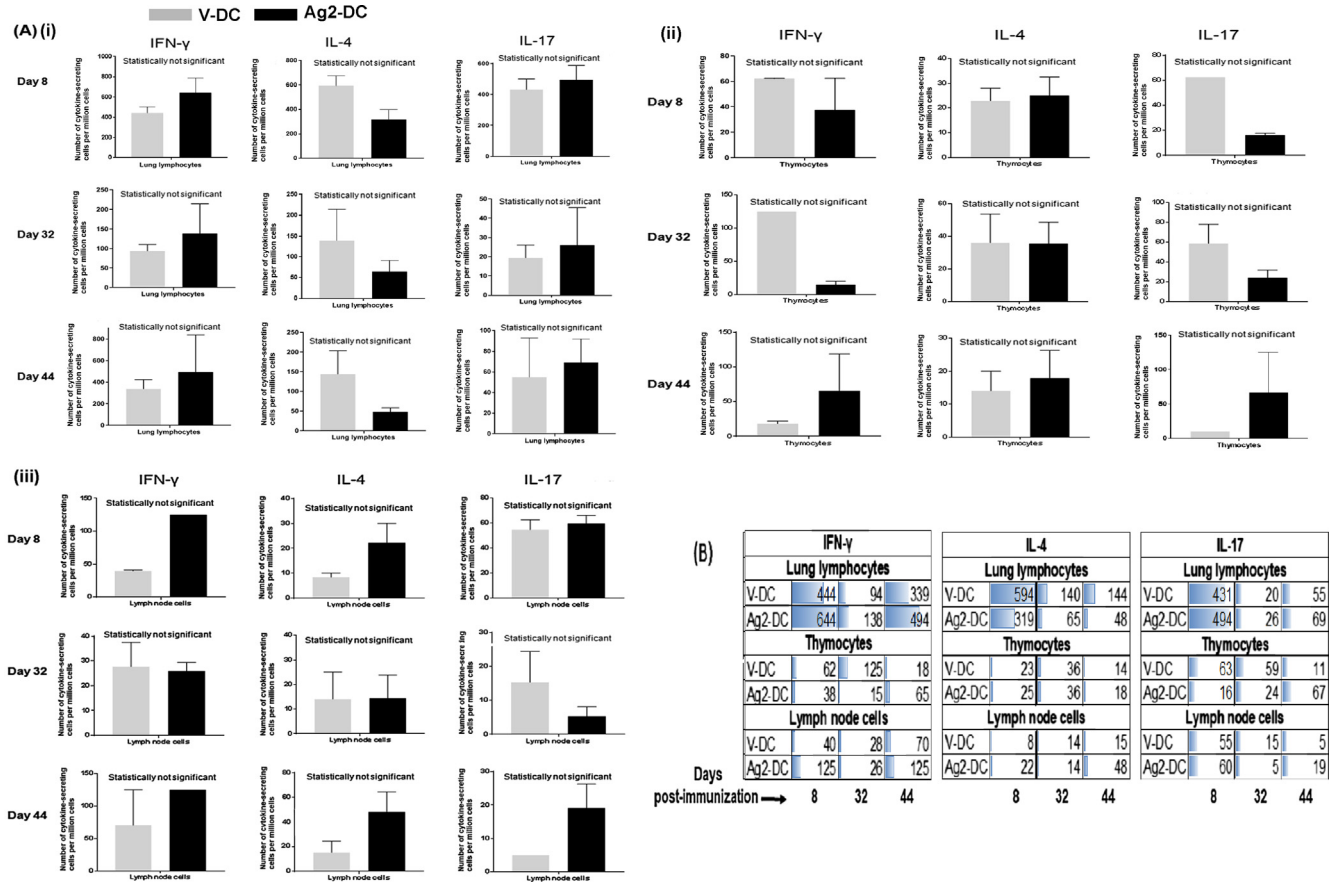


Fig. 3. Ag2/PRA-specific secretion of IFN-γ, IL-4, and IL-17 by lung lymphocytes, thymocytes, and peripheral lymph nodes harvested from mice immunized with Ag2-DC or V-DC. (A) The number of cytokine-secreting cells were calculated per one million lung lymphocytes, thymocytes, and peripheral lymph nodes (harvested from four-to-five mice per group) in response to Ag2/PRA. (B) Time-dependent changes in cytokine-secreting T cells in immunized mice. Cells treated with medium alone or PMA and ionomycin were included as negative and positive controls, respectively. No spot was detected in antibody-coated wells in the absence of cells (background control wells). Results are mean + *SEM* of two independent readings of spots. Small error bars are contained within the bar. Statistical analysis was performed using *t*-test.

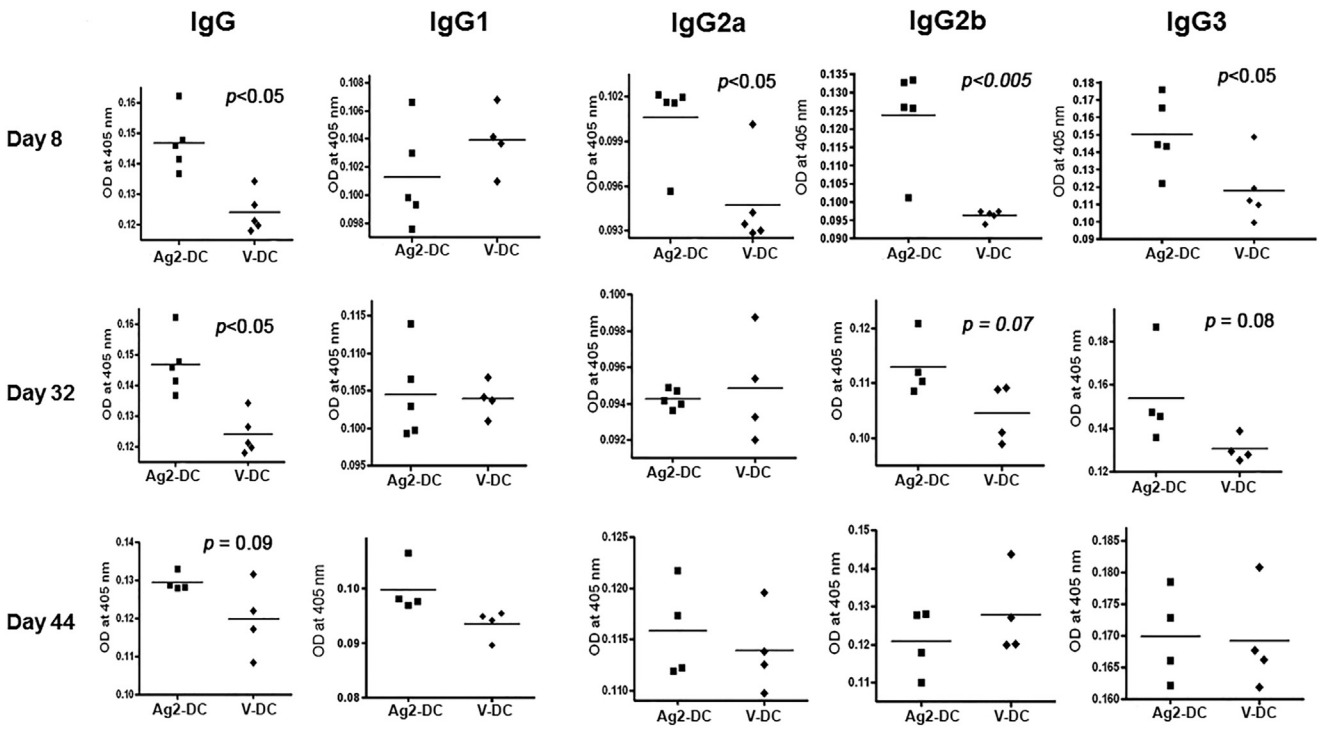


Fig. 4. Levels of Ag2/PRA-specific total IgG, and IgG1, IgG2a, IgG2b, and IgG3 isotypes in serum samples harvested from mice immunized with Ag2-DC and V-DC. Ag2/PRA-specific antibody and bovine serum albumin were included as positive and negative controls, respectively. Individual optical density values obtained from ELISA with 1:100 diluted serum samples are shown as scattered dot plots. Mean values are indicated as straight line. Statistical significance of the results between the groups was analyzed using *t*-test.

of interest because induction of mucosal immunity is critical for protection against lung pathogens, including coccidioidomycosis initiated by inhalation of *Coccidioides* arthroconidia. Inhaled *Coccidioides* arthroconidia develop into endosporulating spherule structures in the lung, and cause pulmonary infection. The spherule structures then rupture and release endospores, which are systemically disseminated throughout the host's body [22].

Intranasal immunization with Ag2-DC demonstrated reduced fungal burden, increased levels of IFN-γ in lung, and improved tissue pathology in the C57BL/6 mouse model of coccidioidomycosis induced by intraperitoneal and intranasal challenge with live *Coccidioides* arthroconidia [11]. Thus, the intranasal route was used for immunization of mice with Ag2-DC for an investigation of T cell cytokine-secreting cells and antibody response over time.

It is important that any therapeutic agent or vaccination does not induce adverse effects. Although the number of DCs was about 0.2–1.0 × 10^6 per immunization (Fig. 1), relatively few compared with the total lung cell population, and the DCs were derived from syngeneic mice with same genetic background and identical immune status, no unwanted immunologic response or adverse effects were anticipated. All mice survived and no signs of sickness or adverse effects were observed. The use of syngeneic mice is beneficial, as there is no contribution of other compounding immune or genetic factors towards immune stimulation. Only selected indicators of lung injury (protein, lactate, and albumin) were included to evaluate potential injury as a result of intranasal immunization with Ag2-DC or V-DC. Increased levels of protein, lactate, and albumin are associated with injury-induced vascular leakage and metabolic changes, and are well-known indicators of lung injury [23]. No significant change was observed in the levels of total lung protein, lactate, or albumin at any time post Ag2-DC or V-DC-immunization (Fig. 2). This finding suggests that the DC-vaccination itself does not cause any vascular leakage or tissue injury.

Activation of T cells is considered a critical step of protective immunity against coccidioidomycosis [24–26]. An increased level of IFN-γ (Th1 response) is associated with vaccine-induced immunity and protection in mouse models [25]. Defects in IL-12/IFN-γ/signal transducer and activator of transcription 3 axes are associated with disseminated coccidioidomycosis [27,28]. Treatment with IFN-γ improved the clinical conditions of patients with coccidioidomycosis [29,30].

While the efficacy of some vaccines has been associated with a combination of Th1 and Th17 responses in mouse models, others have produced only minimal detectable amounts of IL-17. It is possible that different vaccine antigens and epitopes, adjuvants, and vaccine schedules could contribute to diverse cytokine profiles and immune responses. While our previously published studies included prophylactic DC-vaccination [11], investigations from other laboratories have demonstrated immunomodulation or stimulation by DCs in reversing the T cell anergy, as seen in patients with disseminated coccidioidomycosis [7]. In the antigen recall assay, the IFN-γ, IL-4, and IL-17 cytokine-secreting cells were enumerable in lung cell populations of Ag2-DC immunized mice, and few cells in peripheral lymph nodes were also noted to secrete cytokines (Fig. 3). Although IL-4 and IL-17 cytokine levels were not measured, significantly increased levels of IFN-γ cytokine were detected in lungs of mice prophylactically administered Ag2-DC, and were associated with reduced fungal burden and improved lung pathology upon live *Coccidioides* challenge [11].

While the importance of T cells has been emphasized, the protective role of B cells or antibodies in vaccine-induced immunity against *Coccidioides* has not been studied widely. There is no published report available about associations between antibody deficiency and severe disseminated coccidioidomycosis in humans. However, upon immunization with protective formalin-killed spherule-based vaccine, the B-cell-deficient mice demonstrated reduced survival compared with 100% survival in normal mice [31].

Although the mechanism is not known for the presentation of Ag2/PRA by DCs, Ag2/PRA is predicted to encode both MHC class I and MHC class II binding peptides. According to an *in silico* anal-

ysis performed using an interactive program at the Immune Epitope Database and Analysis Resource supported by the National Institute of Allergy and Infectious Diseases, regions within Ag2/PRA protein (194 amino acids) are predicted to have affinity with BALB/c mouse strain-specific MHC I and MHC class II alloantigens (*unpublished results*). An understanding of the mechanism of antigen-presentation by Ag2-DC could help explain the immune profile, including IgG and IgG isotypes, in the vaccinated mice. These findings are of interest, as the DC-vaccines are known to induce B cells and produce antibodies [32].

When antibody levels were examined retrospectively in DC-immunized C57BL/6 mice included in our previously published study, [11] we noted significantly increased levels of Ag2/PRA-specific total IgG (mean optical density + *SEM*: 1.44 + 0.04 in Ag2-DC *versus* 0.18 + 0.007 in V-DC), and IgG1 (0.96 + 0.03 in Ag2-DC *versus* 0.1 + 0.002 in V-DC) and IgG2b (1.33 + 0.04 in Ag2-DC *versus* 0.14 + 0.01 in V-DC) isotypes (*unpublished results*). These results suggest that the Ag2/PRA-specific antibody response is associated with reduced fungal burden and improved lung pathology.

The cellular and humoral immune responses (T cells and antibody levels) described in this report are measures of primary response as a result of DC-vaccination in BALB/c mice. A robust secondary immune response is anticipated upon live fungal challenge. More focused studies are warranted to investigate the mechanism of action and feasibility of DC-immunization as a therapeutic vaccine in high-risk groups, such as bone marrow transplant patients or immunocompromised patients, who are more prone to or who are already suffering from disseminated coccidioidomycosis.

5. Authors' contributions

SA designed and coordinated the study, and compiled the manuscript. PV harvested the bone marrow, cultured DCs, and prepared the Ag2-DC and V-DCs. SA and PV performed intranasal immunization and organ harvest. SA and AC performed immunoassays. NR prepared lung tissue homogenates, and measured protein, lactate, and albumin levels.

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Conflict of interest

None

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