UNIVERSITY OF CALIFORNIA, MERCED

Characterizing Innate and Adaptive Immune Responses to vaccine strain *Coccidioides posadasii (cts2/ard1/cts3Δ)*

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Quantitative and Systems Biology

by

Anh Loan Diep

Committee in charge:

Professor Marcos E. García-Ojeda, Chair

Professor Katrina K. Hoyer, Advisor

Professor Clarissa J. Nobile

Professor Anita Sil

The Dissertation of Anh Loan Diep is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Katrina K. Hoyer, Advisor

_____

Anita Sil

_____

Clarissa J. Nobile

_____

Marcos E. García-Ojeda, Chair

University of California, Merced

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To my K-12 teachers who taught me to dream and believe in myself,

To my parents who helped me to persevere and fight on,

To my friends who encouraged me to fly,

I dedicate this thesis to you.

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LIST OF ABBREVIATIONS

APC, antigen presenting cell

BM, bone marrow

BMDM, bone marrow derived monocytes

BL/6, mouse from the C57BL/6 background

CBC, complete blood count

CD, cluster of differentiation

CFU, colony forming units

CTLA-4, cytotoxic T lymphocyte antigen 4

DC, dendritic cell

DC1, dendritic cell subtype 1

DC2, dendritic cell subtype 2

dLN, draining lymph node

FBS, fetal bovine serum

Foxp3, forkhead box p3

i.n., intranasal

i.o., intraorbital

i.p., intraperitoneal

IFNγ, interferon gamma

IL, interleukin

KO, knockout

LN, lymph node

MFI, mean fluorescence intensity

mLN, mesenteric lymph node

M0, macrophage

M1, classically activated macrophage

M2, alternatively activated macrophage

O.C.T, optimal cutting temperature compound

PBS, phosphate buffered saline

PI, post-infection

PMA, phorbol 12-myristate 13-acetate

RBC, red blood cells

RT, room temperature

Teff, CD4 T effector

Th, CD4 T helper

Treg, T regulatory

UC, University of California

WT, wild type

LIST OF SYMBOLS

α, alpha; antibody against

β, beta

°, degree

Δ, delta; deleted or knocked out

ε, epsilon

γ, gamma

μ, micro

n, nano

+, positive expression

-, negative expression

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Chapter 1: Introduction

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Chapter 2: Macrophage and Dendritic Cell Activation and Polarization in Response to Coccidioides posadasii Infection

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Chapter 3: Regulatory T Cells influence Coccidioides Infection Clearance and Local Immune Cell Activation

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CURRICULUM VITA

EDUCATION AND RESEARCH

2013-2017	Bachelor of Science, Immunology and Microbiology University of California, Merced School of Natural Sciences
2014-2015	University of California, Merced School of Social Sciences, Humanities and Arts Laboratory of Dr. Teenie Matlock
2015-2017	University of California, Merced School of Natural Sciences Laboratory of Dr. Kirk Jensen
2017-2021	Doctor of Philosophy, Quantitative and Systems Biology University of California, Merced School of Natural Sciences Laboratory of Dr. Katrina Hoyer

GRANTS, FELLOWSHIPS AND AWARDS

2017-2021	Teaching Assistant School of Natural Sciences University of California, Merced
2019	GradSlam 2019 People's Choice Award University of California
2019	GradSlam Campus Finalist, University of California, Merced
2018	QSB Summer Research Fellowship University of California, Merced
2017	QSB Summer Research Fellowship University of California, Merced

PUBLICATIONS AND PRESENTATIONS

PUBLICATIONS:

Diep, A. L., Tejeda-Garibay, S., Miranda, N., & Hoyer, K. K. (2021). Macrophage and Dendritic Cell Activation and Polarization in Response to *Coccidioides posadasii* Infection. Journal of Fungi, 7(8), 630.

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PRESENTATIONS:

"Immune factors contributing to chronic disease outcome in Valley Fever"-Poster
Anh L. Diep[1], Dan Davini[1], Clarissa J. Nobile[1], Aaron Hernday[1], Anita Sil[2], Katrina K. Hoyer[1]
Department of Molecular and Cell Biology, University of California Merced[1]; Department of Microbiology and Immunology, University of California San Francisco[2]
Presented at: UCSF/UCB/UCM Immunology Retreat 2018, September 2018
UC Merced's Research Week, March 2019
Bay Area Microbial-Pathogenesis Symposium at UC San Francisco, March 2019

"Immune Differentiation in Response to Coccidioides"-Talk
Anh L. Diep[1], Dan Davini[1], Clarissa J. Nobile[1], Aaron Hernday[1], Anita Sil[2], Katrina K. Hoyer[1]
Department of Molecular and Cell Biology, University of California Merced[1]; Department of Microbiology and Immunology, University of California San Francisco[2]
Presented at: Bay Area Microbial-Pathogenesis Symposium at UC San Francisco, March 2019
63rd Coccidioidomycosis Meeting at UC Davis, April 2019

"Valley Fever and the Immune Response"-Talk
Anh L. Diep[1], Dan Davini[1], Katrina K. Hoyer[1]
Department of Molecular and Cell Biology, University of California Merced[1]
Presented at: Medical Spanish Learning Project Conference II, November 2019
Early Childhood Education Center at UC Merced, February 2020
University Friends Circle Luncheon, February 2020

"Innate immune cell activation and function during Coccidiodes infection"-Poster
Anh L. Diep[1], Kelly Otsuka[1], Lek Wei Seow[1] , Clarissa J. Nobile[1], Anita Sil[2], Katrina K. Hoyer[1]
Department of Molecular and Cell Biology, University of California Merced[1]; Department of Microbiology and Immunology, University of California San Francisco[2]
Presented at: Asilomar MidWinter Conference, January 2020
Research Week Showcase-Poster Presentation, March 2020

"Macrophage and dendritic cell responses to Coccidioides"-Poster
Anh L. Diep[1], Susana Tejeda-Garibay[1], Nadia Miranda[1] Samuel Arda[1], Katrina K. Hoyer[1]

[1]Department of Molecular and Cell Biology, University of California Merced
Presented at: Bay Area Microbial-Pathogenesis Symposium via virtual conference, March 2021
65th Coccidioidomycosis Study Group via virtual conference, April 2021

Characterizing Innate and Adaptive Immune Responses to vaccine strain *Coccidioides posadasii (cts2/ard1/cts3Δ)*

by

Anh Loan Diep

Doctor of Philosophy in Quantitative and Systems Biology

University of California, Merced, 2021

Professor Katrina K. Hoyer

Coccidiomycosis, colloquially known as Valley fever and Desert fever, is a respiratory fungal disease caused by *Coccidioides immitis* and *Coccidioides posadasii*. In the United States this fungus is endemic to the California San Joaquin Valley, most of Arizona, and the American Southwest. Infection cases are increasing but there is still no effective vaccine or new therapeutics against severe chronic and disseminated coccidiomycosis. Tremendous work has been done over the years to elucidate infection pathogenesis, fungal genetics, and fungal immunity. However, more work must be done to deeply characterize effective and ineffective immune responses to *Coccidioides* to further enhance therapeutics and fungal vaccine development. We assess host immune response to *Coccidioides* posadasii (*cts2/ard1/cts3*Δ), an avirulent vaccine strain previously characterized to provide effective protection. We show that avirulent *Coccidioides posadasii* infection in in vitro cell-based assays demonstrate macrophages hold no bias towards pro-inflammatory (M1) or anti-inflammatory (M2) polarization while DCs become proinflammatory (DC1). Macrophages and DCs show decreased MHC-II and CD86 co-expression after culture with avirulent *Coccidioides*, suggesting a novel virulence mechanism by which *Coccidioides* can block immune activation by inhibiting antigen presenting cell (APC) activation and maturation. In vivo infections show a promising mixed DC1/DC2, pro- and anti-inflammatory, response with no changes in APC activation/maturation, suggesting other immune cells contribute to protective immunity. Our adaptive experiments suggests that Tregs play a detrimental role in *Coccidioides* clearance. When adoptively transferred, Tregs increase fungal burden in the lungs and enhance DC2 frequency. These results highlight the complicated nature of vaccine development and suggest that the effective, protective avirulent strain induce immune activation that is inhibited by Treg presence. This work contributes to characterizing vaccine-induced immune responses to *Coccidioides* infection. This thesis work builds a foundation for future immune studies aimed at manipulating host immunity to reduce disease severity.

CHAPTER 1

Background and historical context

frontiers in Cellular and Infection Microbiology

REVIEW
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Host Response to *Coccidioides* Infection: Fungal Immunity

Anh L. Diep[1] *and Katrina K. Hoyer*[1,2,3*]

[1] Quantitative and Systems Biology, Graduate Program, University of California Merced, Merced, CA, United States, [2] Department of Molecular and Cell Biology, School of Natural Sciences, University of California Merced, Merced, CA, United States, [3] Health Sciences Research Institute, University of California Merced, Merced, CA, United States

Coccidioidomycosis is a fungal, respiratory disease caused by *Coccidioides immitis* and *Coccidioides posadasii*. This emerging infectious disease ranges from asymptomatic to pulmonary disease and disseminated infection. Most infections are cleared with little to no medical intervention whereas chronic disease often requires life-long medication with severe impairment in quality of life. It is unclear what differentiates hosts immunity resulting in disease resolution versus chronic infection. Current understanding in mycology-immunology suggests that chronic infection could be due to maladaptive immune responses. Immunosuppressed patients develop more severe disease and mouse studies show adaptive Th1 and Th17 responses are required for clearance. This is supported by heightened immunosuppressive regulatory responses and lowered anti-fungal T helper responses in chronic *Coccidioides* patients. Diagnosis and prognosis is difficult as symptoms are broad and overlapping with community acquired pneumonia, often resulting in misdiagnosis and delayed treatment. Furthermore, we lack clear biomarkers of disease severity which could aid prognosis for more effective healthcare. As the endemic region grows and population increases in endemic areas, the need to understand *Coccidioides* infection is becoming urgent. There is a growing effort to identify fungal virulence factors and host immune components that influence fungal immunity and relate these to patient disease outcome and treatment. This review compiles the known immune responses to *Coccidioides* spp. infection and various related fungal pathogens to provide speculation on *Coccidioides* immunity.

Keywords: *Coccidioides immitis*, *Coccidioides posadasii*, Coccidioidomycosis, Valley fever, host pathogen interactions, fungal immunity

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Marcus De Melo Teixeira, University of Brasília, Brazil
Marley C. Caballero Van Dyke, Northern Arizona University, United States

***Correspondence:**
Katrina K. Hoyer
khoyer2@ucmerced.edu

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INTRODUCTION

Coccidioidomycosis is a fungal lung disease caused by inhalation of *Coccidioides immitis* and *Coccidioides posadasii*. It is a disease endemic to the Southwestern United States, Central America, and South America and is typically transmitted from the soil *via* wind (Johnson et al., 2014). In endemic regions of the American Southwest alone (California, Nevada, Utah, Arizona, and New Mexico) the estimated incidence is 122.7 cases per 100,000 (Benedict et al., 2019). Fungal arthroconidia enter the lungs and differentiate into a spherule state that replicates *via* endosporulation. Subsequent endospore rupture spreads the fungus, resulting in tissue damage

and inflammation (Ternovoĭ and Golotina, 1977). Clinically, coccidioidomycosis is often misdiagnosed as pneumonia or lung cancer (Nguyen et al., 2013; Saenz-Ibarra et al., 2018). When the host immune system is unable to clear infection, it develops into a chronic state sometimes disseminating into other organs (Nguyen et al., 2013). In 60% of cases, infection remains asymptomatic or presents mild flu-like symptoms and is cleared by the host with little to no medical intervention (Saubolle et al., 2007). In 40% of cases, patients present moderate to severe flu-like symptoms that can become chronic. One percent of symptomatic cases develop severe disseminated infection (Saubolle et al., 2007).

In large part due to biosafety regulations, *Coccidioides* has been much less explored than other fungal pathogens. Though first reported in 1892, and with research dating back to the early 1900s, focus on the immune response against *Coccidioides* did not begin until the 1980s (Smith, 1940). A small but dedicated group of immunologists focus on fungal pathogens and host responses, but there is a critical need for further research into host responses to *Coccidioides*. There is little understanding of the immune events and players that contribute to disease resolution or chronic infection. Clinicians currently rely on symptom-based diagnosis, antibody-antigen assays, and imaging (chest x-rays and CT scans) to diagnose *Coccidioides*-infected patients, but these methods are limited in their ability to assess disease progression and host clearance capacity (Johnson et al., 2012; Wack et al., 2015). This review explores the initiation of innate immune responses and the development of adaptive immune responses to *Coccidioides*. Where gaps in *Coccidioides* knowledge exists, closely related fungal pathogens are used to extrapolate.

DISEASE AND EPIDEMIOLOGY

Coccidioides is endemic in regions with heavy intermittent rains along with the hot, arid summers (Johnson et al., 2014; Coopersmith et al., 2017; McCotter et al., 2019) It is found primarily in alkaline soils with high surface salinity (Elconin et al., 1964; Swatek, 1970; Lacy and Swatek, 1974). During wet, rainy months, filamentous threads composed of barrel-like subunits called arthroconidia expand within the soil. Environmental stresses, such as heat or digging, disrupts the soil and aerosolize the arthroconidia, making it airborne (McCotter et al., 2019). Infection occurs when arthroconidia are inhaled into the lungs and temperature and moisture differences trigger morphological change from arthroconidia to spherule to endospore (Johannesson et al., 2006). The fungal spherule develops into an endospore, capable of maturing and bursting to release more spores (Nguyen et al., 2013). As fungi develop, the host presents generic flu-like symptoms including headache, fever, body ache, coughing, and respiratory distress (Johnson et al., 2012; Wack et al., 2015).

Coccidioides infection also occurs in non-human animals, spanning across taxonomical classes from reptiles to mammals (Fisher et al., 2007; del Rocío Reyes-Montes et al., 2016). Animal carcasses are often found positive for *Coccidioides* while the surrounding soil environments test negative for the fungi (del Rocío Reyes-Montes et al., 2016). Originally, animals were believed to be accidental hosts, but genomic analysis indicates that *Coccidioides* code for several animal peptidases and lack enzymes associated with plant-decomposing fungi (Fisher et al., 2007; del Rocío Reyes-Montes et al., 2016; Taylor and Barker, 2019). This suggests that *Coccidioides* infected animals can act as fungal distributors, transporting the fungus from the initial infection site and upon animal death returning the fungus to new soil. Animal carcasses may also act as a protective, nutrient rich nursery for *Coccidioides* growth. Peptidase expression suggests that *Coccidioides* evolved methods to infect and thrive inside a protein rich environment, perhaps contributing to its success in surviving in harsh, alkaline environments. Wind and disturbance from other scavenging animals can then further disseminate the fungus driving human infection.

Agricultural, construction, and fieldwork in endemic regions are risk factors for fungal exposure. San Joaquin Valley (SJV) in California is an agricultural hub, supporting over 180,000 agricultural and 100,000 construction/labor jobs (Garcia and Young; Nicas, 2018). Solar energy field expansion puts solar-panel workers at risk for fungal exposure (Wilken et al., 2015; Laws et al., 2018). Legislative efforts in California have mandated *Coccidioides* risk education and safety protective equipment for at-risk workers in endemic areas (Salas, 2019). In Arizona, disease incidence increases with age, with those over the age of 70 experiencing the highest rate of disease (McCotter et al., 2019). Disease susceptibility for coccidiomycosis has been correlated with increasing age, with the elderly being much more susceptibility to infection and severe disease (Saubolle et al., 2007; Johnson et al., 2012; Nguyen et al., 2013; Wack et al., 2015). The high disease incidence in Arizona has been credited to the steady influx of new settlers over the last few decades and the increasing popularity of the state amongst retirees (McCotter et al., 2019).

Disease impact is further complicated by socioeconomic constraints. In California's SJV, Hmong and Latino minorities make up a large percentage of field workers and soil-based laborers (Johnson et al., 2014). These populations tend to fall into the lowest wealth bracket with little to no access to healthcare, thus representing those with the least availability and opportunity to seek medical care, and the most exposed to *Coccidioides* (Mobed et al., 1992). Health care practioners working in the are often trained outside the endemic region and are initially unfamiliar with disease symptoms (Saenz-Ibarra et al., 2018). In 2007 in Arizona, only 50% of health care providers surveyed were confident in treating *Coccidioides* infection and only 21% correctly answered treatment questions (Chen et al., 2011). Since then, Arizona has implemented specialized coccidioidomycosis continuing medical education for in-state practioners, resulting in health care providers being twice as likely to answer treatment questions correctly compared to their untrained cohort. In California, outreach programs throughout endemic regions are providing disease awareness for physicians and patients, while legislative efforts aim to

mandate coccidioidomycosis centric continuing medical education courses to provide specialized regional training (Salas, 2018).

INNATE IMMUNITY

Innate Detection and Immune Evasion

The lungs maintain many defense mechanisms to survey and eliminate airborne threats. Lung epithelial cells (LECs) secrete anti-microbial peptides, complement proteins, and defensins which enhance granulocyte activity and create a less hospitable environment for *Coccidioides* (Hernández-Santos et al., 2018). To survive, *Coccidioides* must successfully avoid detection from surveying and patrolling innate immune cells. Lung-resident macrophages, also known as alveolar macrophages, comprise up to 95% of pulmonary leukocytes and participate in early immune detection of pathogens and maintain the lung microenvironment (Wynn and Vannella, 2016). In *Aspergillus* infections, tissue-specific neutrophils are recruited by LECs and enter the lung early after infection due to β-glucan and chitin (Dubey et al., 2014). Innate leukocytes control early pathogen invasion *via* phagocytosis and production of reactive oxide and reactive nitrogen species (RNS) (Xu and Shinohara, 2017). β-glucan and chitin are conserved across many fungal species, including *Coccidioides*, so these molecules could interact with epithelial cells and aid in neutrophil recruitment. In cases where host immune responses cannot control infection, disease becomes chronic. Host responses sometimes control infections through granuloma formation in the lung as fungi is walled off instead of destroyed (Nguyen et al., 2013; Johnson et al., 2014; Wynn and Vannella, 2016).

To survive lung defenses and evade innate immune responses, *Coccidioides* expresses virulence factors for immune evasion and survival. Inside the lung, arthroconidia express ornithine decarboxylase, an enzyme implicated during growth from arthroconidia to spherule state (Guevara-Olvera et al., 2000). During transition, the spherule internal cell wall segments bud off into endospores. Lifecycle transition allows vulnerable, easily phagocytosed, arthroconidia to develop into phagocytosis-resistant spherules (Hung et al., 2002; Gonzalez et al., 2011; Nguyen et al., 2013). Arthroconidia are vulnerable to RNS while mature spherules suppress nitric oxide species (NOS) and inducible NOS expression in macrophages (**Figure 1**) (Gonzalez et al., 2011). Mature spherules are too large for most host phagocytic activity, allowing *Coccidiodes* to evade early immune detection (Hung et al., 2002). *Coccidioides* induces host expression of arginase resulting in ornithine and urea production, important components for transition from arthroconidia to spherule (Hung et al., 2007).

In the spherule state, *Coccidioides* secretes metalloproteinase 1 (Mep1) which digests an immunodominant antigen spherical outer wall glycoprotein (SOWgp) on the fungal surface (**Figure 1**) (Hung et al., 2005). Phagocytotic granulocytes rely on pathogen associated molecular patterns such as SOWgp, thus Mep1 secretion prevents detection by innate immune cells (Hung et al., 2005). *Coccidioides* upregulates nitrate reductase during development, an enzyme that converts nitrate to nitrite, thereby enhancing *Coccidioides* survival in anoxic conditions, such as those found inside a granuloma (Johannesson et al., 2006). Early detection to inhaled fungus is critical for host response. Macrophages and neutrophils detect *Coccidioides* arthroconidia and immature spherules *via* receptors Dectin-1, Dectin-2, and Mincle interacting with SOWgp (Hung et al., 2002; Nguyen et al., 2013). Endothelial lung cells use these same receptors to regulate defensin secretion.

Toll-like receptors (TLRs) and c-type lectin receptors (CLRs) interact with major pathogen-associated molecular patterns to detect *Coccidioides* (Romani, 2004; Viriyakosol et al., 2008;

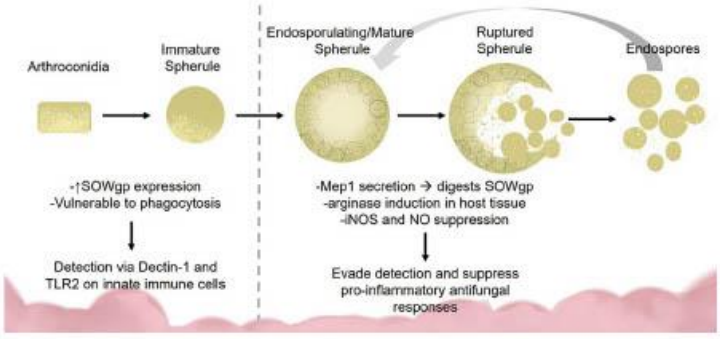

FIGURE 1 | Fungal dimorphism presents challenges for immune detection and activation. Early infection: *Coccidioides* is vulnerable to immune detection during early infection due to the smaller size (2–5 μM) and SOWgp expression which is detected *via* Dectin-1 and TLR2 on innate immune cells. These interactions mediate clearance *via* phagocytosis and reactive oxide species production. Later infection: As *Coccidioides* sporulates, it secretes MEP1 which digests SOWgp from the fungal surface, hampering immune detection. Spherules induce arginase expression in host tissues, suppressing NOS/NO production *via* an unknown mechanism, contributing to immune suppression.

Viriyakosol et al., 2013). Like most fungi, *Coccidioides* expresses β-glucans, chitins, and mannans in the outer cell wall (Nguyen et al., 2013). These cell components are recognized by a variety of TLRs and CLRs and elicit strong inflammatory responses from local immune cells. *Coccidioides* interactions with TLR2 and Dectin-1 on macrophages activate production of reactive oxide species (ROS) and inflammatory cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNFα) (Viriyakosol et al., 2008; Viriyakosol et al., 2013). There are no known nucleotide-binding oligomerization domain-like (NOD-like) receptors yet associated with *Coccidioides* detection.

In humans, polymorphisms in IFNγ/IL-12 signaling pathway result in a STAT1 gain of function mutations that associate with increased disease severity in *Coccidioides*, *Histoplasma*, and *Candida* infection (Sampaio et al., 2013). In disseminated *Coccidioides*, patients with severe disease were found to have a STAT3 mutation (Odio et al., 2015). STAT 3 mediates IL-23 signaling, critical for IFNγ, IL-12, and IL-17 production while STAT1 signaling induces Th1 cell differentiation in response to IL-12 to produce IFNγ; IFNγ, in turn, inhibits Th17 differentiation (Yeh et al., 2014). IL-12β1 receptor deficiency is associated with increased risk of disseminated coccidioidomycosis (Yeh et al., 2014). In chronic mucocutaneous candidiasis, gain of function mutations in STAT1 and STAT3 correlates to more severe disease and poor TH17 responses (Zheng et al., 2015). These observations suggest that STAT1 and STAT3 immune signaling is critical in host control of Th1/Th17 cytokine balance and is required for protection and *Coccidioides* fungal control.

In *Blastomyces dermatitidis* infection, LECs regulate collaborative killing between alveolar macrophages, dendritic cells (DC), and neutrophils (Hussell and Bell, 2014; Hernández-Santos et al., 2018). Upon LECs ablation, *B. dermatitidis* phagocytosis is reduced, and viable yeast numbers increase. Other data suggests that IL-1/IL-1R interactions regulate CCL20 expression in LECs. Chemokine CCL20 strongly recruits lymphocytes and weakly recruits neutrophils (Hernández-Santos et al., 2018). IL-1R-deficient mice express less CCL20 and lung Th17 cells are reduced, suggesting that IL-1/IL-1R signaling in LECs could regulate adaptive immune functions (Hernández-Santos et al., 2018). IL-1R is critical for vaccine induced resistance to *Coccidioides* infection *via* MyD88 induction of Th17 responses (Hung et al., 2014a; Hung et al., 2016a). Though it has not been explored, LECs could mediate early responses to *Coccidioides* through IL-1R, suggesting another innate immune cell role in anti-fungal responses within the lung tissues.

Alveoli structure likely helps shape local immune responses. Three dominant cell types exist within and around the alveoli structure: Type 1 and Type 2 pneumocytes (also known as alveolar epithelial cells, AECs), and tissue-resident alveolar macrophages (Guillot et al., 2013; Hussell and Bell, 2014). Type 1 pneumocytes (AECI) secrete IL-10 constitutively, which bind to IL-10R on alveolar macrophages to maintain an anti-inflammatory state. Type 2 pneumocytes (or AECII) express CD200 which interacts with CD200R on alveolar macrophage to inhibit pro-inflammatory phenotype (Guillot et al., 2013; Hernández-Santos et al., 2018). Alveolar macrophages express TGFβ-receptors that bind to pneumocyte-expressed αvβ6 integrin, tethering them in the alveolar airspace. In inflammatory conditions, AECIs upregulate TLRs and AECIIs increase SP-A and SP-D production (Guillot et al., 2013). These surfactant proteins are known to enhance pathogen opsonization and phagocytosis, and are capable of binding to *Coccidioides* antigen (Awasthi et al., 2004). *Coccidioides* infected mice expressed less SP-A and SP-D protein in their bronchial lavage fluid compared to uninfected and vaccinated controls, demonstrating the pathogen's capability of altering the lung mucosa (Awasthi et al., 2004). AECII secreted production of surfactant proteins may be influenced by *Coccidioides* allowing fungal escape of phagocytosis and prolonged survival.

Neutrophils

Neutrophils are the first responders and most abundant granulocytes in the immune system, making up 40%–70% of the total leukocyte population at homeostasis (Kolaczkowska and Kubes, 2013). Neutrophils destroy pathogens *via* phagocytosis, secretion of anti-microbial peptides, and extracellular traps, and provide signals for monocyte entry to sites of infection (Schaffner et al., 1986; Jain et al., 2016). In a 1:1 neutrophil to *Coccidioides* endospore interaction, human neutrophils readily phagocytose *Coccidioides* endospores and exhibit partial phagocytosis of larger spherules, coined "frustrated phagocytosis" (Lee et al., 2015). Neutrophils from healthy patients and chronic coccidioidomycosis patients exhibit similar neutrophil phagocytosis capabilities; however, high neutrophil presence is associated with chronic *Coccidioides* infection (Lee et al., 2015; Davini et al., 2018). This suggests that expanded neutrophil presence is detrimental for *Coccidioides* clearance perhaps due to their persistence into later stages of infection that may preclude other more effective responses (Davini et al., 2018). This in combination with neutrophil inability to fully phagocytose large endospores may make neutrophils ineffective, allowing prolonged fungal infection.

Neutrophil presence in tissue is typically associated with inflammatory tissue damage and pro-inflammatory cytokine expression such as IL-6 and IL-1β (Kolaczkowska and Kubes, 2013). Neutrophils follow C3a, C5a, IL-8, and IFNγ gradients toward sites of inflammation (Kolaczkowska and Kubes, 2013; Liu et al., 2017). However, chemoattractive molecules have limited stability and diffusion capabilities through tissues, suggestive that high neutrophil recruitment requires robust and/or steady expression of chemokines, which may also cause more inflammatory tissue damage. Until recently, it was believed that neutrophils migrate to infected tissue to mediate pathogen clearance and die within infected tissue after a few hours. Newer evidence suggests neutrophils re-enter circulation from the site of infection and may disseminate inflammation from the original recruitment site (Jain et al., 2016). For *Coccidioides* infection, this suggests a potential novel method for neutrophil dissemination of infection from the lungs where neutrophilia might promote disseminated disease. Depletion of neutrophils in *Paracoccidioides brasiliensis* infection results in decreased IFNγ

and IL-17 with almost all infected mice succumbing to infection (Pino-Tamayo et al., 2016). This highlights the delicate balance of helpful versus harmful responses that pro-inflammatory innate immune cells play during disease clearance. In murine models of disseminated fungal infection with *Blastomyces*, *Aspergillus*, and *Candida*, neutrophils transdifferentiate into specialized hybrid neutrophil-DCs, and *in vitro*, neutrophil-DCs retain microbicidal, neutrophil-like function while also stimulating CD4+ T cell differentiation (Fites et al., 2018). This suggests a dual role for neutrophils in coccidioidomycosis, where too much neutrophil presence could contribute to disseminated disease and tissue damage, while some appropriate level response allows stimulation of adaptive immunity. *In vivo* examination and characterization of neutrophil-DC hybrids may provide a better understanding of innate immune cell influence on adaptive immune cell responses in *Coccidioides* infection. *In vivo* examination of neutrophil migration may unveil dissemination mechanisms, allowing for targeted therapeutics to prevent severe, disseminated coccidioidomycosis.

Macrophages and Alveolar Macrophages

Macrophages engulf fungi and generate ROS and NOS that aid in degrading pathogens (Hussell and Bell, 2014). Fungicidal activity against *Coccidioides in vitro* by murine alveolar macrophages is enhanced in the presence of IFNγ (Beaman, 1987). IFNγ enhances and promotes phagocytosis, oxide species generation, pro-inflammatory cytokine production, and macrophage differentiation into the M1 phenotype for pathogen clearance and recruitment of other pro-inflammatory cells (Gentek et al., 2014). Pathogen recognition receptors on macrophages bind to targets for phagocytosis. Specifically, Dectin-1 and TLR2 interactions with *Coccidioides* facilitates clearance by macrophages by promoting oxide species and pro-inflammatory cytokine production (Viriyakosol et al., 2005; Tam et al., 2014).

Lung resident alveolar macrophages reside in air-exposed tissue compartments of the alveoli. Alveolar macrophages remove and clear particulates such as dust, pollutants, or airborne microorganisms in the respiratory mucosal surfaces (Lohmann-Matthes et al., 1994; Hussell and Bell, 2014). Alveolar macrophage-depleted mice challenged with *Aspergillus fumigatus* had higher fungal burden than wild-type mice (Gonzalez et al., 2011). When alveolar macrophages and DCs are ablated during *Aspergillus* infection, neutrophil infiltration increases (Lohmann-Matthes et al., 1994). This suggests alveolar macrophages and DCs may inhibit neutrophil recruitment during productive immunity to fungal lung infections.

Alveolar macrophages also promote tolerance to commonly encountered lung antigens (Hussell and Bell, 2014). Resting alveolar macrophages closely resemble an M2 or alternatively activated macrophage (Hussell and Bell, 2014). It is theorized that these cells require cooperation between many receptors to override the basal anti-inflammatory, tolerogenic state found in the lungs (Wilken et al., 2015). Once activated, alveolar macrophages exhibit higher respiratory burst, phagocytotic capabilities, and inflammatory cytokine production (Lohmann-Matthes et al., 1994). These cells are regulated through interactions with IL-10R, CD200R, TGFβ-R, mannose receptors, and triggering receptors (TREM1 and TREM2), which all dampen proinflammatory signaling pathways (Hussell and Bell, 2014). Following inflammation caused by high viral infection, murine alveolar macrophages have decreased TLR2 responsiveness, low mannose receptor and high CD200R expression (Hussell and Bell, 2014). Acute inflammation seems to irreversibly change the overall alveolar macrophage responsiveness toward pathogen invaders. This has interesting implications for *Coccidioides* infection in patients with chronic inflammatory lung diseases such as asthma, chronic obstructive pulmonary disease (COPD), or high pollution exposure. In coccidioidomycosis, it is possible that M1 macrophages may be required for pathogen clearance, but instead become M2 due to signals from fungal factors (**Figure 2A**). Studies examining macrophages recruited following *Coccidioides* infection would help characterize the macrophage subtypes needed for fungal clearance. Such data may identify novel macrophage targets to treat *Coccidioides* infection by influencing macrophage differentiation.

Eosinophils

Eosinophils are granulocytes associated with parasite infection, allergy, and asthma (Uhm et al., 2012). They make up 1%–3% of the leukocytes in the immune system and migrate to sites of inflammation *via* IL-5 chemotaxis (Yamaguchi et al., 1988; Uhm et al., 2012). Though not typically associated with an anti-fungal innate response, immunocompromised patients with allergic bronchopulmonary aspergillosis have high eosinophil lung infiltration during fungal infection, suggestive of maladaptive immunity (Chong et al., 2006). In chronic *Coccidioides*, infection correlates with peripheral blood eosinophilia (Harley and Blaser, 1994; Davini et al., 2018). Clinical observations from a *Coccidioides* case study highlight a correlation between asthma and poor fungal clearance, marked by high eosinophil lung infiltrate (Lombard et al., 1987). Increased IL-5 secretion in TNFα-deficient mice results in high eosinophil numbers and decreased IL-17A production, linking eosinophil changes to reduced adaptive responses (Fei et al., 2011). In acute *Paracoccidioides brasiliensis* infection, eosinophil recruitment is modest compared to uninfected mice but upon neutrophil depletion, eosinophil numbers increase significantly in the lungs (Pino-Tamayo et al., 2016). However, even with increased eosinophil presence, these mice still succumbed to infection with higher fungal burden. These data suggest that eosinophils could be recruited in the absence of neutrophils as a compensatory mechanism but unfortunately are not as protective as neutrophils.

Eosinophil presence in pulmonary coccidioidomycosis inversely correlates to neutrophil frequency (Lombard et al., 1987; Lee et al., 2015). In *Aspergillus*-allergy asthma murine models, lung DCs secrete TNFα which preferentially recruits neutrophils over eosinophils in the lung (Fei et al., 2011). This suggests local lung leukocytes influence immune cell recruitment and potentially control *Coccidioides* infection by establishing a different lung microenvironment (Fei et al., 2011). It is unknown whether asthma contributes to poor *Coccidioides* clearance, and asthma

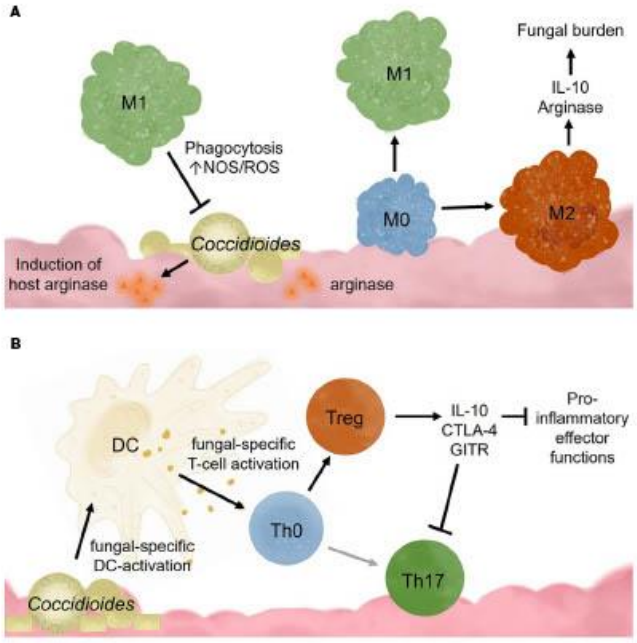

FIGURE 2 | Innate immune cell responses to *Coccidioides* influence adaptive immune cell activation and effector functions. **(A)** *Coccidioides* may evade innate immune cell clearance and influence immune functions. **(B)** Dendritic cells are critical for activating adaptive responses and influencing adaptive immune cell population differentiation.

rates are high in current endemic regions. It could be that eosinophil and neutrophil recruitment varies between acute and chronic coccidiomycosis patients due to predisposed lung conditions such as allergies and asthma. Therefore, exploring pre-existing health conditions and disease progression may provide important clues to define effective clearance mechanisms.

Dendritic Cells

DCs are professional antigen presenting cells, responsible for the initiation, regulation, and maintenance of adaptive immune responses (Desch et al., 2013). Lung-resident DCs must also maintain a balance between activation against invading pathogens and tolerance to continuous antigen exposure. DCs are adept at identifying fungal pathogens and promoting pathogen clearance, due to their powerful ability to activate naïve T lymphocytes (Romani et al., 2002). *Coccidioides* spherule recognition induces human DC maturation and activation resulting in elevated CD40 and CD80/CD86 (B7.1/B7.2) surface expression and heightened T lymphocyte stimulation (Dionne et al., 2006). DCs from healthy human patients, pulsed with *Coccidioides* spherule lysate, induce antigen-specific T cell activation (Richards et al., 2001).

There are many DC subsets with distinct functionality (Segura, 2016). Some promote inflammatory immune activation while others induce tolerance and tissue regeneration. Conventional DCs (cDC) versus monocyte-derived DCs (mDCs), exhibit opposing migration capacity to the lungs (Nakano et al., 2013). cDCs travel into the lung during homeostasis, whereas mDCs only migrate into lung tissue during active inflammation (Segura, 2016). Lung endothelial cells secrete IL-10, which maintains an anti-inflammatory, tolerogenic state and promotes anti-inflammatory DC function (Segura, 2016; Teitz-Tennenbaum et al., 2018). In IL-10 deficient mice infected with *Cryptococcus*, DCs upregulate inducible NOS expression and recruit neutrophils and M1 macrophages to the lungs during infection (Teitz-Tennenbaum et al., 2018). In contrast, IL-10-sufficient DCs express high arginase and CD206 (mannose receptor) which promotes tolerance within the lung by inducing IL-10 expression within endothelial tissues (Teitz-Tennenbaum et al., 2018). Maintaining an anti-inflammatory state within the lungs is critical for preventing unnecessary tissue damage to the delicate airspace architecture. Tolerance and anti-inflammatory signals are thus critical for ensuring that tissue-damaging inflammation does not occur unless pathogenic danger is imminent. Since DCs are responsible for controlling adaptive immune responses, understanding tissue-resident DCs and lung-microenvironmental influences is critical for understanding immune activation choices during pathogen detection and clearance.

DCs possess phagocytotic and pathogen clearance capabilities and act as professional antigen presenters to adaptive immune cells. Like macrophages that polarize into pro-inflammatory or anti-inflammatory subsets, DCs also exhibit polarization and DC polarization skews helper T cell differentiation toward specific subtypes (de Jong et al., 2005). *Cryptococcus neoformans* promotes an anti-inflammatory DC phenotype which suppresses inflammatory innate cells activation, allowing fungal persistence (Teitz-Tennenbaum et al., 2018). IL-10 blockade results in DCs with higher expression of costimulatory molecules and pro-inflammatory cytokines (Segura, 2016). In *Histoplasma capsulatum infection*, CD8α+ pro-inflammatory DCs were found to be critical for fungal clearance by inducing CD4+ T helper 1 (Th1) cells and CD8+ T cells (Lin et al., 2005).

DCs regulate T cell responses and immunological memory generation required for effective vaccines. Creating vaccines for dimorphic fungal pathogens is difficult as T lymphocytes recognize specific antigens and polymorphic fungal pathogens express different antigens throughout their life cycle. DCs have the unique challenge of presenting and mounting an effective immune response against *Coccidioides* regardless of morphological stage. Recent attempts at DC-based vaccinations show promising success in mouse models. Adoptive transfer of *Coccidioides*-antigen loaded DCs reduces murine fungal burden by live, virulent *Coccidioides* challenge (Awasthi, 2007). Disseminated coccidioidomycosis patient DCs loaded with T2K antigen overcame T cell anergy, driving T cell proliferation (Richards et al., 2002). This highlights a potential therapeutic where patients' immune responses could be reactivated for fungal clearance. In murine vaccine studies, *Coccidioides* antigens delivered with glucan-chitin particles (GCP) effectively stimulate DC inflammatory responses. DCs loaded with GCP-antigen complex induce a mixed T helper 1 and T helper 17 response against *Coccidioides* infection, thought to be critical for effective fungal clearance (Hung et al., 2018a). These studies suggest that manipulating DC responses may be a viable route to creating vaccines that induce strong and specific immunity and may overcome pre-existing T cell anergy. The antigen subunit studies suggest effective DC activation requires multi-variant antigen interactions, and that multi-antigen vaccine therapies are promising strategies against *Coccidioides*.

INTRODUCTION TO ADAPTIVE IMMUNITY

Unlike innate immunity, adaptive immunity offers higher pathogen specificity, more powerful pathogen control mechanisms, and the ability to establish memory against future infections. Infection persistence implies a breakdown in immunity effectiveness or host feedback mechanisms protecting against damaging responses. To understand why chronic *Coccidioides* infections occur, we must first understand effective immunity to *Coccidioides*.

B Cells

Protective immunity against *Coccidioides* is mediated predominantly by T cells (Hung et al., 2018a). *Coccidioides*-specific antibodies are observed in human and mouse studies, indicating that B cells also recognize and interact with *Coccidioides* antigen. IgG antibody is the prominent antibody isotype observed in humoral mediated responses to *Coccidioides* infection, indicative that class-switching occurs (Magee et al., 2005). Screening patients infected with *Coccidioides*, *Histoplasma*, and/or *Blastomycoses* with complement-fixing assays identified IgG1 as the predominant isotype generated.

Thirty days post-infection, mice have no neutralizing or complement-fixing antibodies in their sera against *Coccidioides* antigen and adoptive transfer of B cells from immunized mice into naïve mice does not confer protection (Beaman et al., 1979). In a contrasting study, T cell rich, B-cell deficient transfer conferred early protection, but the mice ultimately succumbed to disease 34 days post-infection (Magee et al., 2005). Mice that received whole splenocyte (mixed T cell, B cell, and other immune cell populations) transfers survived the longest. This could be due to the inclusion of B cells or other immune populations in the transfer. Vaccination with formalin fixed *Coccidioides* spherules and secondary intranasal challenge with live, virulent *Coccidioides* in BALB/c mice causes a marked increase in B cell-specific genes within whole lung tissue and generation of *Coccidioides*-specific serum antibodies (Magee et al., 2005). These studies suggest that B cells have a protective role in *Coccidioides* response and may even increase in frequency within the lung as suggested by bulk gene expression analysis data where B cell specific genes increased in expression in *Coccidioides* infected lung tissue compared to uninfected lung (Magee et al., 2005).

IgG generation requires CD4+ T cell-mediated class-switching and may explain the discrepancy between the B cell studies described above: T cells might provide initial protection, but without B cells there is no sustained antibody protection. Some *Coccidioides* antigens stimulate both T and B cell responses (Zhu et al., 1997). SOWgp is the best known immunostimulating *Coccidioides* antigen, eliciting a humoral response and innate immune cell activation (Hung et al., 2000). Disagreement around B cells in protective immunity against *Coccidioides* might be partially explained due to varied use of live-wildtype, live-attenuated, or formalin-fixed *Coccidioides* between studies, and the purity of cell populations utilized. *Coccidioides* is polymorphic and expresses multiple antigen types throughout its lifecycle, thus effective neutralizing antibodies and class-switching may be required at different stages of the immune response. High affinity antibody generation requires affinity maturation *via* somatic mutation, processes reliant on CD4+ T cell help. Considering all these data together, B cells likely contribute to adaptive and humoral immunity against *Coccidioides*, but further work is needed to definitively define the contribution to disease progression and control.

T Cells and Effector Cytokines

Patients that recover from coccidioidomycosis with little to no medical intervention have polyfunctional T lymphocytes in circulation (Nesbit et al., 2010). Upon *Coccidioides* antigen stimulation, peripheral human CD4+ and CD8+ T lymphocytes secrete pro-inflammatory cytokines, such as IL-2,

TNFα, and IFNγ (Nesbit et al., 2010). In humans, HIV and immunosuppression are risk factors for severe, disseminated infection and this risk is associated with decreased CD4+ T cell numbers (Saubolle et al., 2007; Johnson et al., 2012; Wack et al., 2015). In T cell-deficient mice, infection is severe, and effector T cell transfer protects mice against virulent infection (Fierer et al., 2006). CD4+ T cells from immunized C57BL/6 mice transferred into non-immunized CD40-deficient mice confer protection and prolong survival (Zhu et al., 1997). Vaccination with an attenuated *C. immitis* laboratory strain in CD4-deficient mice is protective, suggesting that CD8+ T cells can protect against *Coccidioides* (Fierer et al., 2006). No other studies show direct CD8+ T cells contribution to *Coccidioides* immunity. However, the CD8+ T cell study used an intraperitoneal infection, not intranasal delivery, so translation to pulmonary infection is unclear. In other fungal infections such as *Blastomyces* and *Histoplasma*, IL-17 producing CD8+ T cells provide protection and fungal clearance even in CD4-deficient mouse models (Nanjappa et al., 2012; Hung et al., 2016b). Overall, CD8α+ T cells may contribute to *Coccidioides* immunity, but further work is needed to characterize their anti-fungal mechanisms.

T cell differentiation into subsets allows targeted and tailored immune responses to different pathogen classes. For anti-fungal responses, T helper 1 (Th1) and T helper 17 (Th17) cells are especially critical in *Coccidioides* murine infections (Nanjappa et al., 2012). Loss of either of these T helper subtypes, or their associated cytokines, results in impaired immune responses and impaired fungal clearance. In human *Coccidioides* infection, Th17 cells are protective and acute coccidioidomycosis patients have high Th17 promoting serum cytokine levels (Davini et al., 2018). *In vitro Coccidioides* antigen stimulation of peripheral blood cells from acute disease patients yields robust IL-17 production (Hung et al., 2016b). *Coccidioides*-induced Th1 and Th17 cells secrete cytokines that mobilize innate immune cells to the site of infection, promote the activation and differentiation of immune cells, and induce anti-microbial peptides in endothelial cells (Hung et al., 2016b). This crossroad in innate immunity mediated by Th17 is observed in other fungal pathogens that infect mucosal tissues (Khader et al., 2009). Th1 cells make cytokines that enhance macrophage phagocytosis and ROS generation.

Coccidioides-resistant DBA/2 mice mount strong Th1 responses against *Coccidioides*, with increased serum IL-12 production (Magee and Cox, 1996). IL-12 administration to *Coccidioides*-susceptible BALB/c mice results in lowered fungal burden in lungs and spleen, whereas IL-12 blockage dramatically increases fungal burden across tissues, suggesting that IL-12 is protective against *Coccidioides* (Magee and Cox, 1996). DBA/2 mice express fully formed C-type lectin receptors, Dectin-1, unlike susceptible C56BL/6 mice with truncated Dectin-1. Dectin-1 is critical in fungal pathogen recognition and loss of dectin-1 correlates to increased fungal burden and decreased adaptive immune responses (Vautier et al., 2010) Dectin-1 interacts with *Coccidioides* β-1,3-glucans located in the outer cell wall and induces antibody class-switching and production, and CD8+ T cell activation (Viriyakosol et al., 2013). Dectin-1 binding to its ligand induces antigen presenting cell secretion of IL-1β, IL-23, IL-6, and TGFβ, cytokines necessary for Th17 cell differentiation. It is theorized that CARD9, an adaptor molecule downstream of Dectin-1 signaling, promotes intracellular signaling required for Th17 differentiation. CARD9-deficient mice are unable to clear pulmonary and subcutaneous infections with a highly virulent strain of *Coccidioides posadasii* (C735) (Hung et al., 2016a). These data emphasize the importance of fungal sugar pattern recognition receptor interactions for supporting Th1 and Th17 responses in adaptive immunity. In a multivalent vaccine study, CARD9 mediated Dectin-1 and Dectin-2 interactions were critical for establishing protection against *C. posadasii* infection (Campuzano et al., 2020). Mice not expressing CARD9, Dectin-1, or Dectin-2 all have significantly lower inflammatory cytokine responses and fail to mount Th17 responses within the lung. These data emphasize that though adaptive immune responses are critical, innate-associated receptors are required for establishing adaptive immunity, emphasizing that early innate interactions set the state for later adaptive responses.

C57BL/6 and BALB/c mice infected with an attenuated strain of *Coccidioides posadasii* (ΔT) have increased Th1 and Th17 frequencies and reduced fungal lung burden, further supporting the observation that these T helper responses are necessary for anti-fungal protection (Hung et al., 2016b). In other fungal pathogen studies, IL-17, IL-21, and IL-22 secretion by Th17 cells was vital for protection. IL-17 stimulates neutrophil and macrophage pro-inflammatory abilities and stimulates epithelial cells to secrete β-defensins (Khader et al., 2009). IL-21 acts as an autocrine regulator and promoter of Th17 proliferation, IL-22 induces host-secreted anti-microbial peptides, and TNFα promotes multiple proinflammatory pathways through NF-κβ and MAPK (Khader et al., 2009). These functions make Th17 cells and their effector cytokines very powerful against fungal pathogens. IL-1R deletion results in a significant decrease in Th17 numbers in *Coccidioides*-infected lungs, while Th1 numbers remain unchanged (Hung et al., 2014a). Lung Th17 numbers decrease in *Coccidioides*-infected IL-1R deficient mice relative to WT mice while Th1 numbers remain unchanged. In a human population study analyzing genetic susceptibility to *Blastomyces* infection, researchers found that IL-6 loss of function mutations increases susceptibility against *Blastomyces* infection (Merkhofer et al., 2019). IL-6 knock-out mice had lower Th17 responses and increased fungal burden within the lungs. These data emphasize the importance of Th17 responses against fungal infection while highlighting the complexity of cytokine networks needed to establish and regulate anti-fungal responses.

Th17 cells also participate in memory responses. In chronic pulmonary disease and fungal infection (*C. posadasii*, *H. capsulatum*, and *B. dermatitidis*), vaccine induced Th17 cells are sufficient for overcoming secondary challenge (Zelante et al., 2007). However, in *C. albicans* and *A. fumigatus* infection, Th17 cells are detrimental for fungal clearance, highlighting the variable role Th17 cells play in adaptive immunity. Th17 cells dampen neutrophil function and recruitment but also induce

hyperinflammatory responses depending on the immune context (Zelante et al., 2007). There are two known Th17 subsets: pathogenic (GM-CSF producing) Th17 and non-pathogenic (IL-10 producing) Th17 (El-Behi et al., 2011; Bystrom et al., 2019). While advances have been made in defining effector T cell functions during chronic fungal infection, much more work is needed to understand how chronic inflammation alters function. This may explain why Th17 cells are critical for memory responses, host survival and fungal clearance in some fungal infections but damaging and ineffective in others.

In a *Coccidioides* vaccine study, loss of Th17 immunity increased infection susceptibility while loss of Th1 and Th2 immunity did not, implying that Th1 and Th2 cells are not critical for protection (Hung et al., 2011). The underlying mechanisms for this protection are less clear. For example, Th2 cells can promote alternatively activated macrophages which secrete collagen and assist in tissue repair. Alternatively activated macrophages are often recruited to sites of infection where their collagen production assists in establishing granulomatous structures. Virulent and avirulent *Coccidioides* strains form granulomas *in vivo*, and while morphologies have been characterized. we do not know what cells are recruited to the granuloma, what signals form and maintain the granuloma structure, nor details on the immune microenvironment within the granuloma interior (Narra et al., 2016). Exploring granuloma immunity is imperative for understanding infection chronicity as *Coccidioides* infection often presents with granuloma formation. Such knowledge could inform diagnosis and provide markers that distinguish fungal granulomas from cancer nodules and bacterial granulomas.

CD4+ T cell subset frequency is also correlated with infection outcome in human patients. In a pediatric coccidioidomycosis study, high regulatory T cell (Treg) frequency correlated with chronic disease (Davini et al., 2018). Patients with a similar fungal infection, *Paracocidioides brasiliensis*, have a higher %Tregs than healthy controls, and the Tregs are more suppressive (Odio et al., 2015). In mouse models, Treg depletion after infection with *Paracoccidioides brasiliensis* resulted in decreased fungal burden and enhanced survival (Galdino et al., 2018). Chronic *Coccidioides* patients also express heightened serum IL-10 cytokine levels. IL-10 is an effector cytokine used by Tregs to suppress immune activation and reduce inflammation (Cavassani et al., 2006). In the absence of IL-10, susceptible mice develop a protective immune response and lasting immune memory against virulent *Coccidioides posadasii* (Hung et al., 2014b). Tregs regulate immune responses by controlling immune cell activation to prevent hyperinflammatory, damaging responses (Montagnoli et al., 2002; Wing et al., 2008). The mechanisms underlying Treg association with chronic *Coccidioides* disease outcome are unknown. Elevated Treg frequency may block effector responses by overwhelming effector cells, Tregs may be more suppressive, or Tregs may develop at the expense of Th17 responses (Davini et al., 2018). Treg and Th17 differentiation are inversely regulated; the signals required for Treg development block Th17 differentiation (Khader et al., 2009; Yeh et al., 2014). Under inflammatory conditions Tregs can lose regulatory, and gain effector, functions promoting chronic inflammation (Wing et al., 2008). *Coccidioides*-resistant DBA/2 mice have lower lung Treg frequency that produce less IL-10 upon stimulation *ex vivo* compared to susceptible mice (**Table 1**) (Fierer et al., 1998; Viriyakosol et al., 2008; Hung et al., 2014b). These studies suggest a detrimental role for Tregs in *Coccidioides* dearance and emphasize a need to explore their function and plasticity during *Coccidioides* infection. It is unlikely that gain of effector functionality is occurring during chronic *Coccidioides* infection as T helper cells enhance fungal control. Further work must be done to determine whether Treg presence and functionality direct adaptive immune responses and reduce *Coccidioides* control.

In better studied models of chronic inflammation, such as chronic viral infections and cancer, T cells upregulate inhibitory receptors, reducing effector function, in a process termed exhaustion (Wherry and Kurachi, 2015). Chronic antigen exposure is sufficient to drive T cells toward exhaustion, often marked by elevated PD-1 and PD-L1 surface expression (Wherry and Kurachi, 2015). There is very little work examining T cell exhaustion in the context of chronic fungal infections, but existing works that look at chronic *Candida* sepsis patients supports the general observation that exhaustion is detrimental to host health and fungal clearance (Lázár-Molnár et al., 2008; Spec et al., 2016). In *Histoplasma* infections, mice lacking PD-1/PD-L1 survive severe infection while wild-type do not (Lázár-Molnár et al., 2008). *Candida* sepsis patients have elevated circulating PD-1/PD-L1 high T cell frequency (Spec et al., 2016).

TABLE 1 | Resistance to *Coccidioides* infection in specific strains could be due to immune cellularity differences.

Mouse Background	Susceptibility to *Coccidioides* Infection	Alveolar Macrophage Freq.	Lung-resident DC Freq.	Respiratory Leukocyte TNFα production	Respiratory Leukocyte IL-10 production	Lung CD4+CD25 +FOXP3+ (Treg) Freq.	Reference Numbers
C57BL/6	Susceptible	Base	Base	Base	Base	Base	(Fierer et al., 1998; Viriyakosol et al., 2008; Viriyakosol et al., 2013; Hung et al., 2014b)
BALB/C	Susceptible	No Δ	No Δ	↑↑	↑↑	↑↑	(Fierer et al., 1998; Viriyakosol et al., 2008; Hung et al., 2014b)
DBA/2	Resistant	No Δ	No Δ	No Δ	↓	↓	(Fierer et al., 1998; Viriyakosol et al., 2008; Viriyakosol et al., 2013; Hung et al., 2014b)

The base frequency of cells or cytokine production is in reference to C57BL/6 mice; denoted changes are relative to this strain. Cytokine production was measured post stimulation ex vivo. No Δ, no change from base level; ↑↑, higher than base; ↓, less than base.

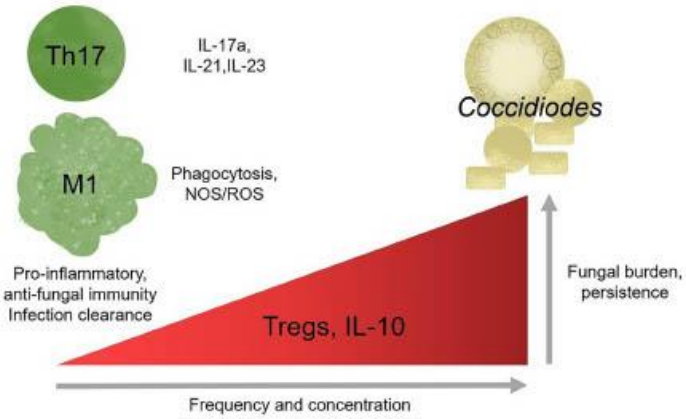

FIGURE 3 | Regulatory responses correlate with chronic disease outcome. Th17 cells and pro-inflammatory responses are protective against *Coccidioides* and loss of responses results in increase susceptibility in mice. Modulating effector and regulatory responses might be a potential therapy for chronic infection.

In paracoccidiomycosis, T cells overexpress PD-1 suggestive of early stage exhaustion during chronic infection (Cacere et al., 2008). It is unknown whether chronic coccidioidomycosis may occur due to T cell exhaustion and maintenance by inappropriately mounted regulatory responses; if so, immune checkpoint blockade could benefit patients with fungal induced T cell exhaustion and promote fungal clearance (**Figure 3**).

VACCINATIONS AND IMMUNE MEMORY

There is currently no fungal vaccine clinically available for humans. Fungal pathogens are eukaryotic, sharing many conserved molecules with humans, making drug and vaccine targeting highly difficult without targeting human cells (Johannesson et al., 2006; Nguyen et al., 2013). *Coccidioides* is dimorphic; the soil and host forms express different surface molecules (Johannesson et al., 2006; Saubolle et al., 2007; Nguyen et al., 2013; Johnson et al., 2014). Thus, effective vaccine strategies must include components that would elicit both a strong and effective immune response without selecting temporal life cycle antigen targets. A *C. albicans* vaccine, NDV-3A, in stage 1b/2a clinical trial that shows promise in protecting patients from recurring vulvovaginal candidiasis (Alqarihi et al., 2019). NDV-3A success demonstrates the major strides made in fungal vaccine research and highlight how much further we have to go. The field has made remarkable progress in identifying adjuvants, antigens, and target cells for vaccine therapies, but further work must be done to demonstrate reliable efficacy between animal models and human use, especially in less studied fungal pathogens such as *Coccidioides*.

Several labs have generated live, attenuated strains of *Coccidioides* that successfully confer protection against secondary challenges with virulent, wild-type *Coccidioides* in susceptible mouse strains (Xue et al., 2009; Hung et al., 2011). The attenuations hinder fungal replication within the host, pausing *Coccidioides* growth at the spherule state. Mice vaccinated with attenuated *Coccidioides* strain ΔT mount Th1 and Th2 effector responses and survive longer than non-vaccinated littermates during post-secondary challenge (Xue et al., 2009). Protein component vaccination with antigen2/PRA (a deglycosylated, proline-rich antigen expressed in *Coccidioides* spherules) is protective when administered subcutaneously or intranasally in susceptible mice (Shubitz et al., 2002). However, this protection decreases at higher fungal doses and only intranasal administration of antigen2/PRA confers protection to both C57BL/6 and BALB/C mice. This component vaccine prolongs survival, but immune cell response to antigen2/PRA has yet to be characterized (Shubitz et al., 2002). Following up on antigen2/PRA as a promising vaccine strategy, mouse bone marrow derived DCs presenting the antigen2/PRA epitope, were intranasally transferred into mice and their immune response analyzed (Awasthi et al., 2019). Lymphocytes and leukocytes from immunized mice express more IFNγ, IL-4, and IL-17 compared to mice that receive control DCs. While this study did not challenge mice with *Coccidioides* post-immunization, it demonstrates the possibility of immune-cell transfer as a vaccination strategy. ΔT vaccination studies show a mixed Th1, Th2, and Th17 response in immunized mice and conferred protection in susceptible C57BL/6 mice (El-Behi et al., 2011). This vaccine's success emphasizes adaptive immunity's importance in protection. Maize provides more efficient generation of antigen2, and a maize-produced subunit vaccine along with the previously discussed glucan-chitin particle delivery

method protected mice against *Coccidioides* and yielded lower fungal burden (Hayden et al., 2019).

The most effective vaccines include adjuvants and additives that enhance immunity and memory. Most notably, adding an agonist of human fragment C5a enhances vaccine immunity against *Coccidioides* infection in mice (Hung et al., 2012). Human C5a added during ΔT vaccination in BALB/c mice, causes heightened Th1 and Th17 responses and circulating effector cytokines; furthermore, humanC5a/ΔT vaccinated mice express higher titers of IgG1 and IgG2 specific to *Coccidioides* and survive longer compared to BALB/c mice vaccinated with ΔT alone (Hung et al., 2012). Ablating IL-10 with ΔT vaccination increases survival and protection against virulent *Coccidioides* infection and increases recall protection post infection (Hung et al., 2014b). These studies further reinforce Th1/Th17 cells in immunity against *Coccidioides* while IL-10 plays a negative role in clearance. This suggests *Coccidioides* immunity requires robust pro-inflammatory responses, while immunosuppression leads to infection persistence.

To complement vaccine research, we need to understand the memory responses vital for sustained immunity. While memory responses have been observed in *Coccidioides* vaccine experiments, tissue-resident memory, effector memory, and central memory subtypes have not been characterized in *Coccidioides* infection. Memory research has made great strides in characterizing cytokine recall responses, but further characterization is required to identify the source of the memory cells. In *Candida* infection, skin-resident memory T cells remain after infection and reactivate upon reinfection (Iannitti et al., 2012). Since many fungal infections occur within mucosal tissues and effective clearance requires tissue specific responses, fungal vaccine development could benefit from exploring tissue-specific memory (Iannitti et al., 2012). In tuberculosis, a bacterial infection associated with granuloma formation, intranasal vaccination with attenuated mycobacterium induces protective CD4+ and CD8+ T cells and mucosa associated lymphoid responses (Walrath et al., 2005). Further analysis revealed tissue resident memory responses from lung parenchyma are critical for protection against virulent mycobacterium (Walrath et al., 2005; Sakai et al., 2014). This validates intranasal delivery as an effective method of vaccination for stimulating tissue resident memory development. While tuberculosis is a bacterial infection, similarities between tuberculosis granulomas and coccidioidomycosis granulomas highlight tissue-resident memory responses as a key for long-lasting immunity in chronic lung infections. Coccidiomycosis starts as a localized respiratory infection, so tissue-resident memory could be critical for providing long lasting protection and warrants further study.

Current work suggests fungal sugar receptors are highly important for the development of anti-fungal recall responses (Shubitz et al., 2002; Cavassani et al., 2006; Hung et al., 2018b). However, much of what we know about memory immunity to *Coccidioides* has used live-attenuated laboratory strains, fungal-derived antigens, and fungal sugar adjuvants to achieve protective immune responses. Characterizing the memory response to live, whole, virulent *Coccidioides* might help to define how memory is generated, or perhaps blocked from generation, in natural infections. Such immunological questions would aid vaccine development, providing a broader context of *Coccidioides*-specific challenges for effective memory responses where our more defined, specific studies are lacking Together, these studies would allow for an elevated understanding of *Coccidioides* immunity as a whole, generating the specific antigen knowledge for vaccine development and broad characterization for better patient treatment.

CONCLUSION: CLINICAL SIGNIFICANCE AND URGENCY

Valley fever research is at a critical point. California's highest endemic region for Valley fever, SJV, has one of the lowest physicians to citizen ratios, adding a barrier to health care access (Petterson et al., 2012). Drugs used to treat chronic Valley fever cause debilitating side-effects in patients such as, but not limited to, headaches, lethargy, seizures, severe hair lost, extreme exhaustion, and nerve pain (Saubolle et al., 2007; Saenz-Ibarra et al., 2018). Due to its generic flu-like symptoms, it is often misdiagnosed as other respiratory infections, leading to late stage diagnosis when more severe symptoms of chronic infection manifest. Since disease clearance or progression determinants are unknown, early diagnosis is invaluable for planning patient treatment. Susceptibility marker identification would help determine those vulnerable to chronic disease. Understanding how the innate and adaptive immune system responds to Valley fever is necessary for optimal diagnoses, treatments, disease progression predictions, and vaccine development. Such studies will inform accurate diagnoses and perhaps provide novel drug targets for therapy. It is unclear whether *Coccidioides* infection becomes chronic because i) the host has high regulatory responses and thus suppresses pro-inflammatory responses, ii) *Coccidioides* is manipulating and influencing host innate immunity to block inflammatory responses, which, in turn, promotes ineffective helper responses, or iii) a combination of host and pathogen influences (**Figures 2A, B**). These observations emphasize the importance of a pro-inflammatory response and suggest that inhibition of pro-inflammatory players promote infection persistence. Tregs seem especially pertinent given supportive pediatric patient and depletion data in *Paracoccidioides* (Felonato et al., 2012). In some settings, Tregs retain their suppressive function yet cannot control T effector responses. This is because T effector cells become refractory to Treg suppression. While this has not been assessed, it is possible that T effector cells could become more sensitive to suppression by Tregs, reducing their effector functionality. Characterizing innate immune cells activation and recruitment of adaptive responses may define how *Coccidioides* eludes clearance and shed light on the role of pro-inflammatory and regulatory responses in disease progression (**Figure 3**).

Until the last 30 years, fungal pathogens have not been as well studied as their viral or bacterial counterparts. The field is at a critical and exciting time as we work together to close gaps in fungal pathogen host immune knowledge. Climate change models predict *Coccidioides'* endemic regions will spread to the American Midwest

by 2050. Thus *Coccidioides* is anticipated to spread significantly beyond the current regions of endemicity (Coopersmith et al., 2017; Gorris et al., 2019). Though severe disease is rare, it is unknown what factors indicate infection susceptibility and disease progression. As antifungal resistance, fungal disease frequency, and regions of endemicity increase, the urgency and need for effective vaccines and better therapeutics also rise. Due to current lack of effective treatment options for chronic disease, the inability to determine likelihood of disease progression toward chronicity at time of diagnosis, and the growing spread of the endemic region, there is a dire need to fully understand host immune response for improved diagnoses and treatment.

AUTHOR CONTRIBUTIONS

AD: conceptualization, literature evaluation, original draft writing, and generated and visualized figures. KH: conceptualization, writing and review, visualization, funding acquisition, and supervision. All authors contributed to the article and approved the submitted version.

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CHAPTER 2

Macrophage and Dendritic Cell Activation and Polarization in Response to *Coccidioides posadasii* Infection

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Macrophage and Dendritic Cell Activation and Polarization in Response to *Coccidioides posadasii* Infection

Anh L. Diep [1], Susana Tejeda-Garibay [1], Nadia Miranda [1] and Katrina K. Hoyer [1,2,3,*]

[1] Quantitative Systems Biology Graduate Programme, University of California Merced, Merced, CA 95343, USA; adiep4@ucmerced.edu (A.L.D.); stejeda-garibay@ucmerced.edu (S.T.-G.); nmiranda8@ucmerced.edu (N.M.)
[2] Department of Molecular and Cell Biology, School of Natural Sciences, University of California Merced, Merced, CA 95343, USA
[3] Health Sciences Research Institute, University of California Merced, Merced, CA 95343, USA
* Correspondence: khoyer2@ucmerced.edu; Tel.: +1-(209)-228-4229

Abstract: Coccidioidomycosis is a fungal, respiratory disease caused by *Coccidioides immitis* and *Coccidioides posadasii*. The host immune responses that define disease outcome during infection are largely unknown, although T helper responses are required. Adaptive immunity is influenced by innate immunity as antigen-presenting cells activate and educate adaptive responses. Macrophage and dendritic cell (DC) recognition of pathogen surface molecules are critical for *Coccidioides* clearance. We characterize the broad innate immune responses to *Coccidioides* by analyzing macrophage and dendritic cell responses to *Coccidioides* arthroconidia using avirulent, vaccine *Coccidioides* strain NR-166 (Δ*cts2*/Δ*ard1*/Δ*cts3*), developed from parental virulent strain C735. We developed a novel flow cytometry-based method to analyze macrophage phagocytosis to complement traditional image-scoring methods. Our study found that macrophage polarization is blocked at M0 phase and activation reduced, while DCs polarize into proinflammatory DC1s, but not anti-inflammatory DC2, following interaction with *Coccidioides*. However, DCs exhibit a contact-dependent reduced activation to *Coccidioides* as defined by co-expression of MHC-II and CD86. In vivo, only modest DC1/DC2 recruitment and activation was observed with avirulent *Coccidioides* infection. In conclusion, the vaccine *Coccidioides* strain recruited a mixed DC population in vivo, while in vitro data suggest active innate immune cell inhibition by *Coccidioides*.

Keywords: *Coccidioides immitis*; *Coccidioides posadasii*; coccidioidomycosis; Valley fever; innate immunity; macrophage; dendritic cell; polarization

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1. Introduction

Coccidioides immitis and *Coccidioides posadasii* are the causative agents of coccidiomycosis, known as Valley fever or Desert fever. Most infected individuals are asymptomatic and clear the infection with little to no medical intervention. Symptomatic disease in about 40% of infections presents across a clinical spectrum from acute pneumonia to chronic lung nodules to disseminated disease [1]. Chronic coccidioidomycosis severely decreases quality of life, and there is no cure for this infection. Broad-spectrum antifungals are currently the only treatment for chronic disease and induce severe side effects such as nausea, skin lesions, hair loss, and nervous system complications [1,2]. *Coccidioides* infections are on the rise, underreported, and often misdiagnosed [3].

Immunity to *Coccidioides* requires T helper (Th)1 and Th17 cellular responses that are trained by early innate recognition of *Coccidioides* in the lung [4,5]. Monocyte/macrophages and dendritic cells (DC) phagocytose pathogens and stimulate adaptive immunity via antigen presentation, cytokine and chemokine secretion and costimulatory signals, shaping the inflammatory and effector response. Macrophages and dendritic cells polarize into different subtypes in response to the tissue-specific signals and pathogen type present.

Pro-inflammatory M1 macrophages induce tissue inflammation and cellular entry into infection sites, are associated with heightened phagocytic and clearance capabilities, and are typically fungicidal [6]. M2 macrophages are associated with post-inflammation wound repair and suppress inflammation by secreting IL-10 and arginase [7]. Tissue resident alveolar macrophages are sentinels and guardians of the immune microenvironment within the lung. The lung microenvironment maintains balance within the delicate airway spaces and alveolar sacs even during high inflammation [8]. Alveolar macrophages have high phagocytotic capabilities but require a high threshold of activation by pro-inflammatory signals. Macrophages have antigen presenting capabilities, but DCs are the professional antigen presenting cells dominantly responsible for activating and educating adaptive immune responses. Much like macrophages, DCs polarize into specialized subtypes in response to different pathogens.

DCs bridge the innate and adaptive immune response by bringing antigen, costimulation and cytokine signals to naïve T cells within secondary lymphoid organs to induce T cell activation and differentiation [9]. DCs loaded with *Coccidioides* antigen confer protection against virulent *Coccidioides* infection in susceptible mice [10]. CD4 T helper subtype differentiation is shaped by surface interactions with DCs and DC-secreted cytokines [11]. Th1/Th17 responses are typically induced by DC1s, while DC2s promote Th2 activation [11,12].

We examine macrophage and DC differentiation and functionality in response to *Coccidioides* to understand how these cells interact with this fungal pathogen. Using in vitro infection, we show that macrophages phagocytose *Coccidioides* arthroconidia poorly; monocytes respond and activate into macrophages (M0), but do not polarize into M1 or M2 subtypes nor upregulate activation markers. DCs polarize into DC1 in response to *Coccidioides* and upregulate maturation and costimulatory proteins. Multiple bacterial and fungal infections have been observed to increase alveolar macrophage and dendritic cell numbers in the lung [13,14]. Our infected lung image scoring reveals a spike in neutrophil counts at day 1 post-infection. In vivo DC1 and DC2 increase in frequency by day 7 post-infection within the lungs. Lastly, complete blood counts identify a spike in white blood cells and a drop in neutrophils at day 1 post-infection before returning to normal by day 7, suggesting that adaptive and immune cell responses mobilize in the peripheral blood following *Coccidioides* infection. Altogether, our data characterize early innate immune skewing of macrophages and DCs during *Coccidioides* infection and suggests that *Coccidioides* may actively inhibit macrophage and DC responses.

2. Materials and Methods

2.1. Mice

Six- to eight-week-old C57BL/6 male and female mice (JAX # 000664, The Jackson Laboratories, Bar Harbor, ME, USA) were utilized for experiments and sex matched whenever possible. Mice were housed and bred within the University of California Merced specific-pathogen free animal facility in compliance with the Department of Animal Research Services and approved by the Institutional Animal Care and Use Committee (protocol AUP18-000 approved 25 April 2018 and protocol AUP21-0004 approved 22 April 2021).

2.2. Fungal Strain and Culturing for Method Arthroconidia Harvest

NR-166 avirulent *Coccidioides posadasii* (Δ*cts2*/Δ*ard1*/Δ*cts3*) laboratory strain derived from parent isolate C735 was used for all infections (BEI Resources, Manassas, VA, USA) [15]. Liquid 2x Glucose 1x Yeast Extract (2x GYE) media (Fisher Scientific, Hampton, NH, USA) was inoculated with frozen fungal stock and cultured at 30 °C in a shaking incubator for 72 h. Liquid culture was streaked onto 2x GYE agar plates and grown to confluency, then desiccated until the agar condensed. To obtain arthroconidia, the fuzzy white growth was scraped off the plate into PBS and filtered through a 40 μM mesh filter. The fungus was vortexed for 1 min to disassociate and centrifuged at 9000× *g* for 30 min at room temperature. The fungal pellet was washed with PBS and the pellet resuspended to the

appropriate concentration in PBS for use. Arthroconidia suspension was stored at 4 °C for up to 3 months. Complete protocol used was from Mead et al. [16].

2.3. Calcofluor White Labeling of Coccidioides

Powdered calcofluor white (Fluorescent Brightener: CFW; Sigma-Aldrich, St. Louis, MO, USA) was reconstituted in PBS at 5 mg/mL. Before each assay, arthroconidia were stained at 5 µg/mL in CFW for 5 min in the dark then washed twice in PBS. CFW-labeled arthroconidia suspensions were stored at 4 °C in the dark until use and any excess discarded [17].

2.4. Cell Line and Culture Conditions

RAW 264.7 mouse macrophage and NR8383 rat alveolar macrophage cell lines were used in the phagocytosis experiments. Bone marrow-derived cells were used in the macrophage and DC polarization experiments. Cells were cultured in phenol red-free DMEM 10% FBS 1% penicillin/streptomycin 1% L-glutamine (DMEM complete media) for macrophages or RPMI 10% FBS 1% penicillin/streptomycin 1% L-glutamine (RPMI complete media) for monocytes. RAW 264.7 were provided by Anita Sil at University of California San Francisco and NR8383 by Laurent Dejean at California State University Fresno.

2.5. Phagocytosis Assay and Data Acquisition

5×10^5 cells were plated in 1 mL per well in a 12 well plate. Lipopolysaccharide (LPS: Millipore Sigma-Aldrich, St. Louis, MO, USA) stimulation was used as a positive control at 5 ng/mL final concentration. CFW-labeled arthroconidia were plated with cells at a 1:1 ratio. Two duplicate plates were created, one incubated at 37 °C and one incubated at 4 °C (negative phagocytosis control). Following a 2 h incubation phagocytosis was halted by putting the plates on ice to prepare for imaging. For adherent cells, supernatant was aspirated from the plates. For non-adherent cells, the plates were centrifuged at 1200× rpm for 5 min in 10 °C. To quench the fluorescence of externally bound *Coccidioides*, each well was washed with a solution of Congo red (Fisher Scientific, San Jose, CA, USA) in PBS at a concentration of 5 mg/mL for 1 min. PBS was added to the plate to dilute the quench and supernatant removed. Each well was replenished with 1 mL of complete media for imaging. In each plate, controls with unquenched wells (no Congo red wash) were included. Cells were analyzed via imaging and flow cytometry.

2.6. Phagocytosis Imaging

Plates were incubated on ice for 20 min to halt the phagocytosis process on the 37 °C plate while imaging the 4 °C plate. Plates were imaged at 20× using the Invitrogen™ EVOS™ FL Digital Inverted Fluorescence Microscope (ThermoFisher, Waltham, MA, USA). Each well was imaged using a cross pattern (top, center, left, right, bottom) for consistency. Scoring criterion was as follows: macrophages in contact with at least one or more *Coccidioides* were counted as participating in an association event; macrophages with internalized *Coccidioides* were counted as participating in a phagocytosis event. Macrophages participating in both events were counted for both events. Dead, out of frame, out of plane macrophages were not included in the total cell count for each image. Phagocytosis and association frequency was determined by (total # of macrophages participating in each event)/(total live cells in each image).

2.7. Antibody Staining and Flow Cytometry

Mouse pulmonary draining lymph nodes (dLN) and lungs were mechanically homogenized and collected in a PBS/1% FBS solution and filtered through a 100 µM mesh filter. Cell suspensions were centrifuged at 1200× rpm for 5 min at 10 °C. Red blood cells were lysed in 1× lysis buffer made from 10× Ammonium Chloride Lysis Buffer Stock (NH_4Cl (ammonium chloride) 8.02 gm $NaHCO_3$ (sodium bicarbonate) 0.84 gm EDTA

(disodium) 0.37 gm in 500 mL Millipore water; (Fisher Scientific, San Jose, CA, USA), washed, and resuspended in PBS/1% FBS. Cells were resuspended at 2×10^6 cells and stained at 50 µL (using antibodies described below from eBioscience (San Diego, CA, USA) unless otherwise noted) for 30 min in the dark at 4 °C. Cells were washed with staining media and fixed for 45 min in the dark at room temperature, washed and resuspended in 100 µL PBS/1%FBS for flow cytometry acquisition. Cells were stained with Fixable Viability Dye eFluor 506 (1:500), anti-CD8α Pe-Cy7 (clone 53–6.7; 1:400), anti-CD11c FITC (clone HL3, BD Biosciences; 1:400), anti-F4/80 PerCP-Cy5.5 (clone BM8; 1:400), anti-MHC-II (I-A/I-E) APC-Cy7 (clone M5/114.15.2, BioLegend, 1:200), anti-CD86-PE (clone PO3.1, 1:200), anti-SIRPα/CD47 APC (clone P84, BioLegend, 1:400), anti-CD38 APC (clone 90; 1:200), and anti-CD206 Pe-Cy7 (Clone MR6F3; 1:400). Data was acquired on a LSRII (BD) and analyzed using FCS Express Version 4 and 7 Research Edition (DeNovo Software, Pasadena, CA, USA).

2.8. Bone Marrow Harvest for Macrophage and DC Polarization Experiments

Bone marrow was harvested from femurs and pooled according to sex. Bones were mechanically crushed using mortar and pestle and washed with RPMI complete media. Cells were filtered using a mesh 100 µM filter and centrifuged at 1200× rpm for 5 min at 10 °C. Red blood cells were lysed for 1 min, washed with PBS, resuspended in complete media, and counted.

2.9. Macrophage Polarization

Bone marrow-derived monocytes were plated in RPMI complete media in tissue culture treated plates with phorbol 12-myristate 13-acetate (PMA) (Fisher Scientific, San Jose, CA, USA) at a final concentration of 1 µg/mL. After 24 h, non-adherent cells were collected and disposed. Adherent cells were scraped into fresh media resuspended at 1×10^6 cells/mL in RPMI complete media and replated in a new 12 well plate. The conditions were as follows: no stimulation, 100 ng/mL LPS, 20 ng/mL IL-4, *Coccidioides* arthroconidia at 1:1 ratio to cells, *Coccidioides* plus LPS, and *Coccidioides* plus IL-4. Plated cells were incubated for 24 h at 37 °C then all cells, adherent and non-adherent, were harvested for flow cytometry (See Supplemental Figure S4 for gating strategies).

2.10. Dendritic Cell Polarization

30×10^6 bone marrow-derived cells were plated in 30 mL of RPMI complete media with final concentration 20 ng/mL GM-CSF on 100 mm non-tissue culture treated plates [18]. On day 3, 15–30 mL of fresh media and GM-CSF was added to each plate. On day 6, non-adherent cells were collected by harvesting the supernatant, incubating plates with 5 mL of 3 mM EDTA/PBS for 1–2 min, washing with media and collecting cells. Cells were centrifuged for 7 min at 1000× rpm at 10 °C. Cells were resuspended in 20 mL RPMI complete media at appropriate concentration for experimental usage. A total of 2×10^6 cells per 2 mL were plated in a 6 well plate and 0.5 ng/mL IL-4 added to the DC2 condition wells. On day 7, 1 µg/mL LPS was added to DC1 condition wells. On day 8, cells were stimulated with or without *Coccidioides* plus LPS or IL-4 as in the macrophage polarization assays above. Cells were incubated for 48 h at 37 °C and harvested for flow cytometry (See Supplemental Figure S5 for gating strategies).

2.11. Dendritic Cell Polarization with Supernatant Assay

Following the methods outlined in Section 2.10 above, BMDCs were prepared, and supernatant was harvested from the wells for cell stimulation in place of direct stimulation. On day 10 post stimulation, plates were spun down at 1200× rpm for 5 min at room temperature to minimize harvesting cells. Supernatant was collected, passed through a sterile 40 µM mesh cell strainer (Fisher Scientific, San Jose, CA, USA), and then syringe filtered using a 0.45 µM filter unit (Merck Millipore, Burlington, MA, USA). Then, 2 mL

media from each condition was added to fresh BMDCs on day 8 for stimulation. Cells were incubated for 48 h at 37 °C and harvested for flow cytometry.

2.12. In Vivo Infection and Tissue Harvest

Mice were intranasally infected by dotting arthroconidia suspended in PBS onto their nostrils and waiting for inhalation before repeating for the entire experimental dose. Experimental conditions were as follows: uninfected, 30 µL PBS mock infection, and 10^5 arthroconidia in 30 µL PBS. Mice were euthanized on day 1 or 7 post-infection for tissue harvest and analysis. Tissues collected were as follows: peripheral blood was collected for complete blood count analysis, whole lung for flow cytometry and immunohistochemistry, lung draining lymph node and spleen for flow cytometry analysis.

2.13. Complete Blood Count

50–100 µL blood was collected via retro-orbital bleeding in BD Biosciences K2E Microtainer tubes (K2EDTA) (BD Biosciences Pharmingen, San Diego, CA, USA) and analyzed on the Drew Scientific Hemavet 950 (Drew Scientific, Erba Diagnostics, Miami Lakes, FL, USA).

2.14. Immunohistochemistry, Imaging, and Analysis

Left lung lobes were embedded in optimal cutting temperature (OCT) compound (Leica Biosystems, Wetzlar, Germany) for histological analysis. Lung samples were tissue sectioned at 10 µM using a Leica CM1860 cryostat and were immediately fixed in ice-cold acetone. Samples were washed with PBS, 1% and 5% blocking buffer (PBS and BSA) at room temperature. Sections were stained with anti-CD11c FITC (eBioscience, San Diego, CA, USA, clone N418, 1:500), anti-Ly-6G Alexa Fluor 700 (BioLegend, San Diego, CA, USA, clone 1A8, 1:50), anti-Siglec F/CD170 PE (BD, clone E50-2440, 1:50), and either anti-F4/80 PE-Dazzle^{TM} 594 (BioLegend, clone BM8, 1:250) or anti-EpCAM/CD326 Alexa Fluor 594 (BioLegend, clone G8.8, 1:500) for 2 h at room temperature then washed and imaged. A Zeiss LSM 880 confocal microscope at 10× (10×/0.45 Plan Apochromat; 420640-9900) and 40× (40×/1.2 LC LCI Plan Apochromat; 420862-9970-799) objectives was used for imaging. Each lung section was imaged in three consistent sections across all mice and conditions: top, middle, and bottom of lobe. Counts from all lobes were combined to give a final total count for each lung. Lung images were blinded and scored by two independent scorers via the criterion guide below. Intermediate monocytes (iMO) were identified as F4/80+SiglecF-Ly6G+, alveolar macrophages as CD11c+SiglecF+F4/80+, neutrophils as F4/80-Ly6G+, dendritic cells as CD11c+.

2.15. Statistics

Experimental data were analyzed using paired Student's *t*-test and all data analyzed for outliers using Grubbs Outlier exclusion analysis with GraphPad Prism v.8 for Windows Software (GraphPad Software, San Diego, CA, USA). Figure legends denote what comparisons took place, if outliers were detected and excluded, and the *p*-value for each figure.

3. Results

3.1. Poor Coccidioides Phagocytosis by Monocytes and Macrophages

To understand macrophage responses to *Coccidioides*, we first assessed macrophage phagocytic function against *Coccidioides* using traditional image scoring methods and a novel flow cytometry approach. Traditional methods for phagocytosis assays require imaging and independent, blinded scoring which are time-intensive and require tight scoring guidelines to ensure consistency and prevent bias between samples. We adapted a flow cytometry analysis-based approach for analyzing phagocytosis by utilizing fluorescently labeled *Coccidioides* and quenching externally bound *Coccidioides* with Congo red dye that binds amyloid proteins (Figure 1A).

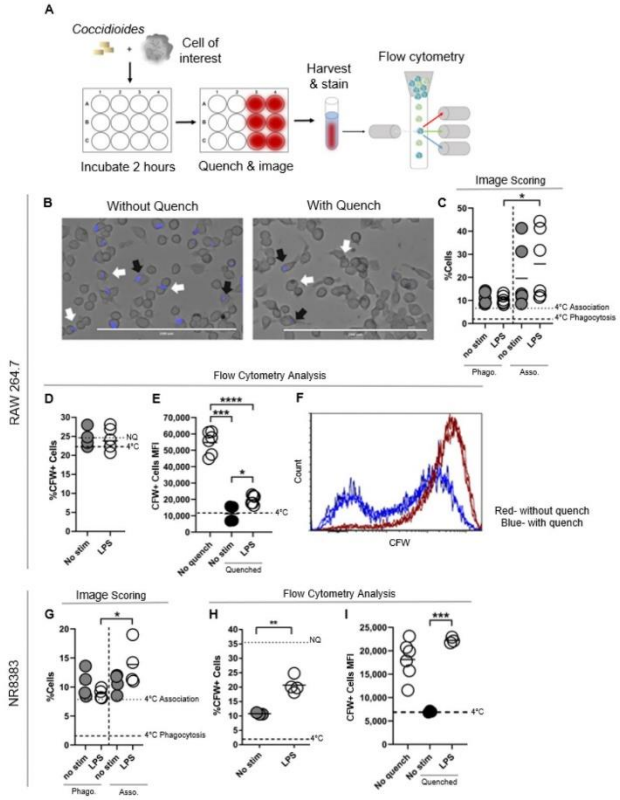

Figure 1. Macrophages poorly phagocytose *Coccidioides*, although mouse macrophages have a stronger association with *Coccidioides*. Phagocytosis analysis via flow cytometry complements traditional imaging assay while increasing output efficiency and allowing analysis of association versus phagocytosis. (**A**) 5×10^5 cells were incubated 1:1 with CFW labeled *Coccidioides* for 2 h then left unquenched or quenched using Congo red dye. Cells were imaged then harvested and processed for flow cytometric analysis. (**B**–**F**) Data from RAW 264.7 mouse macrophages. (**G**–**I**) Data from NR8383 rat alveolar macrophages. (**B**) Bright field image of CFW-*Coccidioides* infected RAW 264.7 cells. Externally bound *Coccidioides* is quenched by Congo red, leaving only the internalized *Coccidioides* visually discernable by the Pacific blue marker for imaging by fluorescence labeling. White arrows indicate internalized (phagocytosed) *Coccidioides*, and black arrows show externally bound or externally associated *Coccidioides* in un-quenched and quenched conditions. (**C**–**I**) Non-quenched controls = cells harvested but un-quenched, No stim = *Coccidioides* only conditions without additional stimulation, LPS = pro-inflammatory stimulant, 4 °C = negative phagocytosis control. (**D**,**H**) Frequency of CFW+ cells gated from Singlet/Live cells. (**E**,**I**) Mean fluorescence intensity (MFI) of the cells from CFW+ population. (**F**) Representative histogram of CFW fluorescence in RAW 264.7 macrophages incubated with CFW-labeled *Coccidioides* at 37 °C under quenched conditions (blue line) indicating *Coccidioides*, or non-quenched (red line) indicating *Coccidioides* association and engulfment. (**C**,**G**) Image scoring data. Left side: frequency of cells with internalized *Coccidioides*; right side: frequency of cells associating with *Coccidioides* externally. $n = 3$–8, data are representative from 2–3 experiments. For all plots, line indicates mean, and each dot is one experimental replicate. Data was analyzed using an unpaired Student's *t*-test and outliers excluded using Grubbs Outlier exclusion analysis. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.00005$.

This method increases the speed and output for phagocytosis analysis, allowing interrogation of multiple cells and conditions simultaneously. We first tested a mouse macrophage cell line, RAW 264.7, using these methods (Figure 1B–F). Representative images of RAW 264.7 cells incubated 1:1 with CFW-labeled *Coccidioides* at 37 °C for two hours show examples of fungal arthroconidia externally bound or closely associated with *Coccidioides* (Figure 1B, white arrows) or internalized (Figure 1B, black arrows). Fungal association refers to cell events where *Coccidioides* is in direct contact with a cell but without clear internalization, while phagocytosis is defined as fungal internalization (Figure 1C). After imaging, cells were harvested and analyzed using flow cytometry (Figure 1D–F,H,I). Without fluorescent quenching of externally bound *Coccidioides*, a representative histogram shows most mouse macrophages interacting with *Coccidioides*, masking our ability to define fungal phagocytosis (Figure 1F, red histogram). However, Congo red quenching results in a bimodal macrophage population, with CFW+ macrophages that have internalized *Coccidioides* and CFW− macrophages bound to *Coccidioides* on the surface (Figure 1F, blue histogram). While on average 19.57% of mouse macrophages interact with *Coccidioides*, only 10.85% successfully internalize *Coccidioides* within two hours (Figure 1C, phagocytosis left column, association right column). With LPS stimulation, 25.83% of macrophages interact with *Coccidioides* but only 10.24% successfully internalize *Coccidioides*.

Thus, although most of these macrophages are associated with *Coccidioides*, few mouse macrophages are successfully phagocytosing *Coccidioides*, even with the addition of a strong stimuli, LPS. We next examined a rat alveolar macrophage cell line, NR8383 where we observed enhanced phagocytosis in the presence of LPS (Figure 1G–I). On average, 10.7% of the raw alveolar macrophages associated with *Coccidioides* and 10.5% phagocytose successfully by confocal fluorescent imaging. With LPS stimulation, 13.9% rat alveolar macrophages associated with *Coccidioides* and 8.9% participated in successful phagocytosis. The interaction to phagocytosis frequency gap is smaller with rat alveolar macrophages (13.9% to 8.9%; 1.6-fold reduction) than mouse macrophages (25.83% to 10.24%; 2.5-fold reduction). However, there was no significance between unstimulated and LPS stimulated alveolar macrophages (Figure 1I, left column).

These differences between the mean fluorescence intensity (MFI) flow data and traditional scoring data can be explained by the fact that the scoring method criterion counts the number of cells that participate in each type of interaction with *Coccidioides* out of all cells within the image frame, while flow cytometry allows for detailed recording of fluorescent intensity as a metric of how many fungi a single cell interacts with. Our phagocytosis data reproduce previous findings for macrophages, where pro-inflammatory stimuli enhance phagocytotic mechanisms [19]. We observed that rat and mouse macrophages poorly phagocytose *Coccidioides* with higher surface association than phagocytosis, but that mouse macrophage association occurs more frequently than rat alveolar macrophage association.

3.2. *Coccidioides* Blocks Monocytes in a Poorly Activated, Non-Differentiated State

We next sought to characterize monocyte differentiation into macrophage subtypes and activation state in response to *Coccidioides*. Immune cell polarization and activation provides targeted pathogen control during early immune response, laying the foundation for adaptive immunity. Bone marrow-derived monocytes were cultured with polarizing stimulants and *Coccidioides*, and polarization assessed by surface protein expression. PMA induces M0 differentiation, LPS stimulates M1 differentiation, and IL-4 promotes M2 differentiation (Figure 2A). Representative flow cytometry plots and gating strategy are shown for *Coccidioides* stimulated monocytes in Figure 2B. On average, 13.9% of monocytes differentiated into macrophages (all F4/80+ cells) in response to PMA stimulation (written as PBS control stimulation in Figure 2C). *Coccidioides* tends to promote macrophage differentiation in BMDMs. Similarly, in the presence of *Coccidioides*, more monocytes trended towards differentiation into M0 macrophages (69.9% with *Coccidioides*, 69.4% for *Coccidioides* plus IL-4 and 65.6% with *Coccidoides* plus LPS), but poorly differentiated into M1 and M2 macrophages (Figure 2D–F). While the presence of *Coccidioides* seems to induce

monocytes to differentiate into M0 macrophages, *Coccidioides* also appears to block M1 and M2 polarization (Figure 2E,F). M1 differentiation decreases in the presence of *Coccidioides* with LPS relative to LPS alone (48.76% to 19.31% (Figure 2E)). Additionally, exposure to *Coccidioides* reduces CD86 and MHC-II co-expression, even in the presence of LPS across all macrophage and polarized subtypes, dropping the activated M1 frequency from 30.67% to 20.77% ($p = 0.1882$) (Figure 2G–J). Overall, these data suggest low macrophage activation and maturation in the presence of *Coccidioides*.

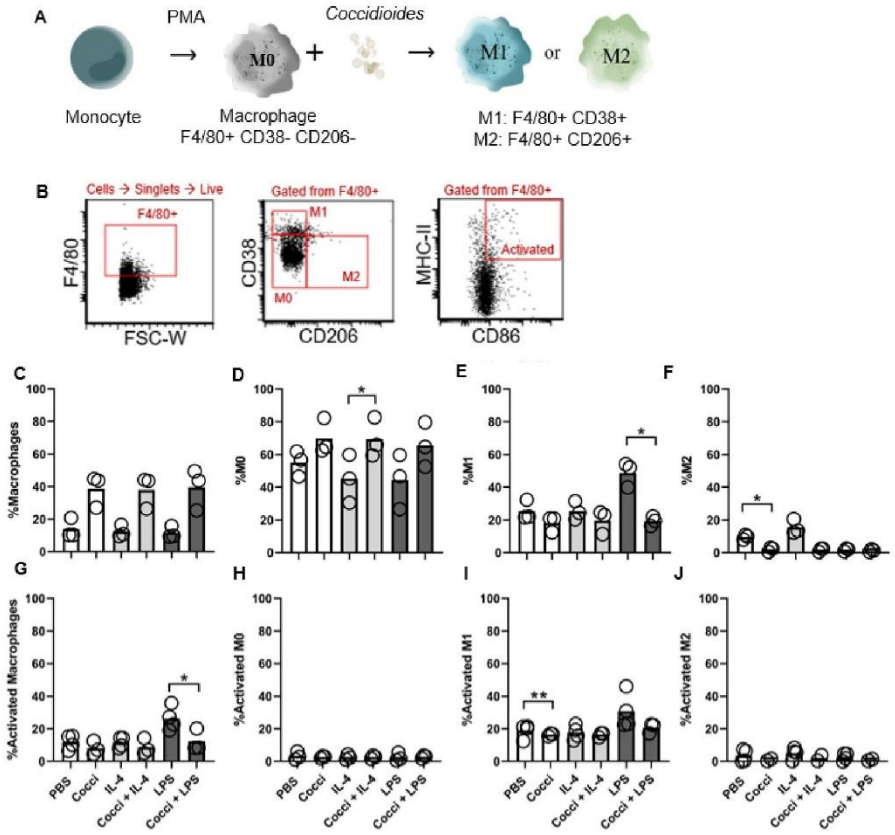

Figure 2. Monocytes activate into macrophage in response to *Coccidioides* but do not polarize into M1/M2 subtypes and largely lack activation/maturation markers. (**A**) Schematic illustrates monocytes differentiation into M0 and polarized M1/M2 potential outcomes with *Coccidioides*. PMA (phorbol 12-myristate 13-acetate), an activator of protein kinase C (PKC), was used as a positive stimulant for M0. LPS (100 ng/mL) acted as M1 positive control and IL-4 (20 ng/mL) as M2 positive control. Cocci = *Coccidioides* added at 1:1 ratio with cells. (**B**) Representative flow plots show the gating strategy for analysis; sample shown was stimulated with *Coccidioides*. See Supplemental Figure S4 for complete gating strategy. (**C**,**G**) macrophages are defined as all F4/80+ cells, (**D**,**H**) M0 defined as CD11b-F4/80+, (**E**,**I**) M1 as CD38+ F4/80+, and (**F**,**J**) M2 as CD206+ F4/80+. (**G**–**J**) Activated cells are defined as CD86+MHC-II+ and are gated off their respective population. $n = 3$–4, data representative of 3 experiments. The bar within each data group indicates the mean, each individual circle represents one biological replicate averaged from all technical replicates within each experiment. Statistics show comparisons between *Coccidioides* and non-*Coccidioides* conditions. Data was analyzed using unpaired Student's *t*-test. * $p < 0.05$, ** $p < 0.005$.

3.3. DCs Favor DC1 Polarization in Response to Coccidioides but Lack Activation and Maturation Markers

We next interrogated DCs to determine if there is a similar polarization and maturation block as is found in macrophages by *Coccidioides*. We cultured bone marrow-derived DCs to evaluate DC maturation and polarization in the presence of *Coccidioides*; representative flow plot shows the gating strategy for DCs (Figure 3A). We observed a block in total CD11c+ DC frequency in the presence of *Coccidioides* (Figure 3B). IL-4 treatment induces DC maturation and induced the highest total CD11c+ frequency (Figure 3B). The addition of *Coccidioides* to IL-4 culture resulted in a significant reduction in CD11c+ cell differentiation from an average of 54.91% to 27.75% (Figure 3B). In the CD11c+ population, all stimulation conditions in the absence of *Coccidioides* induced both DC1 and DC2 differentiation (Figure 3C,D). However, when exposed to *Coccidioides*, only non-polarized and DC1 (CD8α+SIRPα-) differentiation occurred, with DC1 frequency increasing from 4.52% in PBS condition to 42.21% in the presence of *Coccidioides* (Figure 3C). Though not statistically significant ($p = 0.0645$), DC frequency increased from 24.25% with LPS to 43.05% with LPS and *Coccidioides*. Further, little to no DC2 polarization occurred in the presence of *Coccidioides* under any stimulation conditions (Figure 3D).

Strikingly, when in the presence of *Coccidioides*, DCs did not differentiate into DC2, with DC2 frequency dropping from an average of 19.84% in PBS to 0.03% in the presence of *Coccidioides* (Figure 3D). Further, DC1s cultured with *Coccidioides* expressed MHC-II and CD86 at a significantly lower frequency indicating reduced maturation and activation capacity in the presence of *Coccidioides* even when co-cultured with LPS (Figure 3E–G). Total DC activation frequency when stimulated with LPS dropped from 39.34%without *Coccidioides* to 14.49% with *Coccidioides* (Figure 3E). These data suggest that *Coccidioides* inhibits DC maturation, activation and DC2 polarization.

Next, we sought to characterize whether this inhibition was contact dependent. We performed an indirect stimulation assay using supernatant from the direct stimulation assay in Figure 3B–G. Supernatants from PBS, LPS and IL-4 induced similar CD11c+ cells as found in the direct assay. However, the supernatants from the *Coccidioides* treated BMDC did not inhibit DC generation. Unlike the direct polarization assay where the addition of *Coccidioides* caused a significant reduction in DC and DC1 activation, there was no significant change in the supernatant polarization assay (Figure 3K,L). There was significant reduction in the amount of activated DC1 between IL-4 +/− *Coccidioides* indicating some activation blockage by secreted factors under this condition (Figure 3L). Further, we observed a reduction in DC1 frequency when BMDCs were incubated with supernatant relative to arthroconidia, with DC1 frequencies averaging 42.21% in the direct assay and 4.77% in the supernatant assay (Figure 3C and Figure 3I, respectively). These data suggest that DC responses are largely a result of direct contact with *Coccidioides*.

3.4. Lung Immunohistochemistry Reveals Increased Neutrophil Frequency Post-Infection

To evaluate the immune responses in vivo, we intranasally infected mice with CFW-labeled *Coccidioides* and processed the lungs via immunohistochemistry to visualize immune cell quantity and localization patterns in the lung. Figure 4A shows representative lung images taken at 40× magnification of PBS mock infected lungs and *Coccidioides* infected lungs at day 1 and day 7 post *Coccidioides* infection. There were no significant changes in intermediate monocyte (F4/80+Ly6G+), alveolar macrophage (F4/80+Siglec-F+CD11c+), or DC (CD11c+F4/80−) counts between conditions (Figure 4B–D).

Neutrophil (Ly6G+F4/80−) counts increased at day 1 post-infection by 11-fold compared to mock PBS infection, before returning to normal by day 7 post-infection (Figure 4E). The innate cell changes within the lung following fungal infection indicates an inflammatory immune response occurs. However, we were unable to directly ascertain localization patterns of immune cells relative to *Coccidioides* within the lung tissue. This is in part due to the vast tissue survey needed to determine this information and limitations in visualizing

both immune cells and *Coccidioides* by immunohistochemistry at 10× (data not shown) and 40×.

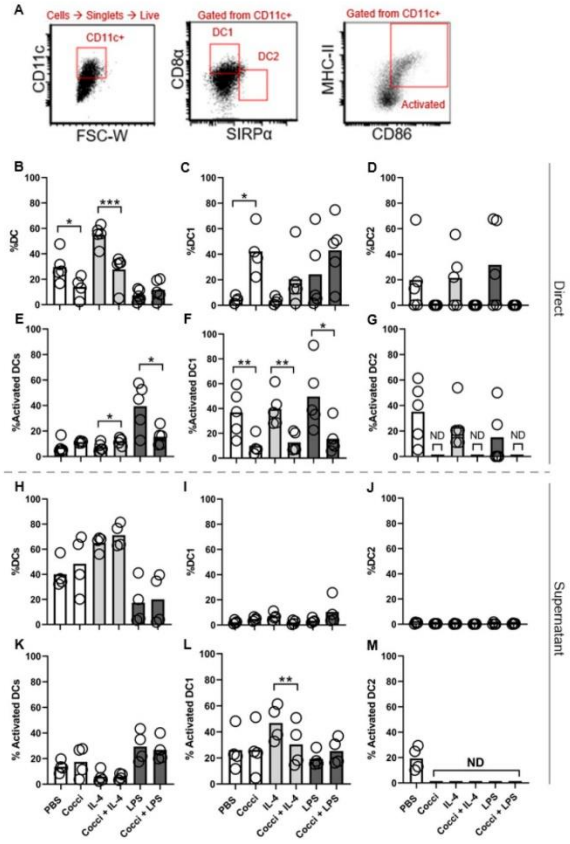

Figure 3. DCs polarize to DC1 in response to *Coccidioides* but do not upregulate CD86 and MHC-II expression. (**A**) Representative flow plots show gating strategy used for analysis; sample shown is stimulated with *Coccidioides*. See Supplemental Figure S5 for complete gating strategy. Using a C57BL/6 bone marrow-derived DC culture system to generate DCs, we assessed DC polarization. Unstim refers to DC culture conditions without cytokine stimulation. LPS (1 µg/mL) was added as a DC1 control and IL-4 (0.5 ng/mL) as a DC2 control. *Coccidioides* was added in a 1:1 ratio with cells. (**B**–**G**) DCs were directly stimulated with *Coccidioides*, LPS and/or IL-4. (**H**–**M**) DCs were stimulated using supernatants generated in (**B**–**G**). (**B**,**H**) DC frequency of all CD11c+ cells including DC1 and DC2. (**C**,**D**,**I**,**J**) DC1 and DC2 populations were gated from total CD11c+ population. (**C**,**F**,**I**,**L**) DC1 are defined as CD8α+SIRPα-CD11c+ and (**D**,**G**) DC2 as CD8a-SIRPa+CD11c+. (**E**–**G**,**K**–**M**) Activated cells are defined as CD86+MHC-II+; activated cells are gated from their respective subtype population. ND = not determined due to lack of cells from previous gate. n = 4–5, data are representative of 5 experiments for direct assay, and 3 experiments for supernatant assay. The bar within each data group indicates the mean, each individual circle represents one biological replicate averaged from all technical replicates within each experiment. Statistics show comparisons between *Coccidioides* and non-*Coccidioides* conditions. Data was analyzed using unpaired Student's *t*-test and outliers excluded using Grubbs Outlier exclusion analysis * $p < 0.05$, ** $p < 0.005$. *** $p < 0.0005$.

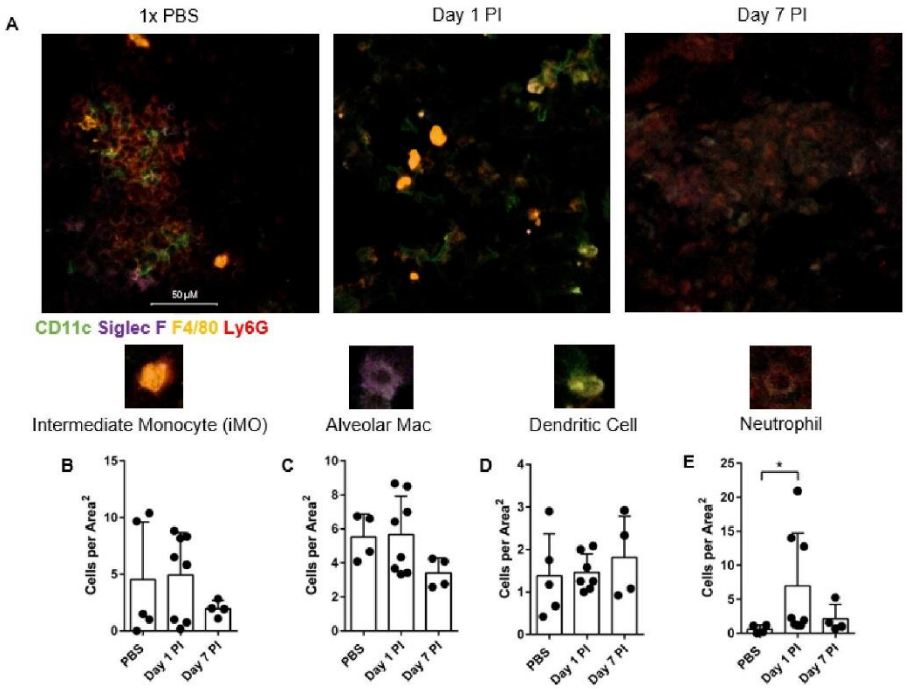

Figure 4. Immunohistochemistry and lung image scoring reveals heightened CD11c positive and Ly6G positive cells in the lung post infection. Lungs were processed at day 1 and day 7 post infection (PI) and fresh-frozen. (**A**) In the representative plot, PBS is the mock infection whereas Day 1 and Day 7 show 10^5 arthroconidia intranasal infection at 40× confocal imaging. 50 µM scale bar applies to all images. (**B**) Intermediate monocytes (iMO) are defined as F4/80+ Ly6G+, (**C**) alveolar macrophages (Mac) as F4/80+ CD11c+ Siglec-F+, (**D**) DCs as CD11c+ F4/80−, and (**E**) neutrophils as Ly6G+ F4/80− CD11c−. Images scored are from 40× magnification. Each image represents 742,819.8969 uM^2 in $Area^2$, calculated from square image of 862 × 862 μm^2. Images were blind scored by two independent counters. For all plots displayed line indicates mean, and each dot is one experimental replicate for each lung sample averaged over 5 images and two blind-scorers. n = 4–7, representative of 4 experiments. Comparisons were made between mock PBS infection and infected at each time point. Data was analyzed using unpaired Student's t-test and outliers excluded using Grubbs Outlier exclusion analysis; * $p < 0.05$.

3.5. DC1 and DC2 Frequency Increase in Lungs Post Infection

We next utilized flow cytometry to analyze lungs and lung-draining lymph nodes (dLN) following intranasal infection to assess DC numbers and functional markers in vivo. First, to confirm that a systemic cellular response occurs following avirulent *Coccidioides* infection, we evaluated peripheral blood cell numbers and frequencies using complete blood count analysis. White blood cell peripheral blood numbers increase on day 1 post-infection and drop below mock-infection levels by day 7 (Supplemental Figure S1A), suggesting that the host recognizes an infection and mounts a systemic response. Neutrophil peripheral blood numbers drop slightly day 1 post-infection before returning to mock infection levels at day 7 post-infection (Supplemental Figure S1C).

No changes are observed in peripheral blood monocyte or eosinophils during avirulent infection (Supplemental Figure S1B,D). CD11c+ cell frequency was normal to slightly elevated in the lung and slightly decreased in the lung-draining LN on day 7 post-infection as measured by flow cytometry (Figure 5A,E). The frequency of activated CD11c+ cells co-expressing CD86 and MHC-II was elevated in the draining LN and lungs on day

7 post-infection relative to day 1 post-infection, returning to mock PBS infected levels (Figure 5B,F).

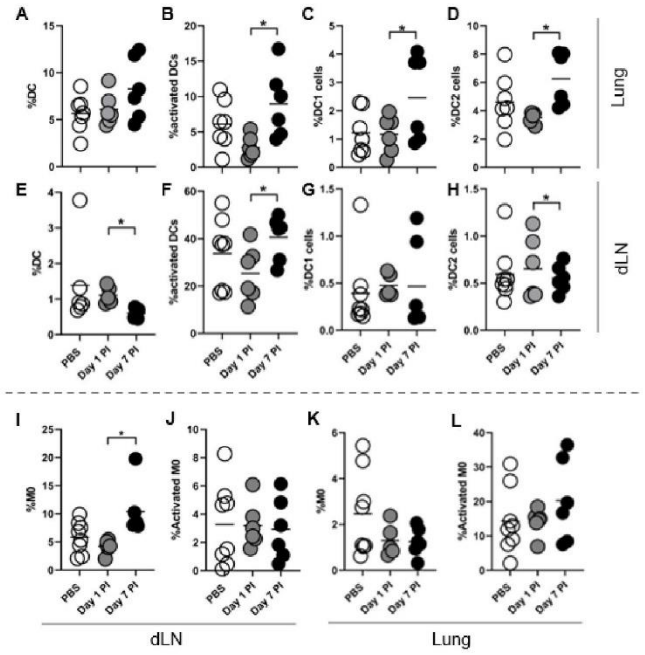

Figure 5. DC1 and DC2 increase in frequency at day 7 post-infection. C57BL/6 mice were intranasally infected with 10^5 arthroconidia or PBS. Cell frequencies are comparable to uninfected mice (data not shown). Both lung lobes were collected and homogenized for flow cytometry analysis. Draining lymph node (dLN) refers to the pulmonary lung-draining LN. DC1 (**C**,**G**) and DC2 (**D**,**H**) populations are gated from CD11c+ population (**A**,**E**). DC1 are defined as CD8a+SIRPa-CD11c+ and DC2 as CD8a-SIRPa+CD11c+. (**B**,**F**) Activated DC frequency accounts for all DCs including DC/DC1/DC2. (**I**,**K**) Macrophages are defined as F4/80+ cells and (**J**,**L**) activated population is gated from the F4/80+ population. Activated cells are defined as CD86+MHC-II+ cells. n = 6–8 individual mice from 3 experiments. For all plots displayed line indicates mean and each dot is one experimental replicate. Comparisons were made between mock PBS infection and infected at each time point. Data was analyzed using unpaired Student's t-test and outliers excluded using Grubbs Outlier exclusion analysis; * $p < 0.05$.

In vitro bone-marrow derived DCs differentiated into DC1 but not DC2 subsets so we next assessed DC subtypes in the lung and draining LN following in vivo *Coccidioides* infection. DC1 and DC2 cell lung frequencies significantly expanded, but with considerable variability (Figure 5C,D). DC subset frequency was unchanged in the lung-draining lymph node except for a mild decrease in DC2 on day 7 post infection relative to day 1 (Figure 5G,H). We next assessed macrophage changes during infection. Macrophage frequency significantly increased in the lungs and tended to decrease in the draining LN at day 7 post-infection (Figure 5I,K). Activated macrophage frequency based on co-expression of MHC-II and CD86 was unchanged following infection (Figure 5J,L). Analysis of macrophages and DCs expressing only MHC-II or CD86+ DCs in the lung and draining LN revealed no significant differences across tissues or time post-infection, except for a decrease in CD86+ macrophage frequency in the draining LN at day 7 post-infection (Sup-

plemental Figure S2B). Total DCs in the draining LN increased at day 1 post infection before dropping at day 7, with no total changes observed in activated DC numbers (Supplemental Figure S3A,B). Together these data indicate a mixed DC1 and DC2 differentiation response within the lung and mild activation of DCs in response to avirulent Coccidioides alone.

4. Discussion

In this study we applied flow cytometry and imaging techniques to study innate immune responses to *Coccidioides*. We demonstrate that murine macrophages poorly phagocytose *Coccidioides*, while rat alveolar macrophages have higher phagocytosis rates. This corresponds to previous work done with macrophages and general observations regarding the high phagocytosis ability of alveolar macrophage [7,19]. We further found that *Coccidioides* induces efficient monocyte differentiation into macrophages, but largely blocks differentiation at the M0 stage, preventing polarization into M2 macrophages, reducing M1 differentiation and decreasing macrophage activation even in the presence of strong activating and differentiating stimuli. *Coccidioides* also poorly activates DCs, inhibiting DC activation by LPS and IL-4. Bone marrow derived DCs preferentially polarize into DC1 with no DC2 differentiation and poorly activate in vitro in response to *Coccidioides*. In contrast, in vivo DCs and macrophages show an increased activation frequency in response to avirulent *Coccidioides* post infection, although activation frequency is rather low, and DCs show a mixed DC1/DC2 recruitment in the lungs. Peripheral blood analysis reveals no changes in blood monocyte counts over infection (Supplemental Figure S1). Altogether, these data suggest innate immune cells respond and recognize *Coccidioides* but there may be undefined virulence mechanisms allowing fungal escape from phagocytosis and impairment of innate immune cell polarization and immune activation in vitro.

Macrophages are typically associated with pathogenic responses for microbial and foreign body clearance. Their ability to polarize into M1 or M2 subtypes allows for targeted, tailored responses. Proinflammatory cytokines and reactive oxygen species are associated with classically activated M1 macrophages with antimicrobial activity [20]. Anti-inflammatory and wound-repairing signals are associated with alternatively activated M2 macrophages with tissue repair properties. Reprogramming of activated M0 macrophages into M1 or alternatively activated M2 macrophages is reinforced by the secreted cytokines and metabolites produced by the developing population. *Coccidioides* secreted factors suppress nitric oxide and inducible nitric oxide synthase by bone marrow-derived macrophages, and similar factors may regulate macrophage polarization [21]. Production of oxide species (OS) is associated with pro-inflammatory M1 subset; however, with *Coccidioides*, iNOS production is not essential for phagocytosis or fungicidal killing. This suggests that *Coccidioides* suppression of OS production in macrophages could be both a functional inhibition as well as specialization inhibition.

The monocyte to macrophage differentiation in response to *Coccidioides* infection, although not previously measured, is consistent with prior studies that found high IL-6, IL-12, TNF and MIP-2 production by *Coccidioides*-infected peritoneal macrophages [22]. Most surprising was the blocking phenomena where macrophages co-cultured with *Coccidioides* and LPS failed to upregulate MHC-II and CD86 co-expression. *Coccidioides* spherules express metalloproteinase 1 (MEP1) which cleaves the spherule outer wall glycoprotein (SOWgp) antigens, decreasing chances of innate immune cell detection via pattern recognition receptors [23]. Our studies utilize *Coccidioides* in the arthroconidia phase, the soil morphology that does not express MEP1, which suggests that *Coccidioides* may have previously undefined virulence mechanisms that block MHC-II and CD86 upregulation. In *Cryptococcus neoformans* infection, productive immune responses within lung macrophages induce heightened iNOS mRNA levels and M1 macrophages inhibit fungal growth more effectively than M2 macrophages [24]. *Paracoccidioides brasiliensis* antigen stimulates strong M1 macrophage polarization within mouse peritoneal-derived macrophages and in vivo studies show M1 macrophages are more critical for fungal clearance than M2 [25,26]. The specialization block induced by *Coccidioides* presence in our in vitro culture suggests a

possible virulence mechanism where macrophage polarization to M1 is inhibited as means of inhibiting host activation of the specialized, pro-inflammatory macrophage function.

In vitro phagocytosis of *Coccidioides* by macrophages is known to be weak. However, our data suggest alveolar macrophages may be more vital than migratory monocytes for *Coccidioides* clearance. Tissue-resident macrophages are one of the first responders in respiratory mediated fungal infections. They play a tolerogenic surveillance role, ensuring immune responses are effective but also not unnecessarily damaging to the delicate airway architecture. Complementing this tolerogenic role, alveolar macrophages, once activated, have a higher phagocytosis capability, higher OS production capacity, and are more adept at pathogen clearance [7]. Early phagocytic studies in rhesus macaque macrophages suggest this may be the case [27]. Alveolar macrophages played a role in *Cryptococcus neoformans* clearance, with some variability depending on laboratory strain used, whereas their interstitial macrophage counterparts are found to harbor the fungi intracellularly [28]. Phagocytosis increases when the alveolar macrophages are stimulated with LPS, suggesting pro-inflammatory signals are particularly beneficial for enhancing pathogen opsonization (Figure 1H). This was shown in previous studies where IFNγ enhanced phagocytosis rates in both peritoneal and alveolar mouse macrophages but only enhanced fungicidal killing within alveolar macrophages [29]. Our data and previous studies suggest that alveolar macrophages play an important role in *Coccidioides* infection control, and their function could be enhanced by strong pro-inflammatory signals. Further work must be done to help further characterize the specific mechanisms by which alveolar macrophages are more efficient killers than their non-tissue resident counterparts.

DCs play a critical role in activating and educating adaptive immune cells for a proper, effective immune response. Chronic disease often implies a breakdown either in adaptive immunity or earlier during innate immunity. With chronic coccidioidomycosis, one theory is that during disease clearance, DC polarization into DC2 would activate a non-productive adaptive immune response against *Coccidioides*, leading to chronic disease. This would indicate that DCs also are not functionally responding to *Coccidioides* despite upregulating the DC1 (CD11c+ CD8a+SIRPa−) phenotypic markers. Previous studies with *Coccidioides* showed that DCs upregulate CD86 and CD80 when co-cultured with *Coccidioides* and *Coccidioides* antigen lysate, however these studies did not look at the co-expression of MHC-II and CD86 to describe DC activation and maturation [30]. DC activation by the vaccine *Coccidioides* strain is protective and antigen primed DCs protected susceptible mouse strains from virulent challenge [10]. Our data and previous studies suggest a partial activation and response from DCs to *Coccidioides*, whether it be whole or antigen lysate components, but our data suggest a novel mechanism of virulence where *Coccidioides* may evade immunity by inhibiting innate immune cell activation and maturation. Given host susceptibility genetics, incomplete DC activation could impact adaptive immunity much more critically for susceptible versus resistant hosts, potentially explaining why despite the lack of co-expression of MHC-II and CD86 in our in vitro studies, the vaccine strain still provides protection for susceptible mouse strains [31]. Further, the in vitro assay creates a high-pathogen interaction that is unlikely to occur in vivo. This high antigen frequency may enhance the influence of *Coccidioides* virulence on innate immune cells, whereas in vivo multiple cell types (lung epithelium, innate lymphocytes) interact with *Coccidioides* likely at lower individual frequencies. The in vitro supernatant DC polarization experiments seem to support this speculation as decreases in activation marker expression frequency occurred only in the presence of *Coccidioides* and not in the indirect assays (Figure 3K–M). Our data suggest that the activation and polarization block is *Coccidioides* contact-dependent and physical interaction between DCs and arthroconidia are needed to inhibit MHC-II and CD86 co-expression. Further, arthroconidia appear to utilize a novel, contact-dependent mechanism to evade immune responses by preventing innate immune cell polarization and activation.

Despite polarizing to a Th1/Th17-favorable pro-inflammatory DC1 subset in response to *Coccidioides*, DC activation and maturation appears impaired. DC1s lacked the typical

MHC-II and CD86 co-expression typical of an activated/matured antigen presenting cell. This suggests that while DCs respond to *Coccidioides* by differentiating, the process is incomplete potentially resulting in impaired functional capacity. One possible explanation for this is that despite receiving activating signals, DCs are prevented from upregulating costimulatory and maturation markers by arthroconidia-specific virulence factors that are expressed during the fungal switch to the spherule phase. We also observed DCs progressing into DC1, whereas their macrophage counterparts could not differentiate beyond M0. While both cell types poorly upregulate activation/maturation markers, DCs seemed to respond better, at least phenotypically, than macrophages. This could be partially explained by the fact that most DCs do not engage in phagocytosis. but rather utilize pinocytosis, while macrophages phagocytose whole pathogen [32]. These different methods of antigen uptake may partially explain why macrophages poorly progress beyond M0. Arthroconidia also remain alive inside mouse macrophages and may continue to influence macrophage activation and maturation [33].

DC1 and DC2 responses slightly increase in the lung by day 7 post-avirulent infection and the DCs are only mildly activated, somewhat replicating in vitro polarization in response to *Coccidioides*. Total DC frequency does not appear to increase within the lung or lung-draining lymph node by flow cytometry, imaging of lung tissue sections recapitulates these data. Immunohistochemistry scoring of lung tissue sections show no significant changes in intermediate MO, alveolar macrophage, or dendritic cell numbers but a statistically significant increase in neutrophils at day 1 post infection. Although these data may seem contradictory, it is known that less than 20% of cells are released from lung tissue by standard dissociation methods, providing a smaller picture of lung cellular changes when measured by flow cytometry. In immunohistochemistry, difficulties of locating *Coccidioides* in the lung due to the physical obstructions and tissue architecture complexities may also impact how truly representative the images are of an infection state. The modest increase in neutrophils based on image scoring aligns with previously observed increases in neutrophils within vaccine studies and pediatric patient data [34–36].

DC1 and DC2 responses increase in the lung in modest capacity and DCs overall upregulate MHC-II and CD86 co-expression by day 7 post-infection while macrophages fail to upregulate co-expression, somewhat replicating the in vitro data. Single-gating analysis shows CD86+ macrophage frequency in the draining lymph node decreases by day 7 post infection (Supplemental Figure S2B). This suggests that the *Coccidioides* inhibitory mechanisms acting against innate immune cells we observed in vitro are also modestly impactful in vivo. While the vaccine strains are protective in mouse models, translation of vaccines to humans has thus far been unsuccessful, although several groups are actively investigating translational strategies. Studies utilizing complement proteins or *Coccidioides* fragments as agonists enhance host immunity, suggesting that adjuvants may provide additional efficacy to live, attenuated vaccine strains [15,34,37]. This study uses an avirulent *Coccidioides* vaccine strain and may not reflect the innate immune activation found against virulent, wildtype strains. Much exciting and illuminating work has been done to demonstrate the protection and recruitment of adaptive immune responses to this avirulent strain. However, innate immune response as shaped by vaccination is equally important, as these cells coordinate and mold the quality and durability of the subsequent adaptive effector and memory subsets. This vaccine strain has been shown to be protective and induces a mixed Th1/Th2/Th17 memory response but the specific early innate immune mechanisms leading to and shaping these responses have not been well characterized [15,37]. Our study shows that macrophages and DCs in vitro appear to be blocked at various polarization phases and fail to upregulate activation/maturation markers CD86 and MHC-II, suggesting a novel virulence mechanism where *Coccidioides* arthroconidia inhibit DC activation and maturation. Our in vivo data showed modest mixed DC1/DC2 presence in the lungs post-infection and overall DC activation in lungs and dLN by day 7 post-infection, recapitulating observed protection capacity for the vaccine strain.

5. Conclusions

We sought to characterize the innate immune responses to NR-166 avirulent *Coccidioides posadasii* (Δ*cts2*/Δ*ard1*/Δ*cts3*) by characterizing how macrophages and dendritic cells response to *Coccidioides*. This strain is used widely in vaccine studies with protective responses in murine models, however, little is known about the innate immune responses to this strain. We found in our studies evidence for a novel *Coccidioides* virulence mechanism where macrophage and dendritic cell maturation/activation is impaired. Macrophage polarization halted at M0 stage with reduced activation/maturation. Dendritic cells polarized towards DC1 subtype but said DC1s had reduced activation/maturation even when co-cultured with LPS. These data suggest *Coccidioides* has a virulence mechanism inhibiting DC activation/maturation by impacting MHC-II and CD86 expression in a contact dependent manner between DCs and arthroconidia. Our in vivo study found a statistically significant overall DC activation in the lungs and draining lymph node by day 7 post-infection and a mixed DC1/DC2 recruitment in the lungs. Though there was an increase in macrophages in the lung at day 7 post-infection, there was no heightened activation. The varied immune cell behavior between in vivo and in vitro experiments likely stems from higher antigen exposure frequency in vitro compared to in vivo infection. This possibly explains why the in vivo data demonstrate mixed DC1/DC2 presence while the in vitro data show a DC1 bias. These findings while interesting, may or not hold up with virulent infection and warrants additional study. The avirulent (Δ*cts2*/Δ*ard1*/Δ*cts3*) strain was used to characterize activation behaviors and polarization biases in innate immune cells during vaccine-induced immune protection. Overall, the data demonstrate the vaccine strain's protective capability and characterized a mixed DC1/DC2 response, a potential explanation for the mixed Th1/Th2/Th17 protection observed in previous vaccine studies. Future studies further characterizing the mechanism of this novel virulence blocking mechanism could yield therapeutic targets for enhancing innate immune cell responses to *Coccidioides* and open further avenues for innate immune cell-based vaccines.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/jof7080630/s1, Figure S1: Short term *Coccidioides* causes subtle changes in peripheral blood. Figure S2: Single-gating of CD86 and MHC-II macrophages and DCs in the lung and dLN reveals subtle changes in population frequencies over short-term infection. Figure S3: Total DCs increase in lung dLN day 1 post-infection then drops at day 7 post-infection. Figures S4 and S5: Gating strategy for in vitro macrophage and DC polarization experiments, respectively.

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Data Availability Statement: Data is contained within the article or supplementary material; additional information is available upon request.

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CHAPTER 3

Regulatory T Cells influence Coccidioides Infection Clearance and Local Immune Cell Activation

CHAPTER 3: Regulatory T Cells influence *Coccidioides* Infection Clearance and Local Immune Cell Responses

Introduction

Coccidiomycosis, colloquially known as Valley fever or Desert fever, is a fungal respiratory disease endemic to the Central Valley in California, Arizona, and most of the American Southwest [1]. Of those who get symptomatic infections, approximately 4% will progress into severe chronic and/or disseminated disease [1]. There is no cure for chronic Valley fever and existing anti-fungal therapeutics have extremely toxic side effects when used long-term [2]. Our pediatric study found that at time of diagnosis, patients with chronic disease outcome had higher Treg frequency in their peripheral blood [3]. There is an urgent and outstanding need for further research into *Coccidioides* immunity.

Regulatory T cells (Tregs) are a specialized population of CD4+ cells that control inflammatory responses by suppressing effector T (Teff) cell and antigen presenting cell (APC) activation. Through this suppression, Tregs prevent unnecessary tissue damage during infection clearance and regulating host immune development to prevent self-recognition [4]. Our lab was the first to show a correlation between heightened Treg frequency in the peripheral blood at time of diagnosis and chronic disease outcome [3]. Though well studied in other fungal models (such as *Paracoccidioides*, *Cryptococcus*, *Candida*), there is very little known about Treg contribution to *Coccidioides* immune responses [5].

There are several potential implications of Treg correlation to *Coccidioides* disease outcome. Hosts with naturally higher Treg frequencies are less likely to clear infection as the Tregs could suppress effector T cell and APC function. Tregs could also directly induce cell death in T cells via granzyme B production [4]. In this case, a naturally high Treg frequency would be detrimental for infection clearance as hosts could be ill-adapted to mount an effective, pro-inflammatory response. Further, Tregs within the lung could be more suppressive. DBA/2 mice, which are resistant to *Coccidioides* infection, have a much lower natural Treg frequency and IL-10 expression in their lungs compared to the susceptible strains, C57BL/6 and BALC/c backgrounds [Chapter 1, Table 1].

Coccidioides could influence the immune microenvironment to recruit more and/or more suppressive Tregs to dampen immune activation. CCR5 expression on Tregs is thought to enhance their suppressive capacity [2]. CCR5+ Tregs are detrimental in murine *Histoplasma* infection, where CCR5 KO mice had less fungal burden and higher Th17 associated cytokines in their lungs versus CCR5-sufficient mice [6]. There is evidence that *Coccidioides* influences the lung microenvironment via induction of arginase production in lung epithelial tissues [7]. It is plausible that *Coccidioides* produces other metabolite compounds that could induce and/or attract Tregs to the site of infection as part of a larger virulence mechanism to inhibit antifungal responses.

We examine Treg impact on fungal burden and APC polarization and activation within the lungs. Using in vivo Treg transfer and avirulent *Coccidioides* infection models, we show that Treg transfer increases fungal burden within the lungs, as measured by colony forming units

(CFU) (Figure 1.) Treg transfer also increased DC2 frequency within the lung draining lymph node (dLN) but had no impact on activated DC frequency (Figure 2). In vivo primary infection with avirulent *Coccidioides* resulted in decreased Treg frequency in the lung by day 14 post-infection (PI) and increases in activated T cell frequencies within draining lymph node and lungs at day 7 PI (Figure 5, 6). Altogether, our data characterizes adaptive immune responses to avirulent, vaccine *Coccidioides posadasii (cts2/ard1/cts3Δ*) and characterizes Treg impact on fungal infection burden.

Materials and Methods

Mice

Six- to eight-week-old C57BL/6 (JAX # 000664) and DBA/2J (JAX #000671) male and female mice were utilized for experiments and sex matched whenever possible. Mice were housed and bred within the University of California Merced specific-pathogen free animal facility in compliance with the Department of Animal Research Services and approved by the Institutional Animal Care and Use Committee.

Tissue Harvest and Dissociation

Mouse pulmonary draining lymph nodes (dLN) and lungs were mechanically homogenized and collected in PBS 1% FBS and filtered through a 100 μM mesh filter. Cell suspensions were centrifuged at 1200 rpm for 5 minutes at 10℃. Red blood cells in lung homogenates were lysed using 1x lysis buffer made from 10x Ammonium Chloride Lysis Buffer Stock (NH_4Cl (ammonium chloride) 8.02gm $NaHCO_3$ (sodium bicarbonate) 0.84gm EDTA (disodium) 0.37gm in 500 mL Millipore water; (Fisher Scientific). Cells were washed, centrifuged, and resuspended in PBS 1% FBS.

Regulatory T cell Sort

Peripheral LNs and mesenteric LNs from 6–8-week-old female donor mice were processed, labeled with anti-CD4 PerCP (Clone: RM4-5; 1:400, BioLegend), anti-CD25 FITC (Clone: PC61.5.3; 1:200, Invitrogen), anti-CD127 PE-Cy7 (Clone: A7R34; 1:400, eBioscience), and filtered through a 70 μM mesh filter. Cells were sorted under sterile sorting conditions on the BD FACS Aria II - Flow cytometer/Cell Sorter. Murine Tregs were identified by gating for live, CD4+, CD127-, $CD25^{hi}$ cells and sorted at >90% purity. Sorted cells were centrifuged and resuspended in RT PBS for injection/transfer. For sorting strategy used, see Liu et. al [8].

Treg Adoptive Transfer

Recipient mice were anesthetized with isoflurane gas (Piramal Critical Care) inhalation using a Rodent Anesthesia Machine (Parkland Scientific) and injected retro-orbitally with 0.5-1 $x10^6$ Tregs suspended in 100 μL PBS. Control mice received 100 μL PBS.

Flow cytometry

Cells were resuspended at $2x10^6$ cells and labeled in 50 µl volumes (using antibodies described below from eBioscience unless otherwise noted) for 30 minutes in the dark at 4℃. Unbound antibody was removed by washing cells in PBS 1% FBS then fixed in 1x Fixation/Permeabilization Buffer (eBioscience) for 45 minutes at room temperature (RT) in the dark. Cells were washed and centrifuged at 1500 rpm for 10 minutes at RT and resuspended in 100 µL PBS 1% FBS for flow cytometry analysis. Data was acquired on a LSRII (BD) and analyzed using FCS Express (DeNovo Software). Cell antibody panels were as follows:

Adaptive T Cell Panel: Fixable Viability Dye eFluor 506 (1:500, eBioscience), anti-CD3ε (clone 145-2C11; 1:200, eBioscience), anti-CD4 PerCP (clone RM4-5; 1:400, Biolegend), anti-CD8α PE-Cy7 (clone 53-6.7; 1:400, Invitrogen), anti-CD25 APC (clone PC61.5, 1:400, Invitrogen), anti-CD69 APC-eFluor 780 (clone H1.2F3, 1:400, Invitrogen), anti-CD62L PE (clone MEL-14, 1:1600, BioLegend), anti-CD44 PE eFluor610 (clone IM7, 1:200, eBioscience), FOXP3 FITC (clone FJK-16s, 1:100, Invitrogen).

Dendritic Cell Panel: Fixable Viability Dye eFluor 506 (1:500, eBioscience), anti-CD11c FITC (clone HL3, BD Biosciences; 1:400), anti-F4/80 PerCP-Cy5.5 (clone BM8; 1:400, eBioscience), anti-MHC-II (I-A/I-E) APC-Cy7 (clone M5/114.15.2, BioLegend, 1:200), anti-CD86-PE (clone PO3.1, 1:200, eBioscience), anti-CD8α PE-Cy7 (clone 53-6.7; 1:400, Invitrogen), anti-SIRPα/CD47 APC (clone P84, 1:400, BioLegend).

Coccidioides culture and harvest

NR-166 avirulent *Coccidioides posadasii (cts2/ard1/cts3Δ*) laboratory strain derived from parent isolate C735 was used for all infections (BEI Resources). Liquid 2x Glucose 1x Yeast Extract (2X GYE) media (Fisher Scientific) was inoculated with frozen fungal stock and cultured at 30℃ in a shaking incubator for 72 hours. Liquid culture was streaked onto 2x GYE agar plates and grown to confluency, then desiccated until the gel condensed. To obtain arthroconidia, the fuzzy white growth was scraped off the plate into PBS and filtered through a 40 µM mesh filter. The fungus was pulse-vortexed for 1 minute to disassociate and centrifuged at 9000 x *g* for 30 minutes at room temperature. The fungal pellet was washed with PBS resuspended in PBS for use. Arthroconidia suspension was stored at 4℃ until use. For complete protocol used, see Mead et. al [9].

CFU Analysis

Whole lung was processed in 2 mL of PBS 1% FBS via mechanical homogenization. Lung homogenate was pulse-vortexed for a minute. 100 µL of lung homogenate was plated on 2X GYE agar plates and spread using glass beads. Plates were checked for CFUs over the course of 4-7 days and final CFU count recorded on day 7. CFUs counted on the plate are calculated to obtain final number of units for the whole 2 mL of lung. $\left(\#\frac{CFUs}{100}\mu L\right) x\ (2000) =$ total CFUs per 2 mL or per whole lung.

Statistics

Experimental data was analyzed using paired Student's *t*-test or unpaired Student's *t*-test, and all data analyzed for outliers using Grubbs Outlier Exclusion analysis with GraphPad Prism version 8 for Windows Software (GraphPad Software). Figure legends denote the comparisons analyzed, if outliers were detected and excluded, and the *p*-value for each figure.

Results

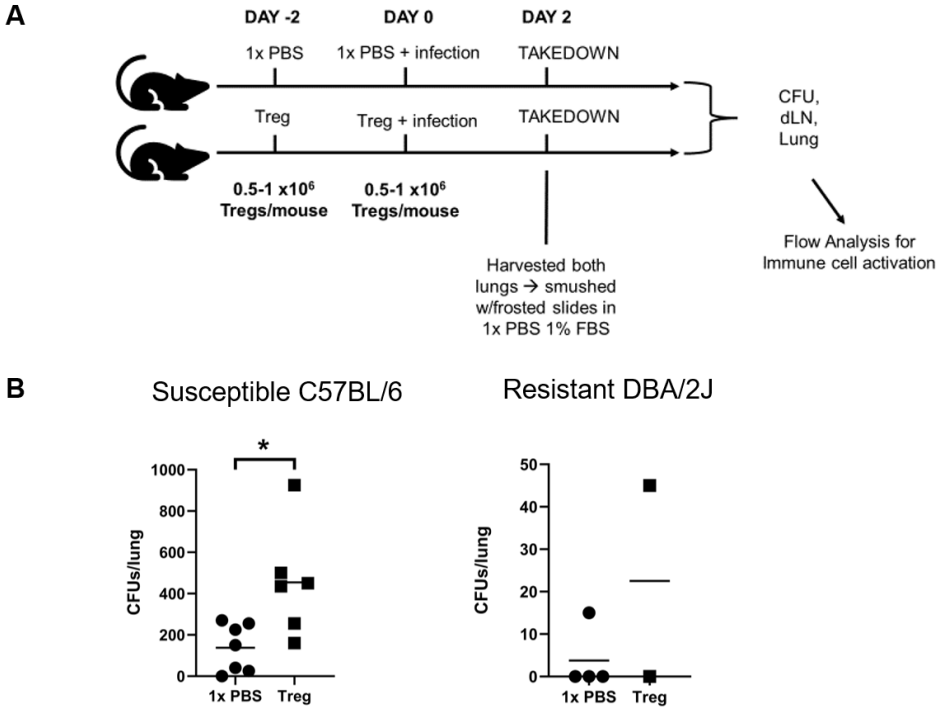

Figure 1. Treg transfer into C57BL/6 mice increase fungal burden within the lungs. (**A**) Sorted Tregs were transferred 2 days before and on the day of infection, and mice were sacrificed 2 days post infection. Draining lymph node and lungs were harvested for immune cell assessment and lung homogenate was plated for colony forming units. (**B**) Fungal lung burden as measured by CFUs per total lung in two murine backgrounds infected with avirulent *Coccidioides* and the presence or absence of Tregs. C57BL/6 data is from 3 experiments, n=6-7 mice, DBA/2J data is from 1 experiment, n=2-3 mice. Each dot represents a biological replicate; line represents the mean. Unpaired Student's *t*-test was used, Grubbs Outlier Exclusion method used to identify and exclude outliers, *p <0.05.

Treg transfer increases fungal burden in lungs

To explore the influence of Tregs on infection clearance, we transferred sorted Tregs into the susceptible C57BL/6 mice infected with the avirulent, vaccine *Coccidioides posadasii (cts2/ard1/cts3Δ)* strain. Tregs were injected two days before and on the day of intranasal *Coccidioides* infection and fungal burden evaluated two days post-infection (Figure 1A). C57BL/6 control mice, infected with *Coccidioides* with no Treg transfer, averaged 108.8 CFUs whereas Treg transfer mice averaged 454.2 CFUs. This represents a 4.2-fold increase in *Coccidioides* burden when additional Tregs are present (Figure 1B). DBA/2J mice had on average 3.8 CFUs with PBS mock transfer and 22.5 CFUs with Treg transfer, a 5.9-fold increase (Figure 1C).

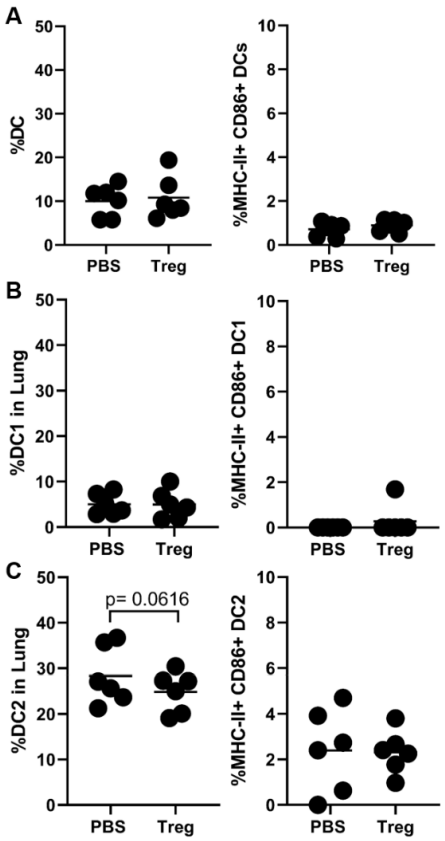

Figure 2. Lung DC2 frequency decrease with Treg transfer. C57BL/6 mice were given Treg transfers as outlined in previous figure (Figure 1) and infected with 10^5 *Coccidioides* arthroconidia. (**A**) DC (CD11c+ cells) frequency does not change with Treg transfer and frequency of activated cells (MHC-II+ CD86+) do not differ. (**B**) DC1 are defined as CD8α+ DCs. (**C**) DC2 are defined as SIRPα+ DCs. Activated frequency obtained by gating off respective subtype population. Each dot represents a biological replicate; line represents the mean. Data is from 3 experiments, n=5-6 mice, Grubbs Outlier Exclusion method used to identify and exclude outliers. Paired Student *t*-test used.

DC frequencies in the dLN and lung shift after Treg transfer

Since Tregs regulate immune responses by directly interacting with antigen presenting cells (APC), we also analyzed APC changes in the lung and dLN post transfer and infection. We characterized Treg impact on DC polarization and activation/maturation by measuring MHC-II and CD86 co-expression on DCs. Contrary to our original hypothesis, there are no changes in dLN and lung DC frequency following adoptive Treg transfer and *Coccidiodes* infection (Figure 2A, 3A). DC1 frequencies remain unchanged while DC2 frequency dropped with adoptive Treg transfer, from an average of 28.3% to 24.9% (p= 0.0616) within the lung (Figure 2B, 2C). In contrast, DC2 frequency increased from an average of 19.0% to 23.3% within the dLN with Treg transfer (Figure 3C). MHC-II and CD86 co-expression frequency remain unchanged across all DC subtypes with *Coccidioides* infection and additional Tregs (Figure 2, 3).

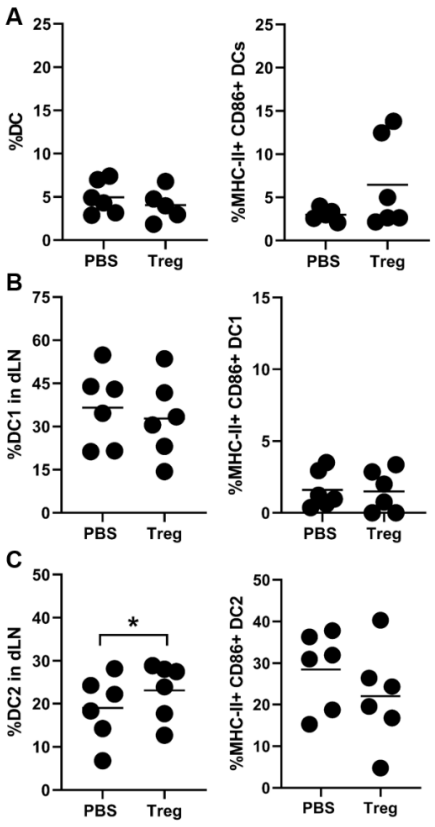

Figure 3. dLN DC2 frequency increases with Treg transfer. C57BL/6 mice were given Treg transfers as outlined in previous figure (Figure 1) and infected with 10^5 *Coccidioides* arthroconidia. (**A**) DC (CD11c+ cells) frequency does not change with Treg transfer and frequency of activated cells (MHC-II+ CD86+) do not differ. (**B**) DC1 are defined as CD8α+ DCs. (**C**) DC2 are defined as SIRPα+ DCs. Activated frequency obtained by gating off respective subtype population. Each dot represents a biological replicate; line represents the mean. Data is from 3 experiments, n=5-6 mice, Grubbs Outlier Exclusion method used to identify and exclude outliers. Paired Student's T-test used, *p<0.05

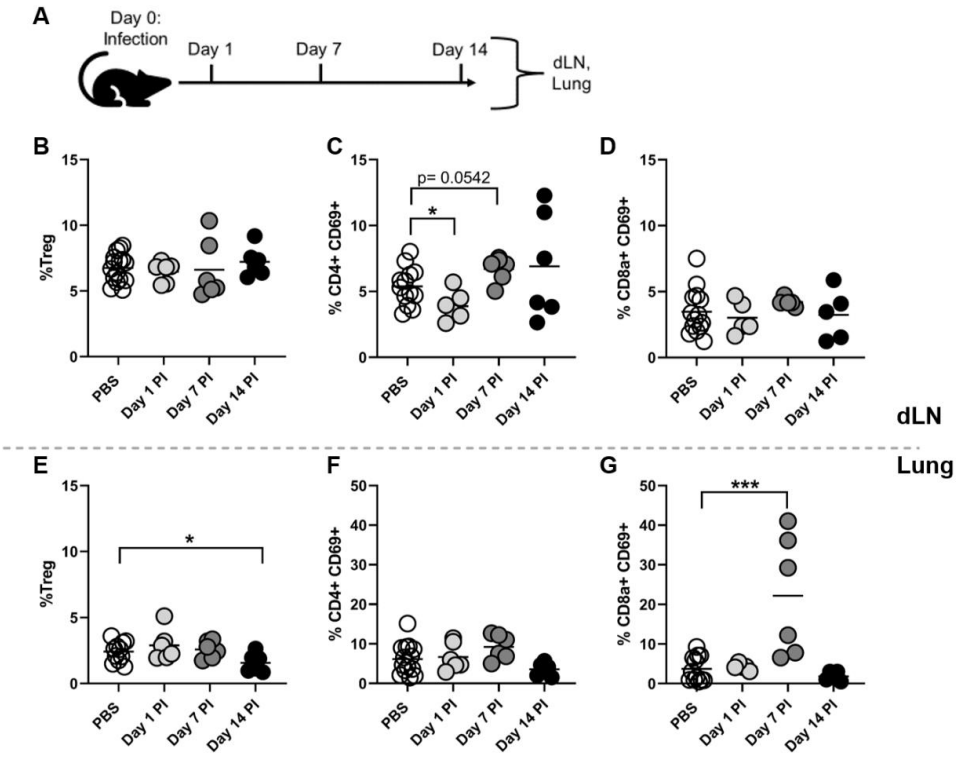

Figure 4. Lung Treg frequency decreases at day 14 PI with avirulent *Coccidioides* infection. dLN and lung activated T cell frequency peaks at day 7 PI. (**A**) Infection timeline overview: mice were infected with 10^5 *Coccidioides* arthroconidia in 30 μL of PBS, mock infection mice received 30 μL of PBS. (**B, E)** Treg frequency was obtained from CD25+ Foxp3+ populations gated off CD4+ T cells. (**C-D, F-G**) Activated T cell (CD69+) frequency gated off of respective CD4+ and CD8α+ populations. Data is from 3 experiments with PBS controls pooled from all experiments. n=5-14 mice, line represents the mean. Unpaired Student's *t*-test used to compare PBS data against infection timepoints. Grubbs Outlier Exclusion method used to identify and exclude outliers, *p<0.05, **p<0.005, ***p<0.0005

Treg frequency decreases in the lungs post-infection

To further characterize adaptive immune responses to the vaccine strain, we measured T cell frequencies within the dLN and lung after avirulent *Coccidioides* infection. C57BL/6 mice were infected with either a mock infection of PBS as our control or 100,000 arthroconidia/mouse intranasally and sacrificed at days 1, 7, and 14 post infection. We analyzed the lung as it is the primary site of infection and the lung draining lymph node as it is the most proximal lymph node (Figure 4A). At day 14 PI, lung Treg frequency dropped to a 1.6% average as compared to the uninfected PBS control's 2.5% (Figure 4E). To assess early T cell activation, we measured CD69+ expression frequency within CD4+ and CD8α+ T cell populations [10]. CD69+ CD4+ T cell frequency averaged 3.9% at 1 day post infection, representing a 1.4-fold decrease compared to the PBS control (Figure 4C). At day 7 PI, dLN CD69+ CD4+ frequency peaked at 6.7% average compared to control 5.4%, with no changes observed within CD69+ CD8α+ frequency (Figure 4C, 4D). Conversely, at day 7 PI in the lung, CD69+ CD8α+ frequency peaked at 22.2% average compared to the uninfected control 3.7% (Figure 4F, 4G). At 7 days post infection, this

represents a 1.2-fold increase in activated CD4+ T cells within the dLN and a 6-fold increase in activated CD8α+ T cells within the lung.

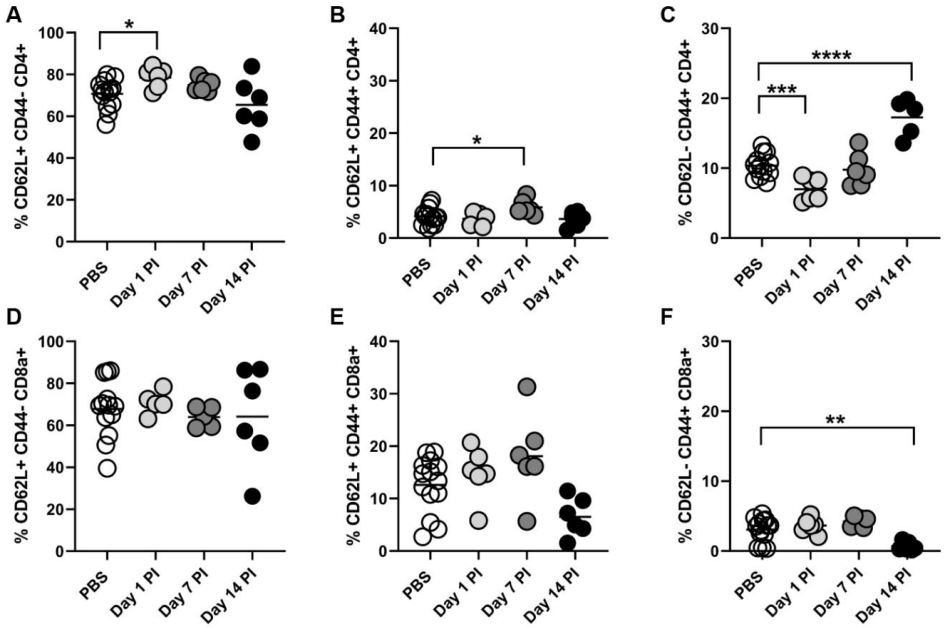

Figure 5. CD62L/CD44 expression in CD4+ and CD8α+ T cells within the dLN. Mice were infected with 10^5 arthroconidia in 30 µL of PBS, mock infection mice received 30 µL of PBS. (**A, D**) Frequency of CD62L+ CD44-, naïve T cells. (**B, E**) CD62L+ CD44+: frequency of activated, effector T cells still residing within dLN. **(C, F)** CD62L- CD44+: frequency of activated, effector T cells ready to leave dLN. Data is from 3 experiments with PBS controls pooled, each dot represents a biological replicate, n=6-14 mice, line represents mean. Unpaired Student's *t*-test used to compare PBS data against infection timepoints. Comparisons Grubbs Outlier exclusion method used to identify and exclude outliers, *p<0.05, **p<0.005, ***p<0.0005, ****p<0.00005

T cells downregulate CD62L and upregulate CD44 expression at day 14 PI in dLN

Though we observed significant activated T cell changes on day 1 and day 7 post infection, CD69 is an early activation marker expressed transiently upon initial activation. As T cells activate and encounter antigen from professional APCs within the lymph node, we assessed T cell responses within the lung draining lymph node by measuring CD62L expression and CD44 co-expression. Naïve T cells express CD62L, or L-selectin, to help facilitate their trafficking into lymph nodes [11]. CD44 expression is associated with effector activation and in some infection models, better infection clearance and heightened inflammation within the lung [12]. In combination, these markers provide cues into the activation and migratory capacity of T cells. Day 1 post infection, CD62L+ CD44- CD4+ T cell frequency averaged 78.6% compared to the PBS control 70.7%, with no changes observed within the corresponding CD8α+ population (Figure 5A, 5D). CD62L+ CD44+ CD4+ T cell frequency peaked at day 7 PI with an average of 5.9% as compared to the 4.2% control (Figure 5B). CD62L- CD44+ CD4+ T cell frequency averaged 7.0% at day 1 PI and 17.3% at day 14 PI, representing a 1.5-fold decrease and a 1.7-fold increase respectively against the 10.4% PBS average (Figure 5C). CD62L- CD44+ CD8α+ T cell frequency had a stark drop at day 14 PI, averaging 0.7% compared to the control 3.3%, a

significant 4.7-fold drop (Figure 5F). Altogether, these data show CD44 expression in CD4+ T cells increase at day 7 PI and by day 14 PI, they begin to downregulate CD62L, suggesting readiness to leave the lymph node.

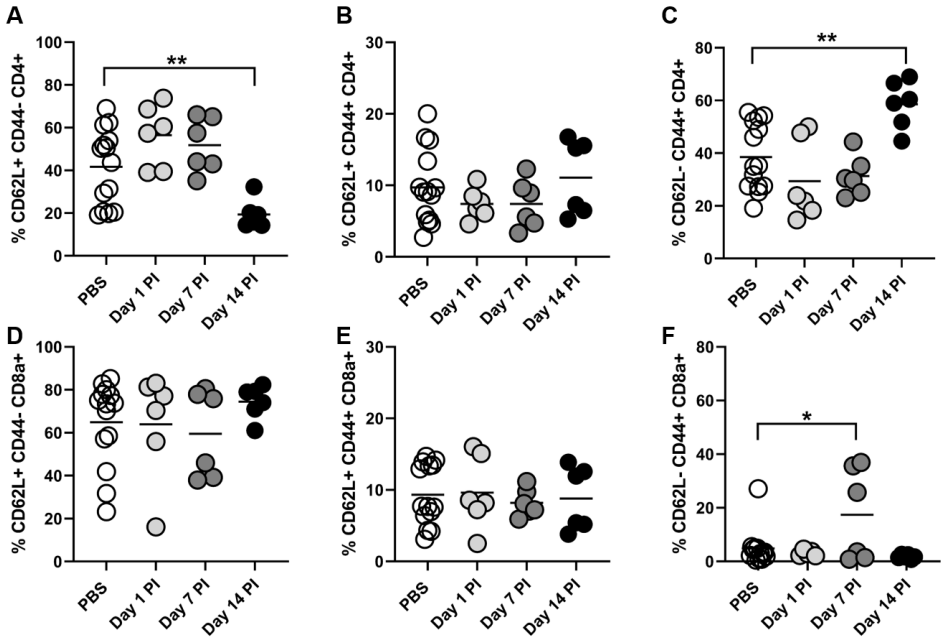

Figure 6. CD62L/CD44 expression in CD4+ and CD8α+ T cells within the lung. Mice were infected with 10^5 arthroconidia in 30 µL of PBS, mock infected mice received 30 µL of PBS. (**A, D**) Frequency of CD62L+ CD44-, naïve T cells. (**B, E)** CD62L+ CD44+: frequency of activated, effector T cells still residing within dLN. (**C, F**) CD62L- CD44+: frequency of activated, effector T cells ready to leave dLN. Data is from 3 experiments with PBS controls pooled, each dot represents a biological replicate, n=6-14 mice, line represents mean. Unpaired Student's *t*-test used to compare PBS data against infection timepoints Grubbs Outlier exclusion method used to identify and exclude outliers, *p<0.05, **p<0.005

Naïve lung T cell frequency decreases while activated T cells increase at day 14 PI

After assessing dLN T cell responses, we examined lung T cell responses as this is the primary site of *Coccidiodes* infection. Activated T cells from proximal lymph nodes would localize to the infection. Thus, one could expect an increase in activated cells within the lung post infection. Frequency of CD62L+ CD44- CD4+ T cells, naïve cells, at day 14 PI averages 20.1%, a 2-fold reduction compared to the control 42.6% average (Figure 6A). No changes were observed in the corresponding CD8α+ population (Figure 6D). CD62L- CD44+ CD4+ T cell frequency increases at day 14 PI at an average of 57% with *Coccidioides* infection as compared to the 37.5% control average (Figure 6C). CD62L- CD44+ CD8α+ T cell frequency peaks at 17.3% average, a 3.4-fold increase compared to the control average of 5.1% (Figure 6F). Lung T cell response analysis revealed an increase in activated CD4 frequency at day 14 PI whereas CD8α frequency peaks at day 7 PI. Together, these data suggest that effector CD8α responses begin 7 days post infection whereas CD4 responses require longer activation time before they traffic to the lungs.

Discussion

Tregs are detrimental to disease outcome in *Paracoccidioides* infection, and their ablation resulted in rescued anti-fungal immune responses and prolonged survival [13]. Our lab observed a correlation between increased Treg frequency in peripheral blood at time of coccidiomycosis diagnosis and a chronic disease outcome [3]. Thus, I hypothesized that Treg transfer would decrease APC activation state and increase fungal burden. Treg adoptive transfer results in increased avirulent *Coccidioides* burden within the lungs, as measured by higher CFU count (Figure 1). However, there were no significant changes in DC frequency or activated/matured DC frequency within the draining LN or lung with Treg transfer (Figure 2A, 3A). DC2 frequency decreased within the lung and significantly increase within the dLN in the presence of adoptively transferred Tregs (Figure 2C, 3C). This Tregs may not inhibit APC activation or maturation during infection but instead influence APC polarization.

Since DCs shape adaptive T cell response, our next experiments focused on characterizing the impact of avirulent *Coccidiodes* infection on CD4 and CD8 T cell populations. We continued avirulent characterization work done in Chapter 2 to further characterize susceptible C57BL/6 mouse immunity to vaccine strain *Coccidioides posadasii (cts2/ard1/cts3Δ*) Our study aims to expand the field's understanding of how this avirulent vaccine strain impacts T cell populations. The work in this chapter demonstrates the first evidence for Treg kinetics post-infection. We observed a drop in Tregs frequency by day 14 PI (Figure 4E). In parallel, early activated CD4 T cell responses within the dLN and lung peak at day 7 PI (Figure 4). We further characterized later immune activation by measuring CD62L vs. CD44 co-expression on T cells. By day 14, CD62L- CD44+ CD4+ T cell frequency increased in both dLN and lung (Figure 5, 6). This increase corresponds to the Treg frequency decrease at day 14 PI (Figure 4E). Together, our interpretation of the data is that as Tregs decrease over time, activated T cells take their place.

Overall, these data suggest Tregs inhibit fungal clearance and could potentially impact adaptive immunity by influencing DC polarization and adaptive T cell frequencies. The absence of IL-10, a Treg associated cytokine, the susceptible C57BL/6 mouse develops protective memory responses to virulent *Coccidioides* challenge [14]. Further work must be done to show whether Treg presence inhibits T cell activation or migration into the lung. To examine Treg influence on adaptive immunity, one could deplete Tregs during *Coccidioides* vaccination and compare protection and survival with virulent secondary challenge against Treg-sufficient controls. Should Treg depletion prove beneficial for survival, clinicians could target Tregs for depletion during vaccination to enhance anti-fungal immunity development. This could result in better adaptive immunity and memory formation, as seen in IL-10 absent murine models of vaccination [14]. Examining Treg influence on DC polarization would also broaden the field's understanding of Treg impact on adaptive immunity through the APCs. CTLA-4 on Tregs competitively bind to CD80/CD86 on APCs, decreasing that APC's ability to interact with naïve T cells for activation [15]. Blocking Treg interaction with APCs could increase the chances of productive T cell activation during vaccination, leading to better adaptive immunity and memory formation. The work in this thesis chapter outlines foundational evidence for Treg contribution to infection persistence and further characterization of adaptive immunity to vaccine *Coccidioides*.

Further work to characterize these responses could revolutionize our understanding of *Coccidioides* disease outcome markers and improve vaccine development.

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CHAPTER 4

Conclusion

CHAPTER 4: CONCLUSION AND FUTURE DIRECTIONS

CONTRIBUTIONS TO THE FIELD

Macrophage and DC Polarization in response to Coccidioides

The work outlined in this thesis explores vaccine strain *Coccidioides posadasii (cts2/ard1/cts3Δ*) influence on antigen presenting cell polarization. Previously, it was known that macrophages are critical for infection clearance via phagocytic function, and DCs are critical for training adaptive immunity activation [1-2]. Pro-inflammatory cytokines such as IFNγ enhance macrophage phagocytosis suggesting a critical role for pro-inflammatory responses and signals in infection clearance [2]. DCs induce immune proliferation and T cell activation in response to formalin-killed *Coccidioides* and lysate [1, 2]. Differentially polarized antigen presenting cells (APC) respond differently to infection and produce differing cytokines [4]. Thus, APC polarization and effector products influence the immune microenvironment which shapes adaptive immune cell recruitment, activation, and maintenance [5]. DC1 subtypes promote pro-inflammatory, antifungal Th1/Th17 responses whereas DC2 subtypes promote non-productive Th2 responses [4-5]. Correspondingly, macrophages follow a similar polarization schema. M1, or classically activated macrophages, are highly phagocytotic and secrete antimicrobial, antifungal cytokines [6]. M2, or alternatively activated macrophages, are associated with wound healing and sometimes granuloma development [7]. M2 are especially detrimental for antifungal responses as their specialized properties suppress inflammation and promotes tissue repair instead of phagocytosis. In *Cryptococcus* infection, M2 cells inhibit clearance by secreting anti-inflammatory, Th2 associated cytokines and their presence promotes fungal dissemination [7].

In Chapter 2, our work characterizes DC and macrophage responses to *Coccidioides*. Bone-marrow derived DCs cultured with *Coccidioides* and DC2 polarizing stimuli, still preferentially polarize into DC1 [Chapter 2]. Bone-marrow derived monocytes become dominantly M0 but do not polarize into M1 or M2 subtypes [Chapter 2]. *Coccidioides* may block M1/M2 polarization to prevent specialized cytokine responses as a means of evading innate immunity. DCs and macrophages cultured with *Coccidioides* also had significantly less MHC-II/CD86 co-expression, suggesting *Coccidioides* is actively inhibiting APC activation/maturation. Supernatant-based experiments suggests this inhibition is contact dependent. In vivo infection data reveals a mixed DC1/DC2 frequency in the lungs and no significant changes to MHC-II/CD86 co-expression. As the lung is a complex immuno-mucosal tissue, there are other immune and epithelial cells that could contribute to fungal detection and pathogen response. Such cells are possibly interacting with *Coccidioides* and lung DCs to promote the mixed DC1/DC2 frequency observed. Our study in Chapter 2 is the first to show evidence for a novel virulence mechanism where *Coccidioides* could evade immune detection and clearance by influencing APC polarization and activation. Other fungal species manipulate macrophage polarization for survival, thus it is likely *Coccidioides* has similar evasion mechanisms [7]. By influencing DC activation and polarization, *Coccidiodes* can impact adaptive immunity and memory formation. Lowered MHC-II and CD86 co-expression on DCs can be interpreted as decreased capacity to activate T cells [4-5].

Coccidioides vaccine research has come a long way and yielded many exciting discoveries about adaptive fungal immunity. The work done in this thesis highlights the importance of defining early innate responses to *Coccidioides* infection for vaccine development. Though previous studies demonstrate T cell activation, proliferation, and mixed T effector differentiation, the mechanisms by which APCs generate these responses are not well characterized. We do not know what signaling pathways, receptors, and interactions between APCs and naïve T cells result in strong antifungal Teff response. Further exploration of APC polarization and activation could yield novel targets for enhancing immunization by targeting the professional APCs that would stimulate adaptive immunity.

Treg Contribution to Infection Clearance

Previous work done by our lab shows a strong correlation between elevated Treg frequency in peripheral blood at time of diagnosis and chronic disease outcome in pediatric patients [8]. Tregs are well characterized in autoimmunity and cancer with their function being crucial for maintaining immune tolerance and regulating inflammatory responses [9]. Treg function in coccidioidomycosis is poorly understood. Our best examples for coccidiomycosis comes from paracoccidiomycosis, caused by *Paracoccidioides brasiliensis*, a distant genetic cousin. In paracoccidiomycosis mouse models, Treg depletion extends survival rates and rescues protective Th1/Th17 responses within the lung [10]. Further, T cells in mice depleted of Tregs and infected with *Paracoccidioides*, produce far more pro-inflammatory cytokines associated with effective fungal clearance than T cells in Treg-sufficient mice [10, 11]. Patients with chronic disease outcome also had elevated IL-10, a regulatory cytokine associated with Treg function [8]. In susceptible mouse backgrounds, the absence of IL-10 resulted in better *Coccidioides* clearance in the lungs as well as protection against virulent *Coccidioides* infection [12]. Though these studies suggest Tregs promote unfavorable disease outcome, further work must be done to characterize the mechanisms by which it occurs.

It is possible that high Treg frequency corresponds to poor *Coccidioides* infection clearance. This idea is supported by the DBA/2 resistance versus C57BL/6 *susceptibility* to *Coccidioides* infection [13]. This resistance is ascribed to the full Dectin-1 receptor of DBA/2 mice versus the truncated version in the C57BL/6 [13, 14]. However, DBA/2 also produce less IL-10 and have a lower Treg frequency in their lungs than C57BL/6; combined with our pediatric study data, this suggests host Treg frequency contributes to infection susceptibility [Chapter 1, Table 1]. Another possibility is that the Tregs have higher suppressive capacity. Unbiased immune parameter analysis found that CCR5+ Tregs associated with chronic disease outcome [9]. CCR5 expression in Tregs is thought to increase migratory capacity to different tissues, thus increasing ability to impact infection outcome [15]. CCR5 KO mice cleared *Histoplasma* infection while CCR5-sufficient mice did not, and CCR5 KO lungs had elevated Th17 associated cytokines and decreased Treg numbers [15.] Together, these data emphasize the likelihood of Tregs, particularly CCR5+ Tregs contributing to chronic disease outcome.

The work detailed in Chapter 3 provides further evidence of Treg detrimental impact on *Coccidioides* clearance. C57BL/6 mice that received adoptive Treg transfer have impaired fungal clearance, as measured by colony forming unit counts (Chapter 3, Figure 1). The mice also have

decreased DC2 frequency in their lungs, suggesting that Tregs can shape DC polarization and/or migration during *Coccidioides* infection (Chapter 3, Figure 2). In *Cryptococcus* infection, IL-10 promotes DC2 polarization and increases fungal burden in the lungs [16]. Our study was performed in the *Coccidiodes*-susceptible C57BL/6 mouse model using avirulent *Coccidioides*, a strain designed to have no replication capacity and therefore little to no virulence within the host [17]. Treg transfer extends avirulent *Coccidioides* persistence within the lung as compared to the control PBS transfer, demonstrating Treg impact on fungal clearance even with an attenuated, weakened strain.

Next, to further characterize adaptive immune responses to *Coccidioides*, we assessed activation and migratory capacities of CD4+ and CD8α+ T cells within the dLN and lung (Chapter 3, Figure 4). CD69+ is the earliest inducible cell surface marker so we assessed its expression to identify early activated T cells [18]. Activated CD4 frequency peaked within dLN and lung at day 7 PI and activated CD8αfrequency at day 7 PI within the lung. As CD69 is a transiently expressed marker and would provide limited insight to activation at later time points, we also assessed CD62L/CD44 expression on T cells over time. CD62L, or L-selectin, is upregulated on T cells to promote trafficking to secondary lymphoid organs where they can interact with DCs and become activated [19]. CD44 expression is associated with effector activation and in *Mycobacterium TB* models, better infection clearance and heightened inflammation within the lung [20]. At day14 PI, CD62L- and CD44+ CD4+ T cells increased in frequency within both the dLN and lung, suggesting activated, effector responses arise at day 14 PI (Chapter 3: Figure 5, 6).

FUTURE DIRECTIONS

Coccidioides research has boomed in the last decade as growing concerns within endemic regions have motivated legislation to enhance research funding and community awareness. Though well studied and characterized by fungal geneticists and ecologists, there are still many questions about *Coccidioides* immunity. A major challenge to developing fungal vaccines are due to the diverse morphologies and antigen expression throughout life cycles [21]. An effective fungal vaccine must educate the adaptive immunity against all possible fungi forms to ensure complete protection. Fungi are also eukaryotes, sharing many common molecules with humans, complicating the search for unique markers to target. To date, most antifungal drugs are derivatives of azoles created in the late 1980s and even these are not suitable for chronic infection treatment as they cause severe, toxic side effects when used long term [22]. Understanding basic immunological responses to *Coccidioides* is crucial for improving future therapeutics and enhancing our knowledge base to create strong, effective fungal vaccines.

The work in this thesis suggests evidence of a virulence mechanism by which *Coccidiodes* is actively inhibiting APC polarization activation (Figure 1A-1C). Future work done with *Coccidioides* and human APCs (such as DCs and macrophages) to characterize innate immune response to virulent fungal challenge would yield patient-relevant data. APC polarization data from patients during acute and chronic infection could help elucidate what early immune events lead to infection clearance or persistence. Further work examining signaling networks within APCs exposed to *Coccidioides* could also provide further details on where APC

polarization is blocked at a transcriptional level. Next steps should include repeating the studies outlined in Chapters 2-3 with virulent *Coccidioides* strains. This would yield invaluable data comparing APC polarization and activation of avirulent, vaccine *Coccidioides* versus wild-type and clinically derived strains.

The Treg data presented in this thesis utilizes the avirulent, vaccine strain on an already susceptible mouse genetic background. Though the data is promising, the most immediate next steps to confirm Treg contribution to infection outcome is to deplete Tregs within a susceptible mouse model and infect with virulent *Coccidioides* strains. To take cue from the *Paracoccidioides* field, Treg depletion during ongoing infection of mice to rescue productive immune responses and increase survival would provide definitive proof that Tregs are detrimental to disease outcome [10-11].

Examining Treg functional state during virulent *Coccidioides* infection and comparing it to avirulent infection could define Treg impact on adaptive immunity and memory development (Figure 1C-1D). Vaccinated C57BL/6 mice develop protective, memory T cell responses to *Coccidioides* in the absence of interleukin-10, a Treg associated cytokine [12]. Depleting Tregs during *Coccidioides* vaccination could enhance anti-fungal immunity development and memory development. Memory T cell subset identification via flow cytometry would also define critical memory subsets for *Coccidioides* protection. Tissue resident memory cells are responsible for protective responses in bacterial and viral respiratory infections, but little is known about their contribution to fungal, respiratory immunity [23-24]. Memory subset identification studies would help to define T cell populations responsible for providing long lasting immunity within the lung.

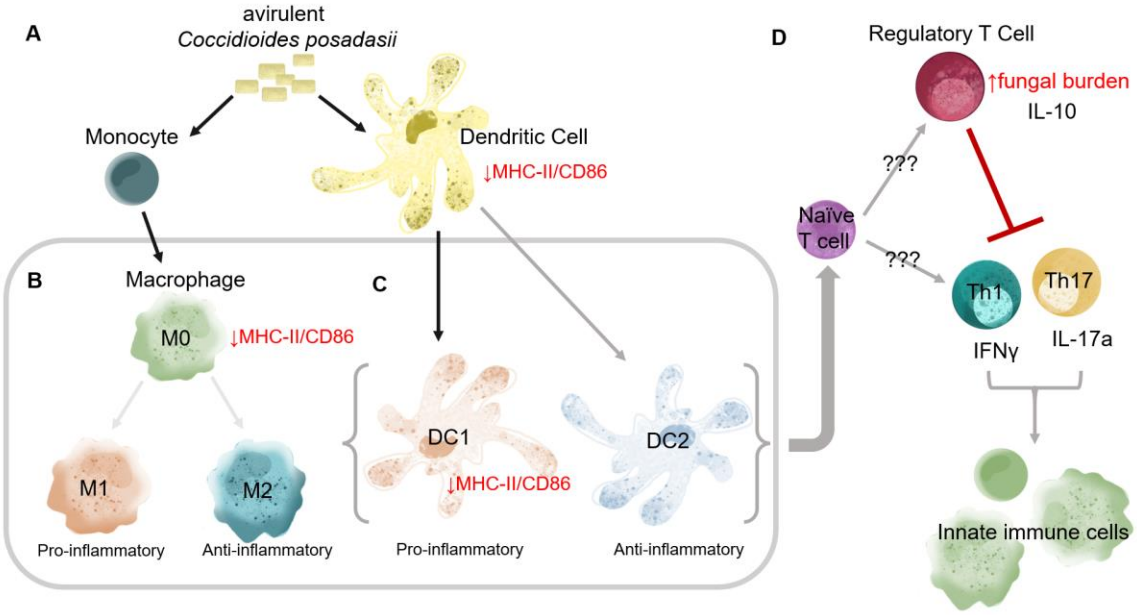

Figure 1. Avirulent *Coccidioides posadasii* induces M0 and DC1 polarization but inhibits MHC-II/CD86 expression. Tregs are detrimental for infection burden as their presence increases fungal burden within lungs. (**A**) In vitro assays with monocytes and DCs defined polarization and activation in response to avirulent *Coccidioides*. (**B**) Monocytes become M0 but do not polarize or activate in response to *Coccidioides*. Grey arrows represent this polarization block. (**C**) DCs polarize into DC1, and exhibit decreased activation in contact-dependent interactions with *Coccidioides*. (**D**) In vivo Treg adoptive transfer with *Coccidioides* infection increases fungal burden within the lung. Tregs suppress T helper 1 (Th1) and T helper 17 (Th17) cells whose cytokine products can shape innate immune cell responses.

SUMMARY

Coccidioides research has come a long way since its original scientific record in the 1800s but many questions regarding fungal immunity remain. This research is the first to assess APC polarization responses and show Treg contribution to *Coccidioides* infection persistence in mouse models. Our Treg transfer work suggests Tregs are detrimental to disease outcome. Further work to understand APC involvement in activating/educating adaptive immunity will be crucial. Activating and promoting the correct APC subtype has implications for adaptive immunity activation. Such knowledge would further our understanding of *Coccidioides* susceptibility in patients and enhance vaccine development. Chronic coccidiomycosis robs individuals of their quality of life and ability to work. Fungal vaccines and new therapeutics for treating chronic infection would change and save countless lives.

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