Title
MyD88-dependent signaling and the host response to the human fungal pathogen Histoplasma capsulatum

Author
Coady, Alison Michele

Publication Date
2014

Peer reviewed|Thesis/dissertation

MyD88-dependent signaling and the host response to the human fungal pathogen *Histoplasma capsulatum*

by

Alison M. Coady

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biomedical Sciences

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

Acknowledgements

The road to a PhD has been a long and challenging journey, and it is one that could not have been completed without the support and help of many people. I am incredibly thankful for the guidance, advice, mentorship and support I have received.

First and foremost, I would like to thank my mentor **Dr. Anita Sil** for the guidance and support I have received in her lab. Anita's enthusiasm for science and dedication to her research and lab is a continued motivation for my own development as a scientist. She encouraged me to think both carefully and independently about my projects, and allowed me to pursue the questions that I was most interested in, even when it involved developing skills and techniques that were far outside of the lab's interests and expertise. I am fully appreciative of the trust she placed in me when she allowed me to start working with mouse models. I am also grateful to Anita for supporting me not only in my scientific endeavors, but also through the emotional rollercoaster that can be graduate school. Her office is a place where I can talk about science as well personal challenges, and I am grateful for the encouragement, care and advice I've received from her.

I would also like to thank my thesis committee. My committee meetings were always a source of inspiration, great ideas and new experiments. **Dr. Joanne Engel** has been supportive of my career from the start, and I have appreciated the conversations we have had about my career choices and future directions. When I asked Joanne to be on my thesis committee she said that she would be, as long as I promised to graduate before she retired. It took me longer than I expected, but I was able to keep my original promise. **Dr. Anthony DeFranco** provided me with a huge amount of scientific advice and support, from giving me the mice that formed the basis of my thesis to patiently answering my naïve immunology questions, and I am thankful that

he has always had the time to discuss science with me. Finally, **Dr. Rich Locksley** provided valuable insight and advice, both during my scientific endeavors and as I've developed my career post-graduate school.

I think that it is a testament to **Anita Sil** that the people that she hires to be in her lab are pretty awesome, and I am thankful that I was able to spend long hours in lab with people who supported my endeavors. Thank you to the entire **Sil Lab** for talking science with me, putting up with my sometimes unwarranted crankiness, and for making the Sil Lab a generally fun place to work. Thank you in particular to **Lena Hwang**, **Charlotte Berkes** and **Diane Inglis** for teaching me how to work with *Histoplasma* and providing scientific advice when I was just starting out as a graduate student and had no idea what I was getting into. Thank you to **Dervla Isaac** for developing the lysis defective mutant screen and allowing me to collaborate with her on this project. **Sarah Gilmore** and **Youngnam Lee** were always available to talk honestly about science decisions and career options, and I appreciated their insight. **Chris Villalta** has a talent for finding cute cat pictures that always brightened my day. **Sinem Behan-Pelvan** is one of the most knowledgeable and determined scientists I know. She has been a mentor to me in how to think scientifically as well as how to handle motherhood as postdoc. **Allen Henderson** is always enthusiastic about science and taught me to view unexpected results as opportunities rather than failures. **Mark Voorhies** is one of the smartest guys I know, and I am glad that he has always had the time to talk statistics with me. I have appreciated being able to share bay space with **Nancy Van Prooyen,** as she is someone who also gets excited about fungal immunology. I also appreciate the experimental help and useful discussions she has shared with me. **Lauren Rodriguez** has made the last year of my graduate school so much more fun, and I'm excited to see what she wrestles out of amoeba. **Davina Hocking-Murray** has been a pillar of support and

advice during my adventures in mouse work. She has always offered her help and support, even when I was a grumpy graduate student. I could not have done all the mouse work that I did without the help of **Johnny Tse**. He has an unwavering dedication to my mouse colony, helped with countless mouse infections, and is always able to do so with a smile. Finally, **Bevin English**, a fellow BMS graduate student in the lab, has an incredible work ethic and enthusiasm for science. In addition, she's been a great friend. I've appreciated our conversations both in the lab and over good beer and I hope we'll continue to hang out even when I'm not in lab.

I would also like to thank the BMS graduate program for their hard work and dedication to making the program such an excellent one. I would particularly like to thank **Lisa Magargal** and **Monique Piazza** for their unwavering support, willingness to help, and ability to quickly solve whatever graduate student problem I could come up with.

My graduate school experience might have been much harder if it hadn't been for a remarkable set of classmates. I was able to stay healthy and sane mostly because **Michelle Toft Graham** is a great motivator. I am grateful for the lofty fitness goals we strived for and the deep conversations we had on the spin bikes. I hope we can do another Muddy Buddy someday. **Linda Lee** has given me so much help and support—both experimental and emotional. I'm looking forward to celebrating us both being finally done with our theses. I was incredibly lucky to be able to live with **Emily Elliott** and **Emily Thornton** for a few years. Thank you for the cats, the New Year's Eve parties, happy cows, Thanksgiving, Cowboy Bebop, and all the other awesomeness. You are the most wonderful roommates and friends a girl could ever hope for. Finally, I am incredibly grateful for the support and love I received from the Book Club Girls**: Lauren Herl Martens, Emily Elliott, Emily Thornton, Renee Vander Laan Greer, Kegan Warner Donlan, Sarah Gierke, Kristen Coakley, Linda Lee, Kate Stewart** and (honorary

classmate) **Kelley Kruze**. Many a bottle of wine was drunk and many a problem of grad school and science solved in the hours I spent with these girls. I treasure the experiences we shared as well as the support we gave each other as women in science and I hope that we'll continue to support each other as our careers progress.

I have been incredibly lucky to have friends from high school and college who I still consider my best friends in the whole world. They have stuck by me through this whole crazy PhD experience by providing me with support and love, hanging out with me even though my ability to communicate with non-scientists was severely perturbed and awkward at times, and have always cheered me on. I couldn't have done it without you guys. **Katie King Schneider** and **Lauren Henderson**--despite how much we've all changed since high school (in good ways), we're always going to be friends. I'm looking forward to seeing where the next 10 years take us. **Annique Bublitz**—I'm so glad we started talking on that ski lift. You taught me how to balance my innate nerdiness with having fun and living life. That came in handy in graduate school. Without **Nena Barnhart,** I wouldn't have come to UCSF. Nor would I have passed organic chemistry. Both things that have directly contributed to my success as a scientist. And finally, **Kelley Kruze**, I am so glad you moved to San Francisco. Thank you for your unwavering support and love and giving. I don't know what I'd do without you.

Without my family, I wouldn't be who I am today. My parents, **Michele and Bill Coady,** ensured that I grew up knowing I could accomplish anything, no matter how great the challenge. Thank you for encouraging learning in every situation and fostering my love of science by enrolling me in summer science classes where I dissected frogs, became a junior zookeeper, and explored space. Having you for parents means that I have always felt loved, supported and cheered for. I am also incredibly lucky to be close to my sibling, **Adam Coady**,

who was my partner-in-crime for many scientific endeavors when we were kids. Our interests have diverged, but we've continued to support each other as we've made our way into adulthood. He's much more hip than me now, but I know he'll always be there for his big sister.

And finally, I have to thank my husband, **Morgan Truitt**. His support and love has been, in large part, what has kept me moving forward and sane during graduate school. Being married to someone who knows exactly what you are going through makes it much easier to handle the challenges and frustrations of graduate school. Thank you for reading all the drafts, thank you for putting up with my meltdowns, thank you for comforting me on bad experiment days and thank you for your unwavering belief in me. I hope that I have and can continue to provide you with as much love and support as you have given me. I look forward to what the next few years bring for us, both inside and outside of science.

Publications and Contributions

The work described in this dissertation was done under the direct supervision and guidance of Dr. Anita Sil.

Chapters 1 and 4 of this dissertation were written by me.

The text of Chapter 2 is a reprint of material as it appears in the journal *Infection and Immunity*:

Isaac DT, Coady A, Van Prooyen N, Sil A. 2013. The 3-hydroxy-methylglutaryl coenzyme A lyase HCL1 is required for macrophage colonization by human fungal pathogen *Histoplasma capsulatum*. *Infect. Immun*. **81**:411–20.

Dervla T. Isaac conceived, designed and implemented experiments, collected and analyzed data and wrote the manuscript. I (Alison Coady) also conceived, designed and implemented experiments, collected and analyzed data, and edited the manuscript. Nancy Van Prooyen designed, implemented and analyzed data for the microscopy experiment. Anita Sil directed and supervised the research, provided financial support, and wrote the manuscript.

The text of Chapter 3 is based on a manuscript that has been submitted to the journal *Infection and Immunity*.

Coady A, Sil A. MyD88-dependent signaling drives host survival and early cytokine production during *Histoplasma* infection. *Infect. Immun.* (in revision).

I (Alison Coady) conceived, designed and implemented experiments, collected and analyzed data, and wrote the manuscript. Anita Sil directed and supervised the research, provided financial support, and edited the manuscript.

Abstract

Histoplasma capsulatum, the causative agent of the disease histoplasmosis, is an intracellular dimorphic fungal pathogen. The organism exists in the environment in a sporulating filamentous form that is easily aerosolized and inhaled by a mammalian host. Inside host lungs, fungal cells convert into a pathogenic yeast form that is able to evade immune defenses by replicating within macrophages. How *Histoplasma* survives and replicates inside a host cell that has evolved to detect and kill invading pathogens is an area of active inquiry. In addition to studying fungal pathogenesis, complementing studies of the host response to this intracellular eukaryotic pathogen allows full understanding of how fungal infection progresses to serious disease, informing the development of treatment options that can both target the pathogen and boost inherent host responses to control fungal growth.

This work describes approaches taken to understand both fungal pathogenesis and host response. First, a high-throughput unbiased screen to identify *Histoplasma* mutants that are unable to lyse macrophages was performed collaboratively, allowing the identification of 26 lysis defective mutants, including a mutant in the *HCL1* gene. The *HCL1* gene encodes an HMG-CoA lyase. Mutants in this gene are unable to grow inside macrophages, exist in an acidified phagosome and display a virulence defect in mice.

Finally, research was undertaken to understand the role of MyD88, a central mediator of the innate immune response, in the response to *Histoplasma* infection. We show that MyD88 signaling is important for host survival, control of fungal growth, cytokine production and appropriate T cell responses, including cytokine production and activation. Using *in vivo* cell-specific MyD88 deficient mice, we determined that MyD88 signaling plays an important role in

the production of cytokines by alveolar macrophages and dendritic cells, potentially influencing the interaction of these cell types with activated T cells.

Table of Contents

List of Figures

Chapter 1: Introduction

Histoplasmosis: Epidemiology and Disease

Histoplasma capsulatum is the etiologic agent of histoplasmosis, a disease first described in Panama by the American physican Samuel Darling. Although found world-wide, it is most commonly observed in the United States and South America (Figure 1.1). In the United States, it is endemic to the Ohio and Mississippi Rivers and is the most common cause of fungal respiratory infections in immunocompetent hosts, with up to 500,000 people being exposed each year (1–3).

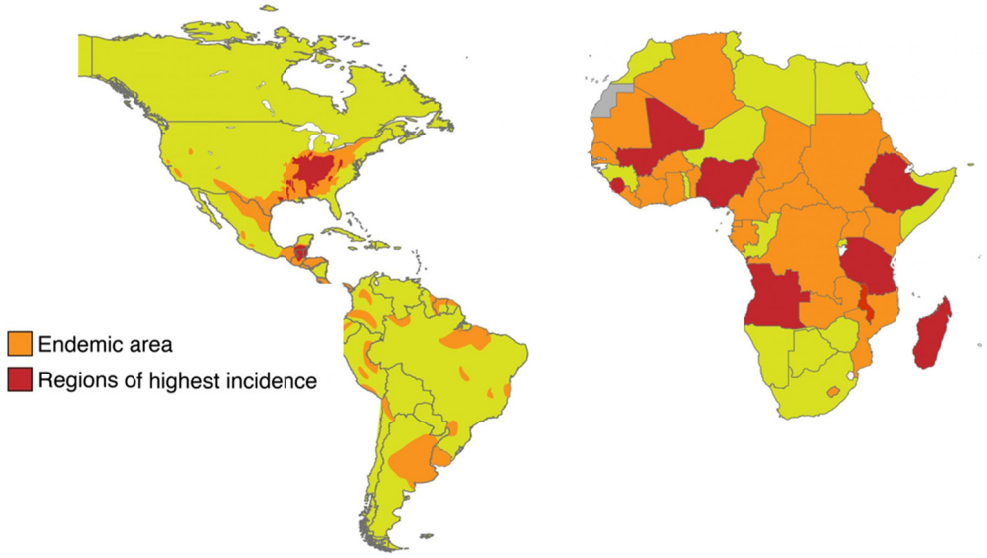


Figure 1.1. *Histoplasma* distribution. Adapted from (150)

A dimorphic fungal pathogen found in the soil, *Histoplasma* grows as mycelia and conidia that are easily aerosolized when disturbed. Once inhaled by the host or at 37^{o}C, *Histoplasma* converts to a budding yeast form that infects and survives inside macrophages, eventually disseminating throughout the body (Figure 1.2). While most people exposed to *Histoplasma* limit infection and develop mild flu-like symptoms, those that are exposed to a large inoculum or who are immunocompromised can develop acute pulmonary histoplasmosis.

In some patients, chronic pulmonary or progressive disseminated histoplasmosis can occur. An adaptive immune response is critical for bringing disease under control, and immunosuppressed individuals, such as HIV/AIDs patients, patients with primary immunodeficiency in the IFNγ/IL-12 axis, and individuals receiving anti-cytokine therapies,

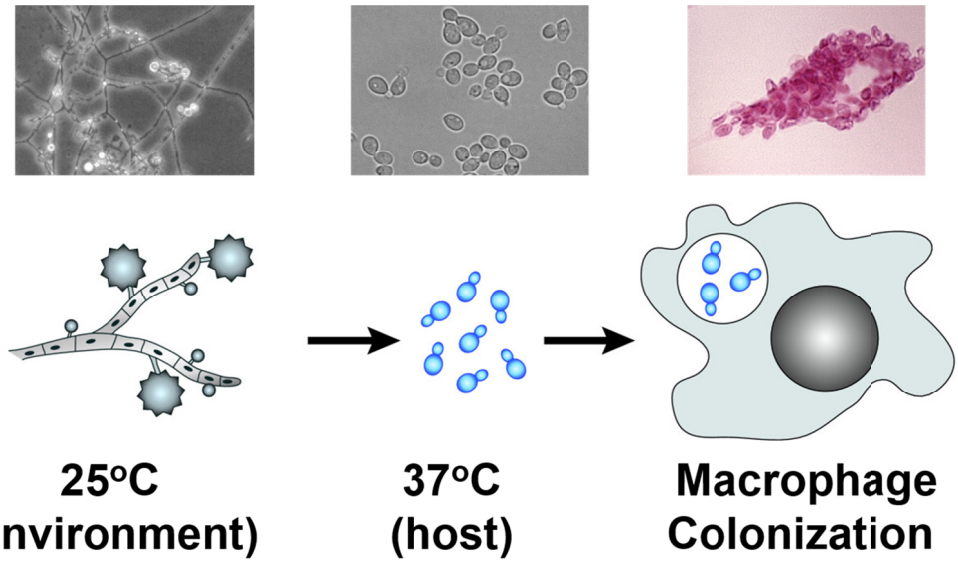


Figure 1.2. *Histoplasma* survives and replicates inside macrophages

frequently fail to contain *Histoplasma* infections and succumb to disseminated disease (4,5).

Histoplasma Pathogenesis

Intracellular pathogens employ a variety of mechanisms to survive inside the host cell, including escape from the phagosome to the cytosol (*Listeria monocytogenes*), prevention/avoidance of phagolysomal fusion (*Mycobacterium tuberculosis*), and modification of the phagolysome itself (*Leishmania*) (6–8). Intracellular survival can provide an environment permissive for pathogen replication, allows for avoidance of host immune mechanisms, and aids

in pathogen dissemination. How *Histoplasma* interacts with macrophage cells to survive and replicate is not fully understood and is an active area of research.

Modulation of the host response to *Histoplasma* may begin as soon as *Histoplasma* interacts with the macrophage. Phagocytosis of *Histoplasma* by macrophages is mediated by the binding of the fungal protein, Hsp60, to the host β2 integrin CD11b/CD18 (also known as CR3 or Mac-1) (9). Binding of CR3, while important for the uptake of multiple pathogens, is also known to negatively modulate protective signaling pathways. By binding the CR3 receptor, it is thought that some intracellular pathogens actively modulate the immune response. For example, CR3 binding diminishes the production of IL-12 and NO during *Leishmania* and *Porphyromonas gingivalis* infection (10, 11). While it is known that *Histoplasma* binds CR3, whether binding of this receptor protects or promotes survival and infection is unknown.

Inside the macrophage, *Histoplasma* remains within the phagosome and displays several strategies for surviving inside this hostile, acidic and nutrient poor environment. While many intracellular pathogens prevent phagosomal acidification by preventing phagolysosomal fusion and/or the recruitment of vacuolar ATPase, whether *Histoplasma* does this is currently unresolved and may be dependent on specific conditions such as host cell type and activation status. Early research observed phagolysomal fusion occurring in infected murine P388D1 cells (12). However, in *Histoplasma*-infected RAW264.7 cells, phagolysomal fusion is inhibited and vacuolar ATPase partially excluded from the *Histoplasma*-containing phagosome (13). Regardless of whether phagolysomal fusion occurs or not, *Histoplasma* is able to neutralize phagosomal pH through an unknown mechanism and the fungus resides in a phagosome that is maintained at a neutral pH of 6.5 (14).

Nutrient acquisition is critical for pathogen growth and the host has evolved a number of ways to restrict metal availability in response to infection (15) . In macrophages, exposure to cytokines such as IFNγ or GM-CSF upregulates anti-microbial processes, including restriction of metals like zinc and iron (16, 17). *Histoplasma* is acutely sensitive to iron availability, as the presence of an iron chelator impedes intracellular growth in the macrophage (18, 19). *Histoplasma* is able to acquire iron from the host using multiple methods, including reduction of iron by secreted iron reductases and the production of sidereophores, high-affinity iron-binding proteins, which allow *Histoplasma* to scavenge protein-bound iron from the host (20–22). Disrupting *SID1*, which encodes the enzyme that catalyzes the first committed step in siderophore production, results in decreased survival within the host (23). Moreover, at pH 6.5 (the pH that *Histoplasma* resides at in the phagosome) host transferrin, an iron-binding protein, is only half-saturated, potentially leaving free iron available for use by *Histoplasma* (18, 22). In addition to iron, zinc levels have recently been shown to be important to fungal replication. Macrophages treated with GM-CSF restrict both iron and zinc, and the addition of a zinc chelator to macrophages reduces fungal growth (16).

The production of reactive oxygen species (ROS) is a well-characterized mechanism the host employs to damage and kill invading pathogens, and many pathogens protect themselves from oxidative stress by producing proteins that neutralize ROS, such as catalases and superoxide dismutase. There is some evidence that *Histoplasma* may actively suppress ROS generation in unprimed macrophages (24, 25). However, *Histoplasma* does induce an oxidative burst in primed macrophages and neutrophils. In order to protect against ROS-mediated killing by primed macrophages and neutrophils, *Histoplasma* utilizes two catalases (CatB and CatP) and a secreted superoxide dismutase (Sod3). Disruption of *SOD3* results in greater sensitivity to

oxidative stress and decreased virulence *in vivo* (26). Disruption of both *CATB* and *CATP* results in increased killing of *Histoplasma* by neutrophils (27).

Finally, several secreted virulence factors have been identified that contribute to *Histoplasma* survival and pathogenesis, Cbp1 and Yps3. Yps3 (Yeast-Phase Specific gene 3) is a protein of unknown function that is highly expressed during yeast phase growth. This protein, which is secreted and found associated with the cell wall, is important for fungal growth within the mouse (28, 29). Cbp1 (Calcium Binding Protein 1) is a homodimeric protein that is also abundantly secreted during yeast form growth. Originally identified during a screen for calcium binding proteins, to date no function has been identified for its calcium binding properties (30–32). It has been predicated to be structurally similar to a mammalian lipid-binding protein called saposin B, suggesting a role for its interaction with the host phagosome (33). Cbp1 is essential for macrophage lysis and virulence in the host (31). Mutants in the *cbp1* gene fail to cause disease within the mouse and are associated with a lower fungal burden in organs. Interestingly, while these mutants can grow inside the macrophage to the same extent as wild-type *Histoplasma*, *cbp1* mutants fail to lyse macrophages, suggesting that *Histoplasma* actively directs macrophage lysis and that macrophage lysis is critical for *Histoplasma* pathogenesis (Dervla Isaac, personal communication).

Host Immune Response to *Histoplasma*

The activation of an adaptive immune response is necessary for successful protection against *Histoplasma* infection. The T cell response, especially the production of IFNγ by CD4+ T cells, is crucial in the resolution of infection (34–36). In order to initiate an adaptive immune response, the innate immune response must recognize and respond to *Histoplasma* early during infection. Phagocytic cells, such as macrophages, dendritic cells and neutrophils, can recognize

Histoplasma, produce inflammatory cytokines, control fungal growth and present antigen to lymphocytes to activate cellular immunity. However, the specific host pathways that mediate recognition and response to *Histoplasma* are not fully understood.

Innate Immune Response

Interaction of *Histoplasma* with Phagocytes

Although all phagocytic cells have a demonstrated robust interaction with *Histoplasma*, macrophages, dendritic cells and neutrophils each respond differently to *Histoplasma* infection, indicating distinct roles for each cell type during the immune response. While dendritic cells are able to rapidly kill and degrade *Histoplasma* after ingestion, macrophages, unless activated by certain cytokines such as IFNγ, TNFα or GM-CSF, cannot control *Histoplasma* growth. Neutrophils can efficiently kill yeast, but only after opsonization (37, 38).

Due to the ability of *Histoplasma* to survive inside macrophages and the availability of well-characterized macrophage models, the response of macrophages to *Histoplasma* has been the most studied. Upon phagocytosis of *Histoplasma* via recognition of the fungal protein Hsp60 by the CR3 receptor (CD11b/CD18), macrophages enact a series of events associated with the inflammatory response and anti-microbial defense (9, 24, 39). Infected macrophages secrete pro-inflammatory cytokines such as TNFα , IL-6, IL-1β and CCL2 (39, 40). Although the response to *Histoplasma* is robust, macrophages, unlike neutrophils and dendritic cells, cannot control *Histoplasma* growth without additional activation by cytokines, such as IFNγ, IL-17 or GM-CSF (16, 41–43). Activation of macrophages results in an increase in nitric oxide (NO) production and increased metal restriction (iron and zinc), which is associated with the anti-*Histoplasma* activity of the macrophage (16, 44, 45).

In contrast to macrophages, dendritic cells are able to degrade *Histoplasma* without additional stimuli. As professional antigen presenting cells, dendritic cells are more efficient at presenting antigen and activating T cells to proliferate and produce cytokine than infected macrophages (38, 46). Although very little is understood about differences between macrophages and dendritic cells that lead dendritic cells to control fungal growth, it is known that phagolysomal fusion does occur in *Histoplasma*-infected human dendritic cells, as compared to human macrophages, where phagolysomal fusion is inhibited by *Histoplasma* (47). In addition, dendritic cells recognize and phagocytose *Histoplasma* via a different receptor than macrophages: dendritic cells utilize the fibronectin receptor VLA-5, which recognizes the fungal surface protein cyclophilin A (38). These data suggest that the method by which *Histoplasma* is recognized and phagocytosed may influence the intracellular fate of the fungus.

The interaction of *Histoplasma* with neutrophils has not been thoroughly studied. Whereas unopsonized *Histoplasma* can be phagocytosed, opsonization is necessary for both induction of the respiratory burst and fungistatic activity (37, 48) . Fungistatic activity has been associated with the activity of azurophil granules containing anti-microbial peptides, probably defensins (49, 50). In fact, when macrophages were engineered to produce neutrophilic defensins, they were able to control fungal growth more readily (51).

Cytokine Response

During *Histoplasma* infection, the production of pro-inflammatory cytokines and chemokines drive both the innate and adaptive immune response. Early production of monocyte and neutrophil recruiting chemokines, such as CCL2 and KC/CXCL1, leads to recruitment of inflammatory cells to the lung, which is in turn critical for the induction of an innate immune response (52). While the role of KC has not been investigated in the context of *Histoplasma*

infection, CCL2$^{-/-}$ mice display a reduced recruitment of monocytes and dendritic cells and a higher fungal burden in the lungs and spleen during infection, although survival of the mice during infection is similar to wild-type mice (53).

Additional pro-inflammatory cytokines, such as IL-1β, TNFα , IL-6, IL-12, and GM-CSF are also produced in the lungs early during infection and contribute to both the recruitment of inflammatory cells and the activation of the adaptive immune response. Depletion of some of these cytokines (IL-1β, TNFα , IL-12, and GM-CSF), either through the use of monoclonal antibodies or knockout mice, results in increased susceptibility to infection and increased fungal burden (54–57). The depletion of these cytokines causes significant defects in TNFα and IFNγ production, suggesting that both of these cytokines are downstream of the initial immune response and ultimately drive control of fungal growth. This is supported by the fact that both TNFα and IFNγ have been shown to directly influence the antifungal state of macrophages (41, 56). Perhaps more significantly, patients given antibodies to TNFα are at a higher risk for severe infection by *Histoplasma*, as are patients with primary immunodeficiencies in the IFNγ/IL-12 axis (58–61). This emphasizes the central role that both TNFα and IFNγ play during control of *Histoplasma* infection. While inflammatory cytokines and chemokines play an important role in the initiation of the innate immune response by recruiting inflammatory cells to the site of infection, activating anti-microbial properties of innate immune cells, and further inducing cytokine production, they also play a key role in driving the adaptive immune response.

Adaptive Immune Response to *Histoplasma*

Cellular immunity, comprised of a largely Th1 response and less critical Th17 response, is crucial for control of a primary *Histoplasma* infection (62–65). Depletion of CD4+ T cells in mice transforms a nonlethal infection into a lethal infection accompanied by decreased levels of

IFNγ production and overwhelming fungal growth in the lungs, spleen and liver (35). Transfer of *Histoplasma*-specific CD4+ T cells can rescue this defect (66). Similarly, the risk of histoplasmosis in humans with HIV increases as CD4+ T cell numbers drop (67). Depletion of CD8+ T cells in mice results in impaired clearance of *Histoplasma* from infected organs, but does not increase mortality (68). The protective response mediated by T cells is largely driven by the production of IFNγ by CD4+ and CD8+ T cells. Mice deficient in IFNγ succumb rapidly to infection due to overwhelming fungal growth in the lungs, spleen and liver (69). Humans with deficiencies in the IFNγ/IL-12 axis display increased risk of disseminated histoplasmosis, highlighting the importance of this cytokine in controlling fungal growth (58, 59). In contrast, B cells are dispensable for protection against *Histoplasma*, as depletion of B cells does not alter fungal burden or survival during infection (70).

The development of an appropriate adaptive immune response is dependent on the proper production of cytokines in conjunction with the presentation of antigens to drive the Th1 activation of T cells. The cytokines IL-12 and IL-18 are important in the initiation and maintenance of a Th1 response (71), however only the role of IL-12 during *Histoplasma* infection has been described (57, 65). Multiple cell types produce IL-12 after interaction with pathogens, including dendritic cells and macrophages. Mice lacking IL-12 (IL-12p35-/-) are more susceptible to *Histoplasma* infection than wild-type mice (62) and treatment of infected mice with IL-12 increases survival through the induction of IFNγ (57). Interestingly, it has been shown that signaling through the CR3 receptor can dampen IL-12 production, suggesting that *Histoplasma*-infected macrophages, which phagocytose *Histoplasma* via the CR3 receptor, may not produce sufficient amounts of IL-12 to initiate a protective host response (72).

Host recognition of *Histoplasma*

The mammalian host possesses multiple recognition pathways that allow it to sense an invading pathogen. The recognition of specific pathogen ligands is initiated by the binding of these ligands to pattern recognition receptors. Toll-like receptors, C-type lectins, scavenger receptors and galectin-3 are signaling molecules that have been shown to recognize specific fungal pathogen-associated molecular patterns (PAMPs) (Figure 1.3) (73). Signaling through these receptors elicits phagocytosis, facilitates the production of inflammatory mediators, induces the production of anti-microbial cellular responses (such as the oxidative burst), and stimulates maturation of DCs to activate naïve T cells and initiate the adaptive immune response. In the case of *Histoplasma*, the signaling molecules that recognize *Histoplasma* and initiate the immune response are still being elucidated.

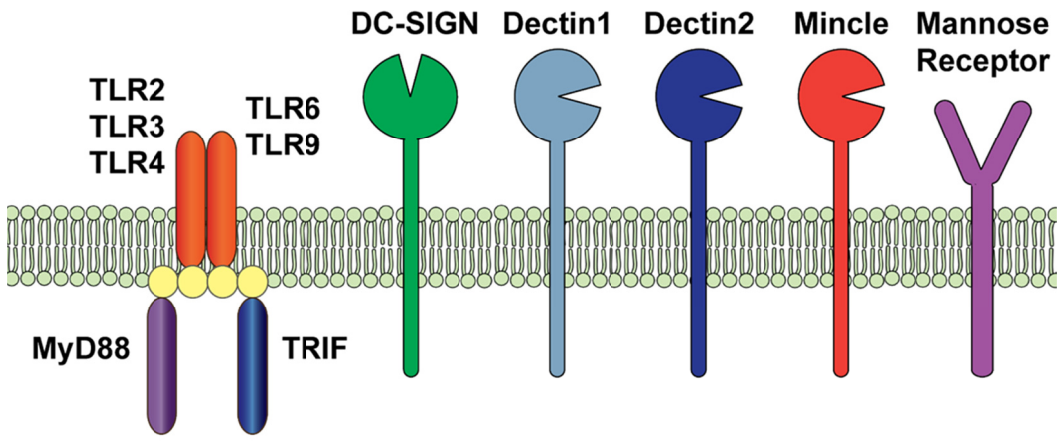


Figure 1.3. Signaling receptors that recognize fungal pathogens.

C-type Lectin Receptors

C-type lectin receptors (CLR) are a class of recently described receptors characterized by the presence of carbohydrate binding domains that recognize and interact with pathogen carbohydrates. CLR signaling is activated by binding to cell wall components such as mannan, glucan and chitin. Several well-characterized C-type lectins known to recognize fungal carbohydrates are Dectin 1, Dectin 2, Mincle, mannose receptor (MR) and DC-SIGN (74). To date, only Dectin 1 and Dectin 2 have been implicated in in the recognition and response to *Histoplasma* (39, 75). These CLRs signal through ITAMs to activate the Src kinases, Syk, and PLCγ, leading to NF-κB and NFAT mediated transcription (76).

Dectin 1 is a primary receptor for fungal β-glucans, a carbohydrate found on most fungal organisms. Multiple studies have shown that Dectin 1 is able to bind and recognize *Histoplasma* (39, 77, 78). In elicited peritoneal macrophages *in vitro*, Dectin 1 signaling contributes to the cytokine response to heat-killed *Histoplasma* (39). More recently, Dectin 2, which binds to α-mannans, has been shown to also recognize *Histoplasma* (75). While the role of Dectin 1 and Dectin 2 in the primary response to *Histoplasma* is not fully understood, both Dectin 1 and Dectin 2 are critical in the development of Th17-mediated vaccine immunity (75) .

Toll-like Receptors

Toll-like receptors (TLRs) recognize a wide variety of highly conserved PAMPs. Transmembrane proteins that are widely expressed in multiple cell types and tissues, TLRs consist of an extracellular domain containing leucine-rich repeats (LRRs) and a cytoplasmic tail containing a TIR domain. Recognition of PAMPs results in TLR dimerization (either with itself or another TLR) and recruitment of TIR domain containing adaptor proteins which mediate downstream signaling, such as NF-κB mediated signaling.

TLRs recognize and mediate signaling in response to a wide variety of fungal species, including but not limited to *C. albicans*, *A. fumigatus* and *C. neoformans* (79, 80). For example, TLR2 recognizes fungal cell wall components such as β-glucans, phospholipo-mannans and glucuronoxylomannan (GXM). TLR4 signaling is activated by O-linked mannans and GXM. The recognition of *Histoplasma* by TLRs is not currently described, although there is evidence that the virulence factor YPS3 engages TLR2-dependent signaling in murine microglia (28).

MyD88

TLR stimulation results in the recruitment of adaptor proteins which transmit the signal to downstream kinases that ultimately lead to activation of transcription factors such as NF-κB and interferon-regulatory factors (IRFs). Most TLRs, with the exception of TLR3, interact with MyD88 to initiate a signaling pathway that results in NF-κB activation. MyD88 not only mediates pathogen recognition through TLRs, it also mediates cytokine signaling through the IL-1 and IL-18 receptors (81, 82). Thus, in many infectious disease models, perturbing MyD88 signaling causes significant defects in early immune events that lead to decreased induction of the adaptive immune response and increased host sensitivity to infection. For example, during infection by fungal pathogens such as *Candida albicans* and *Aspergillus fumigatus*, MyD88 deficiency causes a block in the production of pro-inflammatory cytokines early during infection, decreased neutrophil recruitment, and decreased T-cell activation (83, 84). While IL-1 signaling has been shown to contribute to a protective immune response to *Histoplasma*, the role of MyD88 signaling, which encompasses both TLR signaling and IL-1R/IL-18R signaling has not been studied (54).

Understanding Immunity to *Histoplasma*

In order to develop effective and efficient treatment against *Histoplasma* infection, it is important to study how the organism survives inside the host and how the host responds to infection. Defining molecular pathways that allow *Histoplasma* to survive inside the macrophage identifies potential mechanisms and targets for drug development. Complementing studies that define protective or damaging host responses can potentially allow for the development of treatments that boost or inhibit host responses that are beneficial or harmful, respectively. In addition to the direct contribution of these studies to our understanding of *Histoplasma* pathogenesis, this work would provide a greater general understanding of pathogen invasion and host response. Identifying and exploring the similarities and differences between immunity to *Histoplasma* infection versus other intracellular pathogens allows us to better comprehend a complex and evolving interaction between microbe and host.

Chapter 2: The HMG-CoA lyase HCL1 is required for macrophage colonization

Abstract

H. capsulatum is a fungal respiratory pathogen that survives and replicates within the phagolysosome of macrophages. The molecular factors it utilizes to subvert macrophage antimicrobial defenses are largely unknown. Although the ability of *H. capsulatum* to prevent acidification of the macrophage phagolysosome is thought to be critical for intracellular survival, this hypothesis has not been tested since *H. capsulatum* mutants that experience decreased phagosomal pH have not been identified. In a screen to identify *H. capsulatum* genes required for lysis of bone marrow-derived macrophages (BMDMs), we identified an insertion mutation disrupting the *H. capsulatum* homolog of 3-hydroxy-methylglutaryl CoA (HMG-CoA) lyase (*HCL1*). In addition to its inability to lyse macrophages, the *hcl1* mutant had a severe growth defect in BMDMs, indicating that HMG CoA lyase gene function is critical for macrophage colonization. In other organisms, HMG CoA lyase catalyzes the last step in the leucine catabolism pathway. Additionally, both fungi and humans deficient in HMG CoA lyase accumulate acidic intermediates as a consequence of their inability to catabolize leucine. Consistent with observations in other organisms, the *H. capsulatum hcl1* mutant was unable to grow on leucine as the major carbon source, caused acidification of its growth medium *in vitro*, and resided in an acidified vacuole within macrophages. Mice infected with the *hcl1* mutant took significantly longer to succumb to infection than mice infected with the wild-type strain. Taken together, these data indicate the importance of Hcl1 function in *H. capsulatum* replication in the harsh growth environment of the macrophage phagosome.

Introduction

Macrophages possess an arsenal of antimicrobial defenses that are designed to neutralize and exterminate invading microbes (reviewed in (85)). During phagocytosis by macrophages, microbes are exposed to highly reactive and toxic radicals. As the phagosome matures, it progressively acidifies due to the activity of host vacuolar ATPases that pump protons into the phagosome. Ultimately, the phagosome fuses with the lysosome, allowing maximal acquisition of lysosomal hydrolases in the resultant compartment. Additionally, the phagosome is thought to be a glucose-poor environment, necessitating that phagosomal pathogens utilize alternate nutrient acquisition pathways to survive intracellularly. In this work, we examine the role of a metabolic enzyme, 3-hydroxy-methylglutaryl CoA (HMG-CoA) lyase, in promoting the survival and replication of the fungal pathogen *Histoplasma capsulatum* within the macrophage phagosome.

H. capsulatum is a dimorphic intracellular fungal pathogen that parasitizes macrophages. Endemic to the Ohio and Mississippi Valley regions of the United States, *H. capsulatum* is one of the most common causes of fungal respiratory infection, with up to 500,000 new cases arising each year(86). Infection is initiated when mammalian hosts inhale infectious spores and mycelial fragments that have aerosolized from the soil. Upon entering the alveolar space of the lung, these infectious particles undergo a morphological conversion to budding yeast cells, which parasitize alveolar macrophages. Remarkably, these yeast cells are able to survive and replicate to high levels intracellularly, ultimately lysing host immune cells.

Much of the previous work examining the interactions between *H. capsulatum* yeast cells and the host has characterized fungal survival strategies that promote colonization of its intracellular niche. For example, *H. capsulatum* yeast cells fail to stimulate the production of

toxic reactive oxygen species during phagocytosis by resting murine macrophages (24). The phagolysosome containing *H. capsulatum* does not acidify, and instead remains at a near-neutral pH of 6.5 (13, 14). Presumably, lysosomal hydrolyases are not maximally active at this pH, allowing survival and growth of *H. capsulatum* within the phagolysosome. Additionally, the near-neutral pH of the *H. capsulatum* phagolysosome is thought to promote release of iron from host transferrin, thereby facilitating iron acquisition by the pathogen during its intracellular growth (18, 45).

To identify molecular factors that mediate *H. capsulatum* survival, growth, and pathogenesis, we performed an unbiased genetic screen for *H. capsulatum* insertion mutants that were unable to lyse host macrophages. One of the lysis-defective mutants identified in this screen contained an insertion in an *H. capsulatum* gene that encodes the metabolic enzyme HMG-CoA lyase. This protein has been studied in *Pseudomonas* species, *Aspergillus* species and humans, and is known to catalyze the last step in leucine catabolism. Here we show that the *H. capsulatum* HMG CoA lyase (*HCL1*) is required for full virulence of *H. capsulatum* in host macrophages and in mice.

Materials and Methods

Strains and Culture Conditions. *A. tumefaciens* strain LBA1100 was a kind gift from Thomas Sullivan and Bruce Klein with permission from Paul Hooykas (Leiden University, Leiden, The Netherlands). *H. capsulatum* strain G217B *ura5*Δ (WU15) was a kind gift of William Goldman (University of North Carolina, Chapel Hill). The wild-type, *hcl1* mutant, and complemented strains were generated in this study as described below in the Materials and Methods. Yeast cells were grown in either *Histoplasma* macrophage media (HMM) or minimal media (3M) (87).

Liquid cultures were grown in an orbital shaker at 37°C with 5% CO_2. Stock cultures were maintained by passaging every 2-3 days with a 1:25 dilution. HMM agarose or 3M agarose plates were incubated in a humidified chamber at 37°C with 5% CO_2.

For both macrophage and mouse infections, an overnight, mid-log culture of yeast cells (OD_{600} = 5-7) was prepared. Approximately 18 hours prior to the infection, a two-day late log/stationary phase culture (OD_{600} = 10-12) was diluted 1:5 into HMM media. The diluted cells were then incubated at 37°C with 5% CO_2 overnight to obtain mid-log cultures at the time of infection. Culture ODs were measured using an Eppendorf BioPhotometer.

Genetic screen of *H. capsulatum* lysis defective mutants. Mutant FE6-C3 (the *hcl1* mutant) was initially identified in a genetic screen for mutants that did not lyse macrophages (Isaac et al, manuscript in preparation). Briefly, *H. capsulatum* insertion mutants were generated using *Agrobacterium*-mediated transformation of G217B *ura5*Δ (WU15) as previously described (88). We selected for hygromycin-resistant transformants on HMM plates containing 400 μg/ml uracil, 200 μg/ml hygromycin B and 200 μM cefotaxime. 14,000 insertion mutants were screened for their ability to lyse BMDMs and J774.1 macrophages. Forty-seven mutants were defective for macrophage lysis, including mutant FE6-C3.

Complementation of the *hcl1* mutant. pAC04, an integrating complementation plasmid was generated. This complementation construct contained the *HCL1* ORF, 900 bp of 5' flanking sequence and 886 bp of 3' flanking sequence. pAC04 was transformed into the *hcl1* mutant using ***Agrobacterium*-**mediated gene transfer as previously described (88), generating the *hcl1+HCL1* strain that is designated as the "complemented strain" for the experiments in this

paper. Briefly, the *A. tumefaciens* strain LBA1100 was transformed with pAC04 and induced overnight with 200 μM acetosyringone (AS, Sigma-Aldrich). *H. capsulatum* yeast cells were harvested from 4-day patches on HMM+uracil agarose plates and diluted to 5×10^8 cells/mL. Equal volumes of the *H. capsulatum* and *A. tumefaciens* cultures were mixed, and 400 μL of the mix was spread onto Biodyne A nylon membranes on IM agarose plates containing 200 μM AS and 200 μg/mL uracil. Co-cultivation plates were incubated at 28°C for 3 days. The membranes were then transferred onto HMM plates with no added uracil and incubated at 37°C for 2 to 3 weeks. The *hcl1* mutant was also transformed with pVN47, the parental plasmid for pAC04, which contains the *URA5* gene but does not carry *HCL1*. The resultant strain is designated as the "*hcl1* mutant" for the experiments described in this paper. The G217B *ura5*Δ parental strain, WU15, was also transformed with pVN47, generating a Ura^+ wild-type control, which is the strain designated as "wild-type" for the experiments described in this paper. Several wild-type, mutant, and complemented transformants were analyzed in each experiment, but only one representative strain is shown.

***HCL1* expression analysis.** *Northern Blot*. Total RNA was isolated from logarithmically growing yeast cells using a guanidinium thiocyanate lysis protocol as previously described (89). 4 μg of total RNA from all strains was separated on a 1.5% formaldehyde agarose gel and transferred to a positively charged nylon membrane (Roche 11209299001). The membrane was then subject to Northern Blot analysis according to the Digoxigenin (DIG) Northern Blot protocol (Roche Applied Sciences). DIG-labeled probes were generated from WU15 genomic DNA using the PCR DIG probe synthesis kit (Roche 11636090910). The membrane was then exposed to film and developed.

Quantitative RT-PCR. Total RNA was treated with DNaseI (Promega) and cDNA was synthesized using Stratascript reverse transcriptase (Stratagene) and oligo-dT. Quantitative PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems) and 200 nM of primer on the Mx3000P QPCR system (Stratagene) with Comparative Quantitation program, using actin (*ACT1*) as the normalizing transcript. Primer sequences used are as follows: *HCL1* (*HCL1* sense: 5'-aagacgggattgaccacgattgag; *HCL1* antisense: 5'- atgcgatcgagtgagatgacttgg) and *ACT1* (*ACT1* sense: 5'-gaaggagattaccgctctcg; *ACT1* antisense: 5'-cgacaacaacgaaaaccttaga). All primers spanned an exon-exon boundary.

Cytotoxicity/ LDH Release Assay. Bone marrow derived macrophages (BMDMs) were isolated as described previously (23). In 24-well tissue-culture treated dishes, 2 x 10^5 BMDMs were infected, in duplicate, with *H. capsulatum* strains at a multiplicity of infection (MOI) = 2 yeasts per macrophage. In preparation for the infections, logarithmically growing *H. capsulatum* cultures were pelleted, resuspended in Dulbecco's Modified Eagle Medium (DMEM) without phenol red, sonicated for 3 seconds on setting 2 using a Fisher Scientific Sonic Dismembrator Model 100, and counted by hemacytometer. Colony-forming units were enumerated before and after sonication to confirm that sonication did not affect yeast-cell viability (data not shown). After a 2-hour incubation period, the medium was removed from the infected BMDMs and the monolayers were washed twice with DMEM without phenol red. 750 µl Bone Marrow Macrophage medium (BMM) without phenol red (consisting of DMEM High Glucose without phenol red (UCSF Cell Culture Facility), 20% Fetal Bovine Serum, 10% v/v CMG supernatant (conditioned medium obtained from CMG cells (3T3 fibroblasts transfected with the murine CSF-1 cDNA, kindly provided by Mary Nakamura, UCSF)), 2 mM glutamine, 110 mg/mL

sodium pyruvate, penicillin and streptomycin) was added to each well. The infected macrophages were then incubated at 37^{o}C with 5% CO_2. Approximately 48 hours post-infection, 250 µl fresh BMM was added to each well. At various timepoints post-infection, lactate dehydrogenase (LDH) levels in the infected-macrophage supernatants were measured to monitor BMDM lysis. At each time point, the volume in each well was brought up to 1 ml with BMM. 200 µl of the culture supernatant from the infected macrophages was transferred to two wells of a 96-well plate. To measure the total LDH, two mock-infected BMDM monolayers were lysed at 2 hours post-infection (hpi) with 1ml of lysis solution (1% Triton X-100 in DMEM without phenol red). 200 µl of this mock-infected macrophage lysate was transferred to two wells of the 96-well plate, which was then centrifuged to pellet any cells or debris that might be present in the supernatants or lysate. 20 µl of the clarified culture supernatant was transferred to a fresh 96-well plate containing 30 µl DMEM-phenol red in each well. 60 µl of complete LDH solution was then added to each well. Complete LDH solution is made from equal volumes of the following solutions: (1) 2 mg/ml INT (IodoNitroTetrazolium chloride) in PBS, (2) 36 mg/ml Lithium L-lactate in 10 mM Tris, pH 8.5, and (3) 1x NAD+/diaphorase in PBS containing 1% BSA (diluted from a 10x stock solution (13.5 U/mL diaphorase, 3 mg/mL NAD+, 0.03% BSA, and 1.2% sucrose in PBS). The plate was then incubated for 30 minutes in the dark. 40 µl of 1 M acetic acid was added to stop the reaction. The OD_{490} was then measured using a Molecular Devices Spectramax Plus 384 plate reader. The % BMDM lysis at each time point was calculated as the percentage of the total LDH from lysed uninfected cells at 2 hpi. Since wells of infected macrophages also contain uninfected BMDMs that continue to replicate over the course of the experiment, the total LDH at later timepoints is greater than the total LDH from the 2 hr time point. This increase in total LDH results in an apparent % lysis that is greater than 100% when

normalized to the total LDH from the 2 hr timepoint. We confirmed that *H. capsulatum* G217B cells did not give rise to any LDH activity when subjected to this LDH release assay (data not shown).

Intracellular Replication Assay. In 24-well tissue culture treated dishes, 2 x 10^5 BMDMs were infected, in duplicate, with *H. capsulatum* strains at an MOI of 2. After a 2-hour incubation period, the culture supernatants were removed, the monolayers were washed twice with DMEM and then 1 ml BMM was added to each well. The infected macrophages were then incubated at 37^{o}C with 5% CO_2. The media was changed at 48 hours post-infection and every day thereafter. At various timepoints post-infection, the media was removed from each well and 500 µl ddH_2O was added. After a 5-minute incubation at room temperature, the infected macrophages were mechanically lysed by vigorous pipetting. The lysate was collected, sonicated, diluted in HMM and plated for *H. capsulatum* colony-forming units (CFUs) on HMM-agarose plates at 37^{o}C. CFUs were counted 14 days later.

Microscopic analysis of intracellular growth. 2 x 10^5 BMDMs per well were seeded in 24-well tissue-culture treated dishes containing 12 mm glass coverslips. Approximately 18 hours later, these BMDMs were infected with yeast cells at an MOI=0.1, centrifuged for 5 minutes at 800 x g, and incubated for 2 hours. The media was then removed from the infected BMDMs, the monolayers were washed twice with DMEM and 500 µl BMM was added to each well. The infected macrophages were then incubated at 37^{o}C with 5% CO_2. The media was changed at 48 hpi and every day thereafter. At various timepoints post-infection the media was removed from each well and the monolayers were fixed with 1 ml 3.7% formaldehyde in 100% ethanol for 1

minute. The fixative was then removed and cells were washed twice with PBS before being stored in 1 ml PBS at 4^{o}C. Coverslips were then stained with Periodic acid-Schiff reagent (to highlight fungal cells) and methyl green (to counterstain macrophage nuclei) as previously described (90). Microscopic images were taken with the Leica DM 1000 microscope.

Bioinformatics Analysis. The protein sequence of the predicted gene that was disrupted in mutant FE6-C3 was searched against the non-redundant (nr) protein database with the BLASTP program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (91). Upon identification as an HMG-CoA lyase homolog, the corresponding *H. capsulatum* protein, designated Hcl1, was aligned using ClustalW2 (http://www.clustal.org/clustal2/) (92). with its homologs in *Aspergillus fumigatus, Pseudomonas aeruginosa, Pseudomonas mevalonii, Bacillus subtilis, Brucella melitensis ,* and *Homo sapiens*.

Analysis of Growth in Leucine. To monitor growth in the presence of leucine on solid media, stationary-phase yeast cells from a four-day culture were washed and resuspended in PBS. Two µls of 10-fold serial dilutions, in PBS, were plated on agarose plates containing either HMM, 3M (with glucose), or 3M without glucose supplemented with 10mM leucine. Plates were then incubated at 37^{o}C, 5% CO_2 for 16 days. Images were taken with a Nikon digital camera. To monitor growth in liquid culture, yeast cells were washed, pelleted, and resuspended to an OD_{600} = 0.01 – 0.05 in either HMM, 3M (with glucose), 3M-glu, or 3M-glucose+leucine. 500 µl samples were removed from each culture, vortexed for 30 seconds to break up clumps, and analyzed to determine their OD_{600} using the Molecular Devices Spectramax Plus 384 plate reader. Of note, as cultures go from early logarithmic growth to stationary phase, an increase in

OD_{600} is observed with both the Spectramax plate reader and the Eppendorf Biophotometer described above (Strain and Culture Conditions section). However, the absolute OD_{600} numbers differ for the two instruments, with stationary phase registering as OD 12 on the Biophotometer and approximately OD 2-3 on the plate reader.

***In vitro* pH modulation**. To monitor the ability of the *H. capsulatum* strains to modulate the pH of culture broth, stationary-phase yeast cells from a four-day culture were washed and resuspended in PBS. Cells were diluted to OD_{600} = 2 in 30 ml pH-HMM (HMM without HEPES, containing 40 μg/ml bromocresol purple, pH 4.5). Cultures were incubated at 37°C with 5% CO2 in an orbital shaker. At each timepoint, three 1-ml samples were removed from each culture and the pH was measured using a digital pH meter. The optical density of each culture was measured using the Molecular Devices Spectramax Plus 384 plate reader.

Monitoring of phagosomal pH. Acidification of the phagosome was assessed by use of the acidotropic dye LysoSensor Green DND-189 (Invitrogen; pk_a=5.2). In a 12-well plate, $2x10^5$ BMDMs per well were infected in duplicate with mid-logarhythmic phase *H. capsulatum* strains (wild-type, the *hcl1* mutant, the complemented strain, or fixed wild-type cells (the wild-type strain fixed with 4% paraformaldehyde)) at an MOI of 2 in the presence or absence of 50 nM bafilomycin A1 (Sigma), an inhibitor of the vacuolar ATPase. Cells were slowly centrifuged at 1,200 rpm for 5 min at room temperature. At 1hpi, extracellular yeast cells were removed by washing. Thirty min prior to each timepoint (3, 6, 12, and 24 hpi), infected BMDMs were loaded with LysoSensor (1 μM) at 37°C. After this 30 min incubation, macrophages were washed with PBS and fixed in 4% paraformaldyde for 30 min. Fixed macrophages were blocked

with 2% FBS and 0.2% saponin in PBS for 1 hour at room temperature, then stained at 37°C for 1 hour with rabbit anti-Hc (kind gift from Joe Wheat), which detects *H. capsulatum* yeast cells, and rat anti-LAMP-1 (Invitrogen, 1D4B). Cells were then washed and stained with rabbit Alexa 405 and rat Alexa 546 secondary antibodies for 1 hour at 37°C. Cells were mounted in Gel-Mount (Biomedia) and imaged using a Nikon TiE inverted microscope with Yokogawa spinning disk CSU-X1. Images were taken using Nikon Perfect Focus and unbiased gridding of 50 points per samples of 0.11 μm/pixel. Images were brightened in Adobe Photoshop by applying the identical adjustments to all images.

Mouse infections. Nine week-old female C57Bl/6 mice (Charles River Laboratories) were anesthetized with isoflurane and infected intranasally with wild-type *H. capsulatum*, the *hcl1* mutant, or the complemented stain. In preparation for infection, mid-logarithmic cultures of these *H. capsulatum* strains were washed once with PBS, sonicated for 3 seconds on setting 2 using a Fisher Scientific Sonic Dismembrator Model 100, and counted by hemacytometer to determine cell concentration. Colony-forming units before and after sonication were enumerated to determine that sonication did not affect yeast-cell viability (data not shown). To monitor mouse survival, 15 mice were infected intranasally with 1.25×10^6 yeast cells in approximately 25 μl PBS. At 4 hours post-infection, the lungs were harvested and homogenized from 5 mice infected with each strain. These homogenates were plated for *H .capsulatum* CFUs on Brain-Heart Infusion agar (BHI) plates and incubated at 30°C. The remaining mice were monitored daily for symptoms of disease (i.e. weight loss, lack of activity/response to stimulus, panting, lack of grooming). Mice were sacrificed after they exhibited 3 days of sustained weight loss greater than 15% of their maximum weight in conjunction with one other symptom of disease.

To determine the fungal burden of infected mice when they succumbed to the infection, lungs and spleens were harvested from 4 mice infected with each *H. capsulatum* strain. Additionally, lungs and spleens were harvested from three mice infected with the *hcl1* mutant strain at 7 dpi to determine fungal burden. Lung homogenate was plated for CFUs on BHI plates at 30^{o}C. Statistical analysis for survival and colonization experiments was performed using Prism (GraphPad Software, San Diego, CA). Survival curves were compared using the log rank (Mantel-Cox) test. *In vivo* fungal burden of different *H. capsulatum* strains was compared using the Mann-Whitney-Wilcoxon test. Two tailed p-values were calculated.

All mouse experiments were performed in compliance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the University of California San Francisco.

Results

The HMG-CoA lyase *HCL1* was identified in a genetic screen for lysis-defective mutants.

Wild-type *H. capsulatum* is able to replicate within the macrophage phagosome, ultimately triggering host-cell lysis. We performed a genetic screen to identify *H. capsulatum* genes that are important for intracellular pathogenesis. A library of 14,000 *H. capsulatum* insertion mutants generated by *Agrobacterium*-mediated transformation (93) was screened to identify those mutants with defects in lysis of primary murine bone-marrow derived macrophages (BMDMs) and the macrophage-like cell line, J774. One of the mutants, FE6-C3, was unable to lyse both BMDMs and J774 cells, and was subjected to further study.

To determine which *H. capsulatum* gene was disrupted in the FE6-C3 insertion mutant, we isolated genomic DNA from the mutant strain and performed inverse PCR from the left and right borders of the *Agrobacterium* T-DNA insertion. Sequence analysis of the resulting PCR products indicated that the *H. capsulatum* gene 'HISTO_DA.Contig93.Fgenesh_histo.97.final_new' contained an insertion in the open reading frame, approximately 300 bp downstream from the predicted translational start site (Figure 2.1 A). A BLAST search of the corresponding protein sequence against the nr protein database revealed strong similarity to the HMG-CoA lyase enzyme from multiple organisms (Figure 2.2). We named the corresponding gene *HCL1* (HMG-CoA Lyase 1), and hereafter refer to mutant FE6-C3 as the *hcl1* mutant. Quantitative RT-PCR and Northern blot analysis of fungal cells grown *in vitro* confirmed the loss of *HCL1* expression in the *hcl1* mutant and the restoration of *HCL1* expression when the wild-type gene was re-introduced into the insertion mutant (Figure 2.1 B, C).

HMG CoA lyase enzymes have been studied in several organisms, including humans. Biochemical characterization of the human HMG CoA lyase, HMGCL, has identified several active site residues that are critical for enzymatic activity, including Arg-41, Asp-42, Glu-72, His-233, and Cys-266 (94–97). Using ClustalW, we aligned the *H. capsulatum* Hcl1 sequence with the uncharacterized HMG-CoA lyase for the fungus *Aspergillus fumigatus* (which shares 80% amino acid sequence identity with *H. capsulatum* Hcl1), as well as the functionally characterized enzyme from *Pseudomonas aeruginosa* (47%), *Pseudomonas mevalonii* (46%), *Bacillus subtilis* (36%), *Brucella melitensis* (42%), and *Homo sapiens* (44%) (numbers in parentheses indicate shared sequence identity with *H. capsulatum* Hcl1). We found that these catalytic active site residues were conserved in all examined HMG CoA lyases, including *H. capsulatum* Hcl1 (Figure 2.2).

The *hcl1* mutant has severe lysis and intracellular growth defects in macrophages.

To quantify the severity of the lysis defect of the *hcl1* insertion mutant, we infected BMDMs with wild-type *H. capsulatum* (WT), the *hcl1* insertion mutant (*hcl1*), or the complemented strain (*hcl1 +HCL1*), where the wild-type *HCL1* gene was re-introduced into the mutant strain. (The precise genotypes of these strains are described in the Materials and Methods.) We then monitored the kinetics of host-cell lysis by measuring release of the cytosolic enzyme lactate dehydrogenase (LDH) from host macrophages into the supernatant at the indicated timepoints after infection (Figure 2.3 A). Whereas a considerable amount of LDH was present in the culture supernatant of BMDMs infected with wild-type *H. capsulatum* and the complemented strain, no LDH release was observed in supernatants from macrophages infected with the *hcl1* mutant.

To further explore the role that *HCL1* plays in *H. capsulatum* pathogenesis, we examined the intracellular growth of the *hcl1* mutant during macrophage infection. BMDMs were infected with wild-type *H. capsulatum*, the *hcl1* mutant, or the complemented strain. At various timepoints post-infection, intracellular yeasts were released from BMDMs by osmotic lysis and colony-forming units (CFUs) were enumerated. Wild-type *H. capsulatum* and the complemented strain showed a 16-fold increase in the intracellular CFUs within 48 hours of infection, after which host-cell lysis ensued. In contrast, the *hcl1* mutant only grew a modest 4-5 fold over the entire 5-day infection, which was indicative of a strong intracellular growth defect (Figure 2.3 B). Microscopic examination of infected macrophages corroborated the growth defect observed by CFU enumeration. In these experiments, BMDMs were infected at a very low MOI to maximize the period of intracellular growth before wild-type cells lysed the macrophages. Macrophage monolayers were stained with Periodic Acid Schiff (PAS) reagent at 2 hrs and 5

days post-infection to reveal intracellular *H. capsulatum* yeasts (Figure 2.3 C). At 5 days post-infection, macrophages infected with the *hcl1* mutant contained significantly fewer yeast cells than macrophages infected with wild-type *H. capsulatum*. Notably, the growth kinetics of the *hcl1* mutant were indistinguishable from that of wild-type *H. capsulatum* when it was cultured *in vitro* in standard medium (Figure 2.3 D).

***HCL1* is required for growth when leucine is the major carbon source.**

HMG-CoA lyases are metalloenzymes that catalyze the final step in the breakdown of leucine, cleaving HMG-CoA into acetyl CoA and acetoacetate. Since fungi can then assimilate acetyl CoA into carbohydrates through the glyoxylate cycle, leucine can be utilized as the sole carbon source for growth. Mutants that lack HMG-CoA lyase in several bacterial and fungal species, including *Pseudomonas aeruginosa* and *Aspergillus nidulans*, are unable to grow when leucine is the sole carbon source (98, 99). We tested the ability of the *hcl1* mutant to utilize leucine by replacing glucose with leucine in minimal media (Figure 2.4). The growth of the *hcl1* mutant was comparable to that of wild-type *H. capsulatum* and the complemented strain when it was grown on rich media (HMM) and on minimal media (3M) where glucose is the main carbon source (Figure 2.4 A, B). In minimal medium that lacks glucose (3M – glu), cells of all three genotypes showed limited growth (Figure 2.4 C). However, only the *hcl1* mutant showed severe growth restriction when leucine was substituted for glucose in minimal medium (Figure 2.4 A, D). Taken together with the macrophage infection data, these *in vitro* data suggest that *HCL1* could contribute to the ability of *H. capsulatum* to replicate in the glucose-poor nutritional environment of the phagosome.

Hcl1 deficiency results in acidification of *H. capsulatum* culture medium and the *H. capsulatum*-containing phagosome. In humans, HMG CoA lyase deficiency results in the

accumulation of upstream acidic intermediates in the leucine catabolic pathway, causing concomitant metabolic acidosis and aciduria (100). Similar effects are observed in fungi, where acidic intermediates accumulate in culture supernatants of cells that lack HMG CoA lyase (101). We were particularly interested in this phenotype, since inhibition of phagosome acidification is thought to be vital for the survival of *H. capsulatum* within macrophages. Unlike many microbes, wild-type *H. capsulatum* is able to neutralize the pH of culture medium (102). We predicted that the *hcl1* mutant would be able to neutralize acidic medium *in vitro*, but might be unable to maintain neutral pH due to the accumulation of acidic species. To test this hypothesis, we transferred the wild-type, *hcl1* mutant, and complemented strains into unbuffered, acidic medium (pH 4.5). We then allowed the cells to grow and monitored the pH over time (Figure 2.5). Within 24 hours, all three strains had neutralized the culture medium to pH = 7, indicating that Hcl1 is not required for the establishment of neutral pH. Wild-type and complemented strains maintained a near-neutral pH (pH = 6.2 to 6.5) for the duration of the experiment. However, the pH of the culture medium of the *hcl1* mutant dropped to 5.6 after 120 hours. The three strains displayed identical growth kinetics during this experiment as determined by monitoring optical density (data not shown). These data indicate that Hcl1 is required for maintenance of a neutral culture environment by *H. capsulatum*.

Wild-type *H. capsulatum* is known to inhibit phagolysosome acidification during infection of macrophages (13, 14). Acidification of the phagolysosome is thought to be important to activate lysosomal hydrolases, which have potent degradative and anti-microbial activities. Since the *hcl1* mutant acidifies its surroundings, we hypothesized that the macrophage phagolysosome containing the mutant cells might also acidify with time. To monitor acidification of the phagolysosome during infection, we used the acidotropic dye Lysosensor Green DND-189 which

has a pK_a of 5.2 and accumulates within acidic compartments. We first determined that mid-logarithmic cells from the wild-type, *hcl1* mutant, and complemented strains did not stain with Lysosensor Green (data not shown). We then infected BMDMs with fixed wild-type cells, or live, mid-logarithmic wild-type, *hcl1* mutant, or complemented cells, and assessed acidification of the phagosome at 3, 6, 12, and 24 hours post-infection (hpi). At each timepoint, macrophages were pre-treated for 30 minutes with Lysosensor Green, and then washed, fixed, permeabilized, and subjected to confocal immunofluorescence microscopy to detect internalized *H. capsulatum*, the lysosomal protein LAMP-1, and Lysosensor Green accumulation (Figure 2.6 A). By 3 hpi, we observed that virtually all internalized *H. capsulatum* cells (in all samples) localized to a LAMP1-positive compartment. In the case of fixed wild-type cells, 75% co-localized with Lysosensor Green by 3 hpi (Figure 2.6 B), indicating that they were present in an acidic compartment. By 6 hpi, nearly 100% of fixed wild-type cells co-localized with Lysosensor Green, indicating that, as expected, fixed *H. capsulatum* cells could not counter the ability of macrophages to acidify the phagolysosome. (At subsequent timepoints, fixed wild-type cells were degraded by macrophages and could no longer be assessed.) In contrast to fixed wild-type *H. capsulatum* cells, only 25% of live wild-type *H. capsulatum* cells co-localized with Lysosensor Green at 3 and 6 hpi. By 12 and 24 hpi, this number dropped to approximately 10%. Notably, 60% of the *hcl1* mutant cells, but not the complemented cells, co-localized with Lysosensor Green by 3 hpi, and upwards of 85-90% of the mutant cells co-localized with Lysosensor Green in subsequent timepoints. Lysosensor Green appeared to accumulate within and around the *hcl1* mutant cells. These data indicate that the *hcl1* mutant cells were present in an acidified environment within the macrophage whereas wild-type cells were not. Since the *hcl1* mutant can accumulate acidic species in culture (Figure 2.5), we suspected that acidification of

phagosomes containing the *hcl1* mutant was driven by the mutant itself rather than by the host vacuolar ATPase, which promotes phagosome acidification. Treatment of macrophages with bafilomycin A1, an inhibitor of the host vacuolar ATPase, abrogated acidification of phagosomes containing fixed *H. capsulatum* cells, but only had a mild effect on the percentage of *hcl1* mutant cells that co-localized with Lysosensor Green (Figure 2.6 B). Similarly, the *ura5* mutant parental strain lacking a complementing *URA5* gene did not grow in macrophages (data not shown) and accumulated in acidic phagosomes over time, but, unlike phagosomes containing the *hcl1* mutant, phagosome acidification was inhibited by treatment with bafilomycin (Figure 2.6 C). These data indicate that acidification of the vacuole containing *hcl1* mutant cells was not dependent on the host vacuolar ATPase, and strongly suggest that the *hcl1* mutant itself was promoting vacuolar acidification.

***HCL1* is required for maximal virulence in the mouse model of histoplasmosis.**

To determine if *HCL1* is required for virulence of *H. capsulatum* in the mouse model of histoplasmosis, we infected female C57Bl/6 mice intranasally with a lethal dose (1.25×10^6 yeast cells/mouse) of wild-type *H. capsulatum*, the *hcl1* mutant, or the complemented strain (Figure 2.7 A). Mice were monitored daily for symptoms of disease, including weight loss, panting, and lack of grooming. Mice infected with the wild-type or complemented strains became symptomatic by day 3, and the majority succumbed to infection between days 7 and 8. In contrast, mice infected with the *hcl1* mutant strain started to show mild symptoms at day 7, and the majority of the mice succumbed to infection by day 10-11. The overall median survival times for each group were 7.5 days (wild-type), 7 days (complemented strain) and 10 days (*hcl1* mutant), yielding a statistically significant difference in virulence for the *hcl1* mutant compared to wild-type ($p < 0.0001$). CFUs were enumerated from the lungs (Figure 2.7 B) and spleens

(Figure 2.7 C) of mice infected with the wild-type, *hcl1* mutant, and complemented strains at day 7. Surprisingly, even though the *hcl1*-infected mice were only mildly symptomatic, their pulmonary fungal burden was not significantly different from mice infected with the wild-type strain at day 7 ($p = 0.4$). At day 10, when mice infected with the *hcl1* mutant were succumbing to infection, the fungal burden was not significantly different from the fungal burden in wild-type mice at time of death (day 7, $p = 0.5$), or *hcl1*-infected mice at day 7 ($p = 0.4$). These data confirmed that the *hcl1* mutant was able to grow *in vivo* despite its severe restriction within bone marrow-derived macrophages *in vitro*, and suggested that some other aspect of infection, such as host response, might contribute to the decreased virulence of the *hcl1* mutant. In sum, these data highlight that Hcl1 function is required for full virulence in the mouse model of *H. capsulatum* infection.

Discussion

H. capsulatum is an intracellular fungal pathogen that colonizes the macrophage phagolysosome, ultimately triggering host-cell lysis. Here we used a forward genetic approach to identify an enzyme, HMG CoA lyase, which is required for growth within macrophages and host-cell lysis. Interestingly, due to the metabolic defects that ensue in response to HMG CoA lyase deficiency, it is indirectly required for maintenance of phagolysosomal pH. Studies in other organisms have shown that HMG CoA lyase catalyzes the final step in leucine catabolism; thus HMG CoA lyase deficiency results in the inability of microbes to grow when leucine is the sole carbon source. Additionally, both microbes and humans with HMG CoA lyase deficiency accumulate acidic species that arise due to the block in leucine metabolism. In the case of *H. capsulatum*, we observed that Hcl1 is dispensable for growth in standard laboratory medium

supplemented with glucose, but required for growth on minimal medium when leucine is substituted for glucose as the major carbon source. Additionally, growth of *H. capsulatum* in unbuffered medium revealed that Hcl1 is required to prevent the accumulation of acidic species in the culture supernatant. During colonization of the phagolysosome of macrophages, the *hcl1* mutant is present in an acidified compartment, suggesting that these acidic species can lower the pH of the phagolysosome, which, in turn, could potentially activate lysosomal hydrolases and restrict microbial growth.

These studies highlight the challenges faced by intracellular microbes that grow within the phagosome of host cells, reviewed in (85). The initiation of phagocytosis of microbes by macrophages is normally accompanied by a superoxide burst catalyzed by the NADPH complex. Subsequently, the normal course of phagosome maturation involves transient interactions between the phagosome and a variety of intracellular organelles, ultimately culminating in fusion with lysosomes. As this maturation process occurs, phagosomal pH drops and the phagosome acquires lysosomal hydrolases that are active at acidic pH. *H. capsulatum* counters normal phagosome function and maturation in at least two key ways. First, *H. capsulatum* fails to trigger a superoxide burst in resting macrophages (24), and produces an extracellular superoxide dismutase for protection against the reactive oxygen species generated upon phagocytosis by activated macrophages and polymorphonuclear leukocytes (103). Second, although the *H. capsulatum* phagosome undergoes fusion with the lysosome (in murine macrophages), the fungus uses unknown means to block acidification of phagolysosomes (13, 14). It has been assumed that the ability of *H. capsulatum* to inhibit phagolysosome acidification is critical for survival of the fungus within host cells. Our data demonstrate that the *hcl1* mutant is competent to neutralize acidic pH *in vitro*, but cannot maintain neutral pH as it grows, presumably due to

the production of acidic species. Correspondingly, in the macrophage we observed that the *hcl1* mutant, unlike wild-type cells, is present in an acidified phagosome, suggesting that the phagosome containing the mutant cells may be more hydrolytically competent than the phagosome containing wild-type *H. capsulatum*. Consistent with this model, the *hcl1* mutant is clearly restricted for growth within macrophages. We hoped to determine whether neutralization of the phagosome with the weak base chloroquine (18) could rescue the intracellular growth defect of the *hcl1* mutant; if so, these results would strongly suggest that the intracellular growth deficiency of the mutant Is due to acidification of the phagosome. However, these experiments were not possible in bone marrow-derived macrophages for technical reasons (data not shown).

The inability of the *hcl1* mutant to grow within macrophages may also reflect the nutritional environment of the phagosome. Studies of other phagosomal pathogens such as *Leishmania major* and *Mycobacterium tuberculosis* have shown that enzymes required for gluconeogenesis or the glyoxylate shunt, respectively, are required for colonization or persistence in macrophages and mice, suggesting that the phagosome is a glucose-poor environment (104, 105). Similarly, the fungal pathogen *Candida albicans* upregulates its expression of isocitrate lyase, a key glyoxylate cycle enzyme, after phagocytosis by host cells, and requires this enzyme for full virulence in the mouse model of pathogenesis (106). HMG CoA lyase in other organisms is required for growth when leucine is the sole carbon source, since HMG CoA lyase is a key enzyme in leucine catabolism. A similar result was observed for *H. capsulatum*, as the *hcl1* mutant could not grow *in vitro* when leucine was substituted for glucose. The levels of leucine or other amino acids in the *Histoplasma*-containing phagolysosome is unknown, though the inability of the *hcl1* mutant to grow within macrophages leads us to speculate that the phagolysosome is a glucose-poor, leucine-replete environment. Interestingly,

leucine catabolism generates acetyl CoA, which can be used as a carbon source when assimilated through the glyoxylate cycle (bypassing the catabolic steps of the standard tricarboxylic acid cycle). Thus the requirement for Hcl1 in macrophage colonization and virulence may correlate with its link to carbohydrate metabolism.

Whether Hcl1 affects other metabolic processes in the *H. capsulatum* cell is unknown. In vertebrates, HMG CoA lyase plays additional roles in energy transfer within and between cells. For example, HMG CoA lyase is required to link fatty acid oxidation in the mitochondria to ketogenesis, which is a critical mode of energy transport in higher eukaryotes (107). Fatty acid oxidation produces acetoacetyl CoA, which is then converted into HMG CoA by mitochondrial HMG CoA synthase. HMG CoA lyase then converts HMG CoA into acetoacetate, which is a "ketone body" that is produced in the liver and used to transport energy to other organs when glucose is not available. However, lower eukaryotes lack mitochondrial HMG synthase, and thus there is no known direct link between fatty acid oxidation and HMG CoA lyase-dependent metabolism in fungi.

Despite the profound growth defect of the *hcl1* mutant in bone-marrow-derived macrophages and the J774 macrophage-like cell line, the mutant was able to replicate in the mouse model of pathogenesis. However, mice infected with the *hcl1* mutant took significantly longer to succumb to infection than wild-type mice, indicating that the mutant is partially attenuated for virulence. Interestingly, the fungal burden in the lungs of mice infected with the wild-type strain at time of death (day 7, Figure 2.7 B) was indistinguishable from the fungal burden in the lungs of mice infected with the *hcl1* mutant at the same day post-infection (Figure 2.7 B), but mice in the *hcl1*-infected cohort did not succumb to infection for another 3 days (Figure 2.7 A). Additionally, there was no significant increase in CFUs between day 7 and day

10 for mice infected with the *hcl1* mutant (Figure 2.7 B). Finally, fungal burden in the spleens of mice infected with either wild-type *H. capsulatum* or the *hcl1* mutant at day 8 was not significantly different ($p = 0.4$, Figure 2.7 C), indicating that the mutant does not have a gross dissemination defect. These data evoke the hypothesis that an aspect of infection other than fungal burden, such as the host inflammatory response, could differ in mice infected with the wild-type vs. mutant strains. Alternatively, it could be the case that *in vivo* growth kinetics of the wild-type and mutant strains differ at earlier time points in infection not examined in this study, which could affect subsequent symptomatology and disease progression. Additionally, these data suggest that despite its profound growth defect in BMDMs, the *hcl1* mutant replicates well within an unknown cell type *in vivo*, such as alveolar macrophages and/or inflammatory monocytes. Future studies of the role in Hcl1 in fungal pathogenesis will continue to utilize the mutant as an informative tool to dissect the nutritional and metabolic requirements of life in the *Histoplasma* phagosome, as well as the relationship between intracellular growth and immune response during infection of the host.

Acknowledgements

We thank L. Joseph Wheat and Patti Connolly for the gracious gift of the anti-*Histoplasma* antibody. We are grateful to Daniel Portnoy, Denise Monack, Jeffery Cox, Alexander Johnson, and members of the Sil laboratory for useful discussion as this work progressed. We are indebted to Davina Hocking Murray for assistance with figure preparation. We thank Davina Hocking Murray and Johnny Tse for technical assistance. We thank Sinem Beyhan, Sarah Gilmore, and Mark Voorhies for comments on the manuscript.

This work was supported by Microbial Pathogenesis and Host Defense Training Grant (NIH T32 A1060537) support to DTI, a UCSF NIGMS fellowship to DTI (1R25GM56847), NSF Graduate Research Fellowship to AC, an A.P. Giannini Medical Foundation Fellowship to NVP, NIH (R01AI066224, PO1AI063302, RO1AI093640) and an HHMI Early Career Scientist Award (http://www.hhmi.org/research/ecs/) to AS.

Figures

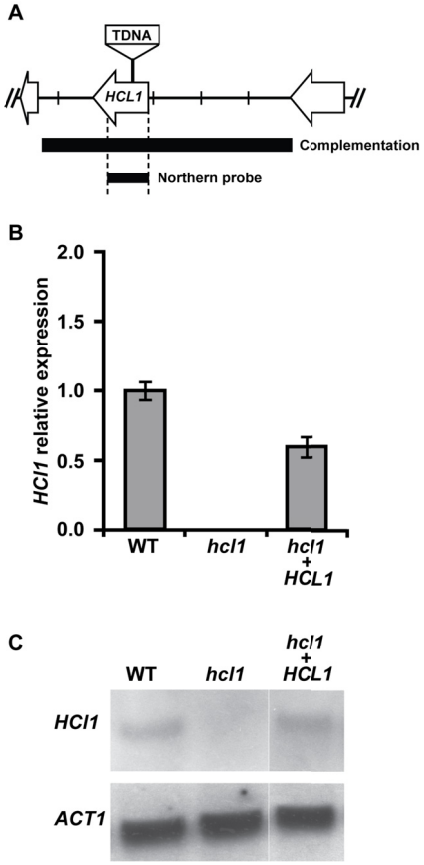


Figure 2.1. The FE6-C3 mutant contains an insertion in the HCL1 open reading frame. (A) Schematic of the *HCL1* genomic locus showing the site of the TDNA insertion in the *hcl1* mutant, the region of the *HCL1* locus that was used to generate the complementation construct, and the region of the *HCL1* gene that was used to probe transcript levels. Tick marks are 1 kilobase apart. (B & C) Total RNA was isolated from wild-type (WT), the *hcl1* mutant, and the complemented strain (*hcl1+HCL1*) grown in broth culture at 37^{o}C. *HCL1* mRNA levels were monitored by (B) quantitative RT-PCR and (C) Northern blot analysis. Primers for qRT-PCR analysis are described in Materials and Methods and located in the coding sequence of *HCL1*.

Figure 2.2. Key catalytic residues are conserved in the *H. capsulatum* HMG-CoA Lyase homolog Hcl1.

ClustalW alignment of *H. capsulatum* Hcl1 with HMG CoA lyases from bacterial and mammalian species. Conserved catalytic residues are marked in red. The asterisk symbol indicates that the residues in that column are identical in all sequences in the alignment, the colon indicates that conserved substitutions between the sequences are observed, and the period indicates that semi-conserved substitutions are observed.

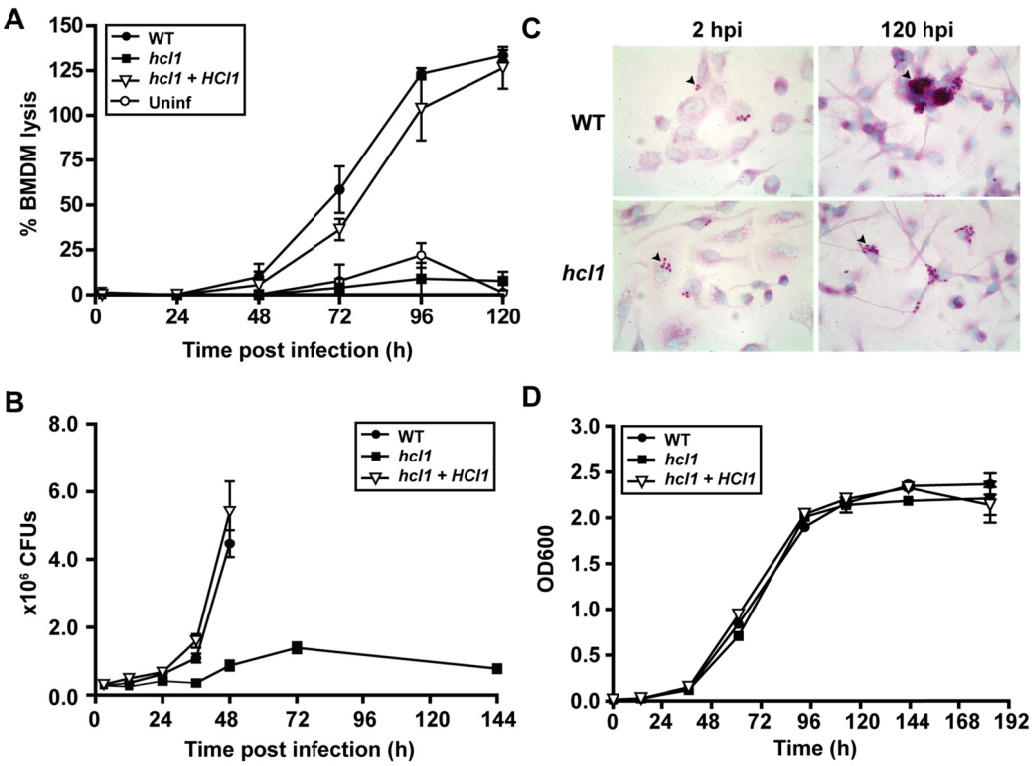


Figure 2.3. **Hcl1 is required for macrophage colonization and lysis during *H. capsulatum* infection.**

(A) BMDMs were mock-infected or infected with either wild-type (WT), the *hcl1* mutant, or the complemented strain (*hcl1+HCL1*) at an MOI = 2. At 2, 24, 48, 72, 96, and 120 hours post-infection (hpi), supernatants were removed from the infected monolayers and lactate dehydrogenase activity was assessed to monitor BMDM lysis. The average % BMDM lysis of four measurements ± standard deviation (from one representative experiment) is shown. (B) BMDMs were infected with wild-type (WT), the *hcl1* mutant, or the complemented strain (*hcl1+HCL1*) at an MOI = 2. At 3, 12, 24, 36, 48, 72, and 144 hpi, BMDMs were osmotically lysed and the lysates were plated for *H. capsulatum* CFUs. Each measurement is the average of 4 platings (duplicate infections/ duplicate platings) ± standard deviation from one representative experiment. In some cases, standard deviation is negligible and is obscured by strain symbols. (C) BMDMs were infected with either wild-type *H. capsulatum* or the *hcl1* mutant at an MOI =

0.1. At 2 and 120 hpi, the infected monolayers were fixed and *H. capsulatum* yeast cells (arrowhead) were stained with periodic acid/Schiff (PAS) reagent. Macrophage nuclei were counterstained with methyl green. Representative images at 100X magnification are shown. (D) *In vitro* growth of wild-type (WT), the *hcl1* mutant, and the complemented strain (*hcl1+HCL1*) was examined in broth culture. Yeast cells were inoculated into HMM at a starting OD_{600} = 0.01 and subsequent OD_{600} was monitored over time. The average of three measurements ± standard deviation is shown from one representative experiment. In some cases, standard deviation is negligible and is obscured by strain symbols.

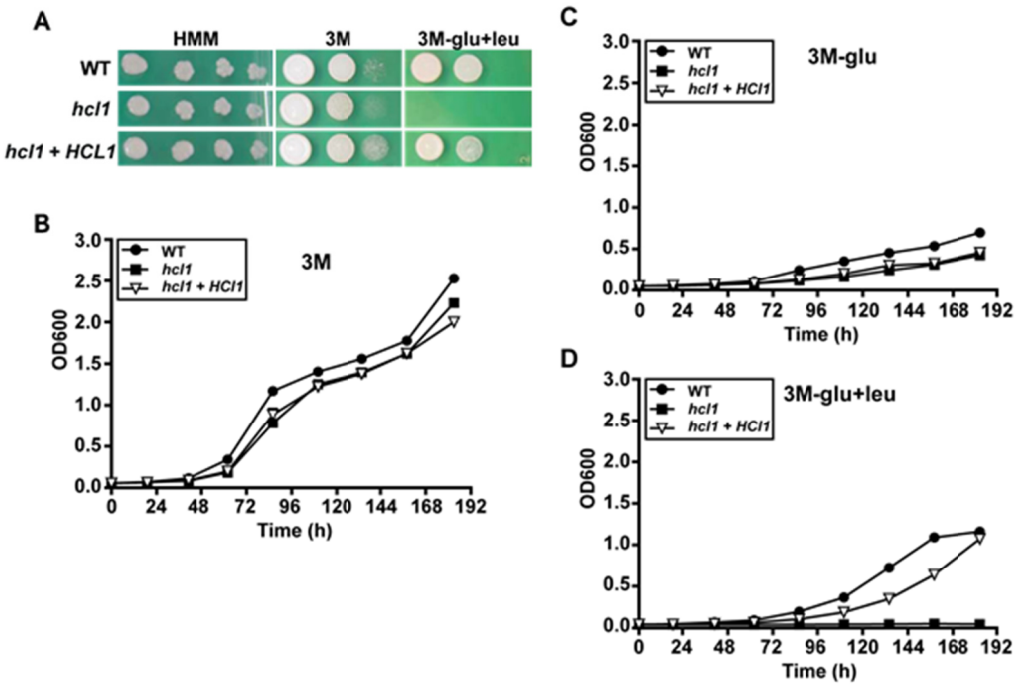


Figure 2.4. *H. capsulatum* Hcl1 is required for growth on leucine as the major carbon source.

(A) Four day cultures of wild-type *H. capsulatum* (WT), the *hcl1* mutant, and the complemented strain (*hcl1+HCL1*) were pelleted, washed with PBS, and resuspended in PBS. Ten-fold serial dilutions were then made on the indicated medium. In the case of 3M-glu+leu, medium was supplemented with 10 mM leucine. Plates were incubated at 37^{o}C, 5% CO2 for 16 days. For the HMM plates, the 1 x 10^{-3} to 1 x 10^{-6} dilutions are shown (due to the enhanced growth on this rich medium). On all other plates, undiluted, 1 x 10^{-1}, and 1 x 10^{-2} dilutions are shown. (B-D) Four day cultures of wild-type *H. capsulatum* (WT), the *hcl1* mutant, and the complemented strain (*hcl1+HCL1*) were pelleted, washed with PBS, and resuspended in either minimal media (3M) (B), minimal media without glucose (3M-glu) (C), or minimal media without glucose, supplemented with 5 mM leucine (3M-glu+leu). At indicated timepoints, OD_{600} was measured in triplicate to monitor growth. Average OD and standard deviation from one representative experiment are plotted, but standard deviation is negligible and is obscured by strain symbols.

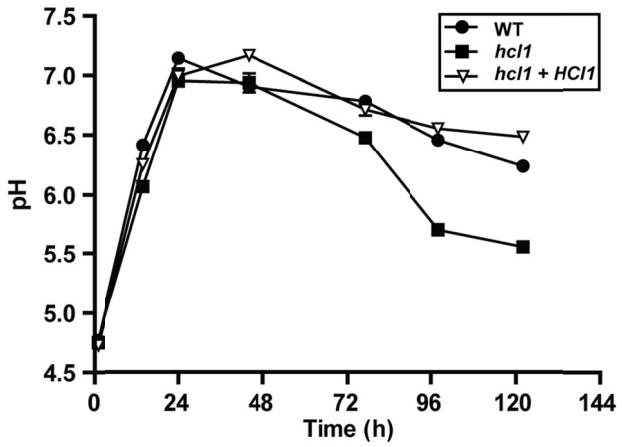


Figure 2.5. Hcl1 is required for maintenance of neutral pH in culture.
Four-day cultures of wild-type *H. capsulatum* (WT), the *hcl1* mutant, and the complemented strain (*hcl1+HCL1*) were pelleted, washed, resuspended in PBS, and diluted into 30 ml unbuffered pH-HMM at OD_{600} = 2.0. The pH of the culture medium was measured at the indicated timepoints. The average of three pH measurements ± standard deviation is shown from one representative experiment. In most cases, standard deviation is negligible and is obscured by strain symbols.

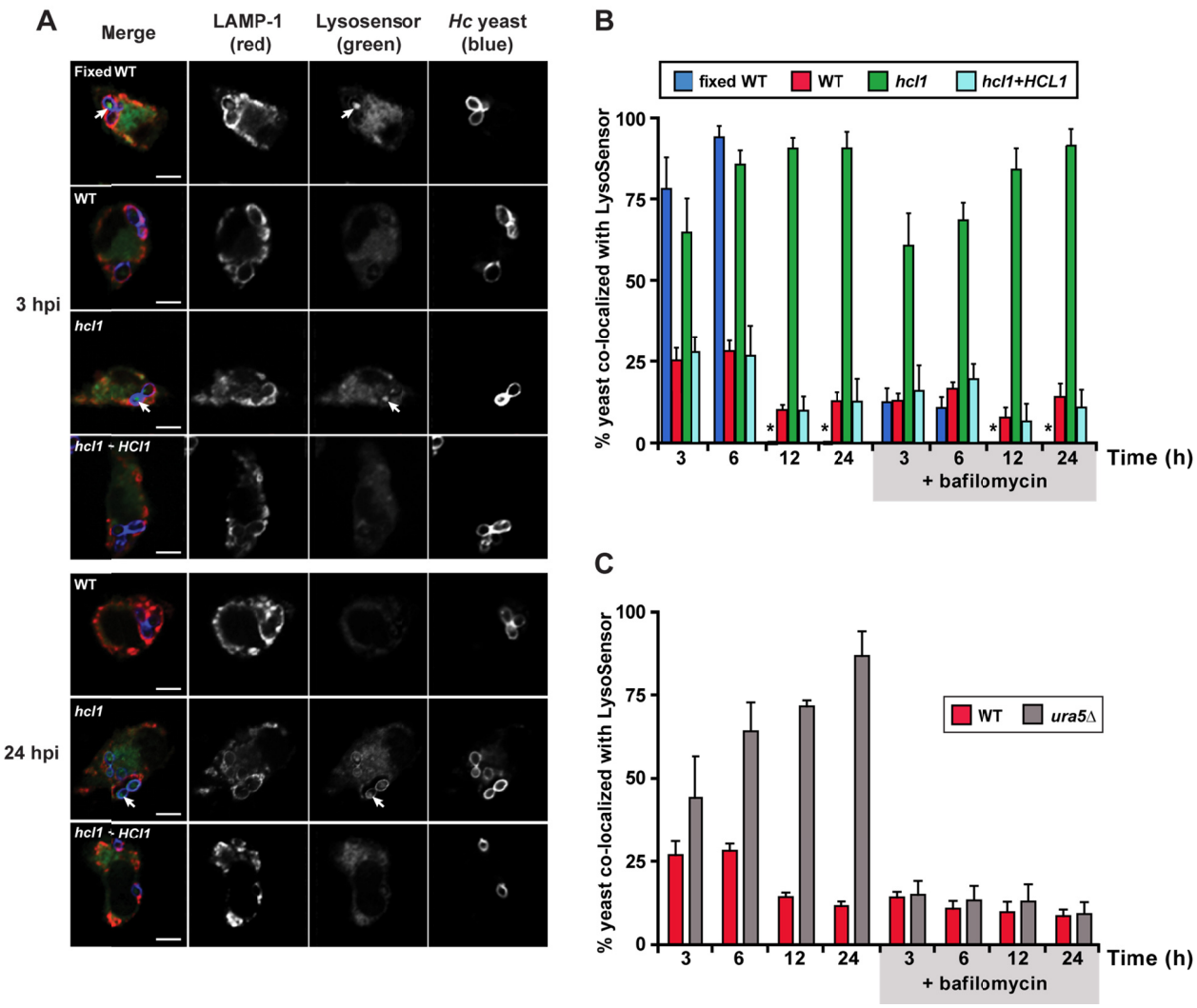


Figure 2.6. Hcl1 is required for maintenance of near-neutral pH in the macrophage phagosome.

(A) and (B) BMDMs were infected with either wild-type (WT), fixed wild-type cells, the *hcl1* mutant, or the complemented strain (*hcl1* + *HCL1*) at an MOI of 2. BMDMs were incubated with LysoSensor Green DND-189 30 min prior to fixation at 3, 6, 12, and 24 hpi. Control experiments were performed in the presence of 50 nM bafilomycin A1. (A) At 3 hours post-infection, the acidic pH of the phagosomes was revealed by bright green fluorescence (LysoSensor; white arrows) which surrounded and impregnated the yeast cells (blue). LAMP-1

straining is shown in red. *hcl1* mutant cells, but not wild-type cells, maintained localization with LysoSensor at later timepoints such as 24 hpi. Scale bars = 5 μm. (B) Quantification of the percentage of yeast cells that colocalized with LysoSensor fluorescence within LAMP-1-positive phagosomes. Fixed wild-type cells were degraded by the macrophage after 6 hpi, and thus could not be quantified after that timepoint. Their absence is indicated by an asterisk. At least 200 yeast cells were counted per sample and standard deviation is shown. (C) BMDMs were infected with either the wild-type strain (WT) or the WU15 G217B *ura5*Δ strain at an MOI = 2 in the absence or presence of bafilomycin as described above. Quantification of the percentage of yeast cells that colocalized with LysoSensor fluorescence within LAMP-1-positive phagosomes was performed. At least 200 yeast cells were counted per sample and standard deviation is shown.

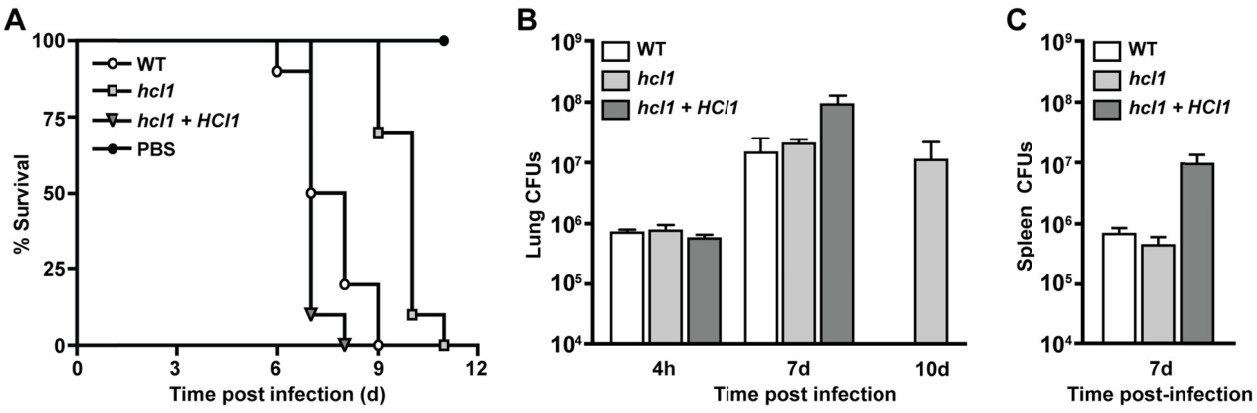


Figure 2.7. The *hcl1* mutant exhibits decreased virulence in vivo.

Female C57Bl/6 mice were infected intranasally with 1.25 x 10^6 CFUs of either the wild-type (WT) (n=15), the *hcl1* mutant (*hcl1*) (n=15), or the complemented strain (*hcl1+HCL1)* (n=15) strain. (A) Kaplan-Meir plots/survival curve. $p < 0.0001$ (log rank test, WT vs *hcl1*) (B) In the case of all three cohorts of mice, lungs were harvested from infected mice and lung homogenates were plated to enumerate *H. capsulatmum* CFUs at 4hrs post infection and at 7 days post-infection, which was time of death for mice infected with the wild-type strain. Lung CFUs were also enumerated at time of death (10 days post-infection) for mice infected with the *hcl1* mutant. Average CFUs/group is plotted. (C) Spleen CFUs were enumerated from all three cohorts of mice at 7 days post-infection. Average CFUs/group is plotted.

Chapter 3: MyD88-dependent signaling drives host survival and early cytokine production

Abstract

The ability of the innate immune system to trigger an adaptive T cell response is critical to resolution of infection with the fungal pathogen *Histoplasma capsulatum*. However, the signaling pathways and cell types involved in the recognition of and response to this respiratory pathogen remain poorly defined. Here we show that MyD88, an adaptor protein vital to multiple innate immune pathways, is critically required for the host response to *Histoplasma*. MyD88 deficient mice ($MyD88^{-/-}$) are unable to control fungal burden and are more sensitive to *Histoplasma* infection than wild-type, $Dectin1^{-/-}$ or $IL\text{-}1R^{-/-}$ mice. We find that MyD88 is necessary for the production of key early inflammatory cytokines and the subsequent recruitment of inflammatory monocytes to the lung. In both our *in vitro* and *ex vivo* analyses, MyD88 is intrinsically required in dendritic cells and alveolar macrophages for initial cytokine production. Additionally, MyD88-deficient bone marrow-derived dendritic cells fail to efficiently control fungal growth when co-cultured with primed splenic T cells. Surprisingly, mice that lack MyD88 only in dendritic cells and alveolar macrophages are competent for early cytokine production and normal survival, indicating the presence of compensatory and redundant MyD88 signaling in other cell types during infection. Ultimately, global MyD88 deficiency prevents proper T cell activation and IFNγ production, which is critical for infection resolution. Collectively, this work reveals a central role for MyD88 in coordinating the innate and adaptive immune response to infection with this ubiquitous fungal pathogen of humans.

Introduction

Histoplasma capsulatum is the most common cause of fungal respiratory infections in immunocompetent hosts in the United States (1-3). The organism exists in the environment in a sporulating filamentous form that is easily aerosolized and inhaled by the mammalian host. Inside the host, fungal cells convert into a pathogenic yeast form that is able to evade immune defenses by replicating within macrophages. In a healthy host, the adaptive immune response is critical for bringing disease under control, and individuals with defects in adaptive immunity frequently fail to contain *Histoplasma* infections and succumb to disseminated disease (1, 4, 5).

Successful activation of an adaptive immune response depends on the early innate events that occur during microbial infection (108). In general, these events are initiated by resident immune cells in the lung, including alveolar macrophages and dendritic cells, which recognize and respond to invading pathogens by directly controlling pathogen growth, secreting anti-microbial products, and producing pro-inflammatory cytokines, ultimately leading to an adaptive T cell response (109). During *Histoplasma* infection, both Th1 and Th17 responses contribute to the activation of macrophages to restrict and control fungal growth (41, 57, 62, 69, 110). If early innate immune events fail to occur and the appropriate immune response is disrupted, infection can continue unchecked and lead to disseminated disease and mortality. In the case of infection by *Histoplasma*, the precise events required to initiate an appropriate innate immune response and curtail disease progression remain poorly defined.

In a healthy host, resolution of *Histoplasma* infection requires a Th1 CD4+ T cell response (35). Depleting either CD4+ T cells or IFNγ leads to rapid dissemination of the pathogen and host mortality (9-13). Recruitment and activation of CD4+ T cells is dependent on the complex cascade of events underlying the innate immune response. Multiple cytokines

including TNFα, IL-12, IL-1β, CCL2 and GM-CSF are all produced early during infection and promote the recruitment, activation and/or maturation of a diverse group of immune cells, including monocytes, neutrophils and T cells (62, 110). While it is known that neutralizing these cytokines exacerbates disease (53–56), the specific cell types and signaling pathways involved in pathogen recognition and subsequent initiation of the innate immune response to *Histoplasma* are still being explored. Recent work implicates the C-type lectin receptors Dectin-1 and Dectin-2 in the recognition of and response to *Histoplasma* (75). Nonetheless, much remains to be understood about signaling pathways that are activated by *Histoplasma* during infection. Since MyD88 is a central adaptor protein in multiple immune recognition and signaling pathways, we chose to explore its role in the host immune response to *Histoplasma*.

MyD88 mediates both pathogen recognition through toll-like receptors (TLRs) and cytokine signaling through the IL-1 and IL-18 receptors (19,20). In many infectious disease models, perturbing MyD88 signaling causes defects in early immune events that lead to decreased induction of the adaptive immune response and increased host sensitivity to infection. For example, in infection models of fungal pathogens such as *Candida albicans* and *Aspergillus fumigatus*, MyD88 deficiency causes a block in the production of pro-inflammatory cytokines early during infection, decreased neutrophil recruitment, and decreased T cell activation (21,22). Here, we demonstrate that MyD88 signaling is critical for mounting the appropriate immune response to *Histoplasma*. In particular, we provide direct evidence that MyD88 is necessary for both the early production of pro-inflammatory cytokines by alveolar macrophages and dendritic cells and the subsequent recruitment of inflammatory monocytes. Ultimately, deficiency in MyD88 severely hinders the development of an appropriate T cell response to *Histoplasma* infection, culminating in increased fungal burden and host mortality.

Materials and Methods

Strains and culture conditions. *Histoplasma* yeast cells were grown in *Histoplasma* macrophage medium (HMM) (87). Liquid cultures were grown in an orbital shaker at 37°C with 5% CO_2. HMM agarose plates were incubated in a humidified chamber at 37°C with 5% CO_2. At the start of these experiments, a large stock of *Histoplasma* strain G217B, designated G217B-AC, was stored at -80ºC in 50% glycerol; cells from this stock were used for all experiments. Cells were inoculated from frozen stock onto HMM plates 3 weeks before each experiment. One week before infection, the strain was inoculated from solid media to liquid HMM and passaged 1:25 every three days. In preparation for infection of both mice and *in vitro* cell cultures, mid-logarithmic-phase cultures were washed once with PBS, sonicated for 3 seconds on setting 2 using a Fisher Scientific Sonic Dismembrator Model 100, and counted using a hemacytometer to determine the cell number.

Mice. Female C57Bl/6J, IL-1R$^{-/-}$ (strain #003245, B6.129S7-Il1r1^{tm1Imx}/J) and MyD88$^{-/-}$ (strain #009088, B6.129P2(SJL)-Myd88$^{tm1.1Defr/}$J) mice were originally purchased from Jackson Laboratory. Dectin1$^{-/-}$ mice were obtained from Chad Steele at the University of Alabama, Birmingham. C57Bl/6Tac, the wild-type control for Dectin1$^{-/-}$ animals, were purchased from Taconic Farms. MyD88$^{fl/fl}$ (Jackson strain #008888, B6.129P2-Myd88$^{tm1Defr/J}$), MyD88 $^{fl/fl}$ xCD11cCre (Jackson strain #008068, B6.Cg-Tg(Itgax-cre)1-1Reiz/J), MyD88 $^{fl/fl}$ xLysMCre (Jackson strain # 004781, B6.129P2-Lyz2$^{tm1(cre)Ifo}$/J) and MyD88$^{fl/fl}$xVavCre (Jackson strain #008610, B6.Cg-Tg(Vav1-cre)A2Kio/J) were a gift from Anthony DeFranco (111, 112). All animals were bred and maintained in a specific pathogen-free facility at UCSF. All mouse

experiments were performed in compliance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee at the University of California, San Francisco.

Mouse infections. 8-12 week old, age-matched female mice were anesthetized with isoflurane and infected intranasally with 1.8×10^4 yeast cells of wild-type (G217B-AC) *Histoplasma*. Mice were monitored daily for symptoms of disease (i.e., weight loss, lack of activity/response to stimulus, panting, lack of grooming). For survival experiments, mice were sacrificed after they exhibited 3 days of sustained weight loss >15% of their maximum weight in conjunction with one other symptom of disease. For fungal burden experiments, lungs and spleens were harvested from mice and homogenate was plated on Brain-Heart Infusion (BHI) agar plates and grown for 10 days at 30°C to enumerate CFUs.

Cytokine analysis. Lung and spleen homogenate from infected mice were homogenized in 1 mL PBS containing 1x cOmplete ULTRA protease inhibitor (Roche). Homogenate was centrifuged at 4^oC and the resulting supernatant was sterilized using a 0.2 uM CoStar Spin-X centrifuge tube (Corning). For cytokine analysis of cells in culture, supernatant was sterile-filtered using AcroPrep 96-well Filter Plates (Pall). Mouse Cytometric Bead Array Flex Sets (BD) were used according to manufacturer's instructions to determine the concentration (pg/mL) of IL-6, TNFα, KC, IL-1β, IFNγ, IL-17, and MCP-1/CCL2. IL-12p70 levels were quantified using a Ready-Set-Go! ELISA kit (eBioscience).

Isolation of lung cells. Mouse lungs were perfused with PBS and digested in Hanks Buffered Salt Solution (HBSS) containing 80 U/mL DNase (Sigma D4527) and 2 mg/mL collagenase D (Roche) for 30 minutes at 37^{o}C. After digestion, lungs were dissociated using a GentleMacs Tissue Dissociator (Milltenyi). Red blood cells were hypotonically lysed and the remaining cells filtered through a 70 μM cell strainer (BD).

Flow cytometry. 2-4 x 10^6 dissociated lung cells were resuspended in PBS and stained with Fixable Viability Dye eFluor 450 (eBiosciences) for 20 minutes, then washed and resuspended in PBS containing 1% heat-inactivated FBS, 1 mM EDTA, 10 ug/mL anti-CD16/32 (Fc Block) and 0.1% sodium azide. Cells were stained for 30 minutes with appropriate antibodies, fixed in 1x BD Stabilizing Fix, and stored at 4^{o}C until analysis on an LSR II (BD). Antibodies used to identify macrophages, neutrophils, monocytes and dendritic cells were as follows: PerCp-Cy5.5-CD11c, Pe-Cy7-CD11b, APC-MHC Class II, PE-Cy7-Gr1 (eBiosciences), PE-SiglecF, PE-Ly6G, APC-Cy7-Ly6C (BD), FITC-CD45.2 (UCSF). Antibodies used to identify T and NK cells were as follows: APC-CD4, PerCp-Cy5.5-CD8, PE-Cy7-CD69, PE-CD69 (BD), PerCp-Cy5.5-NK1.1, FITC-CD3ε, APC-gamma delta TCR (eBiosciences). Flow cytometry data were analyzed using FlowJo version 7.6.5.

Intracellular Cytokine Staining. Dissociated lung cells were prepared as described for flow cytometry experiments, except that 1 uL/mL BD GolgiPlug (brefeldin A) was added to all media until cells were stained with antibodies. Extracellular staining proceded as described above. After staining, samples were fixed in Cytofix/Cytoperm Buffer (BD) for 20 minutes, then stained

with intracellular cytokine antibodies in 1x Perm/Wash buffer (BD) for 30 minutes, and stored at 4^{o}C until analysis. BV786-IFNγ (BD) antibody was used to stain IFNγ producing cells.

Bromodeoxyuridine Staining. Mice were given 1 mg Bromodeoxyuridine (BrdU) in 100 uL PBS i.p. on day 5 and again on day 6 post-infection. Lungs and mediastinal lymph nodes from BrdU-treated mice were harvested 12 hours after the last injection (day 7). Single-cell suspensions from total lung tissue and lymph nodes were stained for surface CD3, CD4 and CD8. Cells were fixed, processed and stained for BrdU using the BrdU Flow Kit (BD Pharmigen) as per manufacturer's protocol.

Generation of bone-marrow-derived dendritic cells. Bone-marrow-derived dendritic cells (BMDC) were derived by culturing bone marrow freshly isolated from femurs of 6- to 8-week old female mice in DC media consisting of RPMI 1640 (GIBCO), 2 mM L-glutaMAX (Life Technologies), penicillin-streptomycin, 50 μM 2-ME (Sigma), 10 mM HEPES, 10% heat-inactivated FBS, 10 ng/ml rGM-CSF (Peprotech) and 1 ng/mL rIL-4 (Life Technologies). Non-adherent cells were harvested on day 6 and CD11c+ cells were purified using magnetic columns and anti-mouse CD11c microbeads (Milltenyi) per manufacturer's instructions. According to flow cytometry analysis, all samples were at least 90% CD11c+ cells. $4x10^5$ cells/well were seeded into 24 well plates in DC media and cultured 48 hours before infection.

Isolation of alveolar macrophages. Alveolar macrophages were harvested by bronchiolar lavage of naïve mouse lungs with 10 mLs PBS containing 1mM EDTA. $5x10^5$ cells/well were plated in 24 well tissue cultures plates in Dulbecco modified Eagle medium (DMEM) containing

20% heat-inactivated FBS, 2 mM L-glutaMAX and penicillin-streptomycin. Non-adherent cells were removed from wells via gentle washing with 1 mL DMEM before infection with *Histoplasma*.

Isolation of CD4+ T cells. Spleens from mice infected with a sub-lethal dose (1 x 10^4 cells) of wild-type *Histoplasma* (G217B-AC) were harvested 15 days post-infection and dissociated in HBSS using the GentleMacs Dissociator. Leukocytes were enriched via Lympholyte-M separation (Cedarlane), and B cells were depleted by incubating the cells on anti-IgM and anti-IgG antibody coated plates for 2 hours. Non-adherent cells were collected and T cells were purified by negative selection using a mouse Pan-T Cell Isolation Kit (Miltenyi). CD4+ T cells were then isolated by positive selection using anti-mouse CD4 microbeads (Miltenyi).

DC-T cell co-cultures. BMDCs were seeded in 96 well plates at a density of 3 x 10^4 cells/well and infected with wild-type *Histoplasma* (G217B-AC) at a multiplicity of infection (MOI) of 1. Immediately after infection with *Histoplasma*, 1 x 10^5 purified CD4+ T cells were added to the co-culture. At various time points post-infection, the medium was removed from each well and 200 µl of double-distilled H_2O were added. Cells were mechanically lysed by vigorous pipetting after a 5-minute incubation at room temperature. Cell lysate was diluted in PBS, plated on HMM agarose, and grown for 10 days at 37°C to enumerate CFUs.

Isolation of CD11c+ cells from lungs. Dissociated lung cells were incubated with anti-mouse CD11c microbeads and purified by magnetic separation on a MACs column (Miltenyi) per

manufacturer's instructions. The purity of lung cell isolates was determined by flow cytometry using antibodies described above. All samples were at least 90% CD11c+ cells.

RNA isolation and qRT-PCR. Immediately after CD11c+ purification, cells were lysed with Qiazol (Qiagen), flash frozen in liquid nitrogen, and stored at -80^{o}C. RNA was harvested using a Purelink RNA mini kit (Life Technologies) following manufacturer's instructions for Trizol reagent-based purification of RNA. cDNA was synthesized using Superscript II (Life Technologies) and a 1:1 mixture of Oligo dT and random primers. Reaction was diluted 1:50 in ddH20 prior to analysis by qRT-PCR. Quantitative PCR was performed using SYBR green Universal Master Mix (Roche) and 200 nM primers on an Mx3000P QPCR system (Stratagene). Relative expression (ΔCt) to an endogenous control, hypoxanthine-guanine phosphoribosyltransferase (HPRT), was determined. Primer sequences were obtained from the Harvard Primer Bank database (http://pga.mgh.harvard.edu/primerbank/index.html) (113–115). Primer IDs are as follows: HPRT (7305155a1), IL-1β (118130747b1), IL-6 (13624310b2), TNFα (133892368b2), and KC (6680109a1).

Statistical Analysis. Statistical analysis for experiments was performed using Prism (GraphPad Software, San Diego, CA). Analysis of variance (ANOVA) with Tukey's post test was used to analyze significance of cytokine expression and flow cytometry experiments. The log rank sum test was used to analyze survival. Statistically significant differences were denoted for p values < 0.05.

Results

MyD88$^{-/-}$ mice are more susceptible to *Histoplasma* infection than Dectin1$^{-/-}$ or IL-1R$^{-/-}$ mice.

MyD88 is a critical downstream mediator of key innate signaling pathways, including Toll-like receptor (TLR) signaling, but the requirement for MyD88-dependent signaling in the host response to *Histoplasma* has not been explored. To determine the role of MyD88 dependent signaling in the immune response during respiratory *Histoplasma* infection, whole-body MyD88 deficient (MyD88$^{-/-}$) and wild-type mice were inoculated intranasally with a sublethal dose of *Histoplasma.* Mice were monitored daily for symptoms of infection, which included weight loss and signs of respiratory distress. All mice began to show symptoms of infection by day 6. Wild-type mice were able to resolve *Histoplasma* infection and recovered within 14-16 days; however, MyD88$^{-/-}$ mice experienced progressively worsening symptoms during this time period and ultimately succumbed to disease 14-20 days after infection (Figure 3.1 A). MyD88 is required for TLR, IL-18, and IL-1 signaling; the latter is already known to contribute to host protection against *Histoplasma* (54). However, we found that MyD88$^{-/-}$ mice were significantly more susceptible to *Histoplasma* infection than IL-1R$^{-/-}$ mice (Figure 3.1 A), implying that deficient TLR and/or IL-18 signaling could contribute to the increased sensitivity of the MyD88$^{-/-}$ mutant. Additionally, we compared the sensitivity of MyD88$^{-/-}$ mice to that of mice lacking the pattern recognition receptor Dectin-1, which can recognize *Histoplasma in vitro* (39, 75, 77, 78, 116). Recently, Dectin-1$^{-/-}$ mice have been reported to have a higher pulmonary fungal burden than wild-type mice during *Histoplasma* infection (75) . In our infection model, we found that Dectin-1, unlike MyD88, was dispensable for host survival (Figure 3.1 A). Overall, these results indicate that MyD88 is required for host survival and suggest that other receptor signaling pathways

upstream of MyD88, such as TLR and/or IL-18 signaling, may also be critical for host recognition and response to *Histoplasma*.

To further investigate how MyD88 signaling affects the progression of disease, we determined the fungal burden in the lungs and spleens of infected mice at 3, 5, 7 and 15 days post-infection (dpi). In wild-type mice, fungal growth in the lungs peaked at day 7 then declined as the mice recovered (Figure 3.1 B). In contrast, the fungal burden in the lungs of MyD88$^{-/-}$ mice was significantly higher than in wild-type mice at 7 dpi (Figure 3.1 B), and continued to increase until the time of death. We monitored dissemination of the fungus from the lungs to the splaeen and determined that the fungal burden in the spleen was significantly higher in MyD88$^{-/-}$ animals than in wild-type mice at 15 dpi (Figure 3.1 C). By this time point, the MyD88$^{-/-}$ animals displayed disease symptoms such as inactivity, hunching, panting, and weight loss (data not shown) and succumbed to infection. Collectively, these results demonstrate that MyD88 is required for responding to and resolving *Histoplasma* infection.

MyD88 is required for the early innate immune response to *Histoplasma*

To determine the role of MyD88 in the immune response to *Histoplasma*, we next investigated cytokine production in the lungs of infected mice during the first seven days of infection. Analysis of cytokines harvested from infected lungs revealed that MyD88$^{-/-}$ mice did not produce normal levels of the inflammatory cytokines IL-6, IL-1β, CCL2 and KC at 5 dpi (Figure 3.2) despite displaying a fungal burden equivalent to wild-type mice. At 5 dpi, we also observed that MyD88$^{-/-}$ mice produced lower levels of IL-12p70, a key cytokine in the initiation of the Th1 response. Notably, not all early cytokine production required MyD88-dependent signaling, as TNFα levels were unaffected by MyD88 deficiency during infection (3 and 5 dpi). Interestingly, by 7 dpi, cytokine levels were higher in MyD88$^{-/-}$ mouse lungs compared to wild-

type mouse lungs, correlating with the significant increase in fungal burden in these mice at this time point.

Since MyD88$^{-/-}$ mice were deficient in the production of cytokines known to recruit monocytes and neutrophils, such as CCL2 and KC, we next investigated the recruitment of inflammatory cells to the lungs of MyD88$^{-/-}$ mice. Flow cytometry analysis of single cell suspensions from the lung revealed a significant block in the overall recruitment of CD45^{+} inflammatory cells to MyD88$^{-/-}$ lungs by 7 dpi (Figure 3.3 A and B). In particular, we found that MyD88$^{-/-}$ mice displayed a decrease in the number of recruited inflammatory neutrophils and dendritic cells by 5 dpi and a significant decrease in the numbers of monocytes and alveolar macrophages by 7 dpi (Figure 3.3 C-F). A similar recruitment defect was observed in IL-1R$^{-/-}$ mice 5 days post-infection, suggesting that recruitment is at least partially mediated by IL-1 signaling (Supplementary Figure 3.1**Error! Reference source not found.**). Thus, MyD88-dependent signaling is required for cytokine production early in infection and for the subsequent recruitment of inflammatory cells known to be important for the host response to and control of *Histoplasma* infection.

MyD88 deficiency results in delayed activation of T and NK cells

A robust adaptive T cell response is critical to resolution of *Histoplasma* infection (35, 66). Since MyD88$^{-/-}$ mice were delayed in the production of key regulators of the Th1 and Th17 response, namely IL-12, IL-1β, and IL-6, we asked if an impaired T cell response might underlie their survival defect. First we examined levels of the cytokines IFNγ and IL-17A, which are produced by activated NK cells, γδ TCR+ cells, CD4+ and CD8+ T cells (117, 118). Whereas wild-type mice consistently produced high levels of these cytokines during *Histoplasma* infection, MyD88$^{-/-}$ mice failed to do so; IFNγ and IL-17A remained low throughout the entire

course of infection (Figure 3.4 A). Next we examined NK and T cell numbers in the lung during infection, as well as their expression of CD69, an early marker of activation (119). The lungs of infected WT and MyD88$^{-/-}$ mice contained roughly equivalent numbers of these cells (with the exception of γδ TCR+ cells at day 7, which were higher in wild-type mice). In contrast, CD69 expression was significantly decreased for γδ TCR+ cells (Figure 3.4 B), NK cells (Figure 3.4 C), and CD8+ T cells (Figure 3.4 D) at both day 5 and day 7 in MyD88$^{-/-}$ mice compared to wild-type controls, indicating a defect in activation of these cells. Similarly, CD69 expression in CD4+ T cells was decreased in MyD88$^{-/-}$ lungs at 7 dpi (Figure 3.4 E). In addition, we found that that while no difference in T-cell proliferation was observed in the lungs at 7 dpi, there was a significant decrease in T cell proliferation in the lymph nodes, as measured by bromodeoxyuridine (BrdU) incorporation (Figure 3.5 A). Importantly, γδ TCR+ cells, NK cells, CD8+ T cells, and CD4+ T cells are all major sources of IFNγ, which is required for activation of macrophages and control of intracellular fungal growth (118, 120). We found that the percentage of IFNγ-producing cells was significantly decreased in MyD88 deficient mice for γδ TCR+ cells, NK cells, and CD4+ T cells (Figure 3.5 B-C). Thus, MyD88 is required for the activation of both innate and adaptive immune cells to produce IFNγ, a cytokine critical for control of fungal growth and resolution of *Histoplasma* infection.

MyD88 mediates intrinsic responses of alveolar macrophages and dendritic cells *in vitro*

During pulmonary infection, *Histoplasma* is found primarily in resident innate immune cells, including macrophages and dendritic cells (121). We hypothesized that MyD88 would be required intrinsically for these innate immune cells to recognize and respond to *Histoplasma*. To test this, we first evaluated the ability of MyD88 deficient bone marrow-derived dendritic cells

(BMDCs) (Figure 3.6 A) and freshly isolated alveolar macrophages (Figure 3.6 B) to respond to *Histoplasma* infection *in vitro.* We found a substantial delay in the production of inflammatory cytokines at 48 hours post-infection for both cell types, suggesting that MyD88 is intrinsically required for the innate immune response to *Histoplasma.* In contrast, IL-1R$^{-/-}$ BMDCs were fully competent for cytokine production in response to *Histoplasma* infection (Supplementary Figure 3.2).suggesting that the defects we observe in the MyD88$^{-/-}$ mutant is not solely due to a failure in IL-1 signaling.

We then used a co-culture model of *Histoplasma*-infected BMDCs and T cells to determine if MyD88 deficiency affected the interaction between these cell types (46). We first infected WT and MyD88$^{-/-}$ BMDCs with *Histoplasma* and monitored intracellular fungal growth by lysing host cells at multiple timepoints over the course of infection and enumerating intracellular yeasts by plating. We observed equivalent fungal growth in WT and MyD88$^{-/-}$ BMDCs when cultured in the absence of T cells (Supplementary Figure 3.3). However, when we cultured infected BMDCs with CD4+ T cells purified from the spleen of infected wild-type mice, we observed that wild-type BMDCs showed greater restriction of fungal growth than MyD88$^{-/-}$ BMDCs (Figure 3.6 C). These data indicate that MyD88 signaling contributes to the response of infected BMDCs to primed T cells.

MyD88 is required for the normal response of dendritic cells to *Histoplasma in vivo*

To extend our *in vitro* observations to *in vivo* infections, we purified CD11c+ cells (consisting of alveolar macrophages and lung dendritic cells) from the lungs of infected WT and MyD88$^{-/-}$ mice. Transcriptional analysis of these cells revealed a requirement for MyD88 in cytokine production as early as 3 dpi (Figure 3.7 A). To determine the relative contribution of MyD88 signaling in these immune cells to the overall host response, we utilized mice carrying a

floxed MyD88 allele and expressing a cell-specific Cre recombinase to delete MyD88 specifically in dendritic cells and alveolar macrophages (CD11c-MyD88) or in macrophages and neutrophils (LysM-MyD88) (111). As we found with global deletion of MyD88, alveolar CD11c+ cells (alveolar macrophages and dendritic cells) from infected CD11c-MyD88 mice were deficient in the early transcription of key cytokines during *Histoplasma* infection (Figure 3.7 B). Surprisingly, however, CD11c-MyD88 and LysM-MyD88 mice did not display any significant defects in survival or control of fungal growth upon infection with a sublethal dose of *Histoplasma* (Figure 3.8 A and B), nor did they demonstrate a robust *in vivo* cytokine deficiency as observed upon global deletion of MyD88 in mice (Figure 8 C and D). These data suggest that MyD88 signaling in other immune cell types can compensate for the defect in alveolar macrophages and dendritic cells. In line with this, Vav-MyD88 mice, which are MyD88-deficient in all hematopoietic cells, displayed a significant survival defect when infected with a sublethal dose of *Histoplasma* (Figure 3.8 E*)*. In sum, MyD88 is required intrinsically in both dendritic cells and alveolar macrophages for their normal response to *Histoplasma* infection, but defects in these cell populations alone are insufficient to fully recapitulate the effects of global MyD88 deficiency. These data implicate a role for MyD88-dependent signaling in additional hematopoietic cells during *Histoplasma* infection.

Discussion

In this study, we demonstrate that MyD88 signaling is a crucial component in the defense against *Histoplasma*. Using MyD88$^{-/-}$ mice, we show that MyD88 is required for control of fungal burden in both the lung and spleen and demonstrate that MyD88$^{-/-}$ mice fail to resolve infection by *Histoplasma*, instead succumbing to disseminated disease. During early infection of the lung, MyD88$^{-/-}$ mice fail to produce inflammatory cytokines, including CCL2 and IL-1β, that

are known to trigger critical events for controlling *Histoplasma* infection, such as the recruitment and activation of neutrophils and monocytes (53, 54). This early innate immune response appears to be partly mediated by a cell-intrinsic requirement for MyD88 in the recognition and response of alveolar macrophages and dendritic cells to infection with *Histoplasma*. Upon detailed analysis of dendritic cell responses *in vitro*, we found that MyD88 signaling contributes to cytokine production and control of intracellular fungal growth. In addition to early cytokine responses and recruitment of inflammatory cells, other key events during the host response to *Histoplasma* infection include an increase in CD4+ T cell activation, and IFNγ/IL-17 production. Recruitment of inflammatory cells to the site of infection is associated with control of fungal growth, and production of IFNγ/IL-17 is necessary for the restriction of *Histoplasma* growth by activated macrophages (41, 53, 122). In the $MyD88^{-/-}$ mouse, production of the cytokines involved in inducing both a Th1 and a Th17 response is delayed. Despite a higher fungal burden in the $MyD88^{-/-}$ mouse, the Th1 response is muted, as IFNy production by both innate (γδ TCR+ and NK cells) and adaptive (CD4+ T cells) cells is decreased, indicating that MyD88-dependent pathways contribute to these responses.

The requirement for MyD88 varies widely for different fungal pathogens in murine infection models. MyD88 is required for host survival, inflammatory cytokine production, and T cell activation during infection with *Candida, Cryptococcus* and *Paracoccidiodes* (123–126). In contrast, during respiratory infection with *Aspergillus*, MyD88 is important for production of TNFα and early pulmonary responses, but is only required for survival if the host is immunosuppressed (83, 125). Importantly, multiple studies have demonstrated a requirement for MyD88 in directing T cell responses to fungal pathogens, which is critical since a robust Th1 and Th17 response is often important to achieve a productive resolution to fungal infection. Indeed,

while MyD88 plays only a moderate role in the recruitment and proliferation of T cells during infection with *A. fumigatus*, it is critical for full IFNγ production by these cells (127). Interestingly, in this context MyD88 does not seem to be intrinsically required within T cells but rather is important for the ability of resident lung cells, such as dendritic cells, to influence the T cell response. Correspondingly, MyD88 has been shown to mediate both T cell-dependent IFNγ production as well as the T cell-DC interaction during infection with *C. albicans* (128). Here, we show that MyD88 signaling is important for both T cell activation and dendritic cell cytokine production during *Histoplasma* infection, suggesting that during MyD88 deficiency, the interaction between these two cell types may be impaired. In line with this, we show that the ability of infected dendritic cells to restrict fungal growth when co-cultured with primed T cells is MyD88-dependent. However, the precise molecular mechanisms which drive this interaction remain unknown. Accordingly, future studies to clarify the role of MyD88 during the T cell response to *Histoplasma* may indicate that MyD88 signaling in dendritic cells is an important mediator of T cell activation.

Numerous dendritic cell responses were MyD88-dependent in our infection models, including optimal growth restriction of *Histoplasma* and timely cytokine responses. Studies have shown that dendritic cells are activated in response to pathogen infection both directly through pathogen-associated molecular pattern (PAMP) recognition as well as indirectly through exposure to the cytokine milieu (129). Both recognition of pathogen PAMPs and exposure to cytokines such as TNFα, IFNγ and IFNα/β increases the upregulation of T cell activation ligands such as MHC II and CD86, the production of inflammatory cytokines, and the processes involved in the control of pathogen growth, such as the production of reactive oxygen and nitrogen species (130–132). The delayed cytokine production and increased fungal growth we

see in MyD88$^{-/-}$ dendritic cells may be a reflection of a failure to recognize *Histoplasma*. Alternatively, MyD88$^{-/-}$ dendritic cells may fail to respond appropriately to the cytokine milieu created by primed T cells, thus causing defects in dendritic cell maturation, activation and fungal killing.

The ability of the host to mount an appropriate immune response to *Histoplasma* depends on its capacity to recognize and respond to this pathogen; however, the panel of immune receptors responsible for the initiation of this response to *Histoplasma in vivo* is still being uncovered. Indeed, the plethora of pattern recognition receptors and downstream signaling pathways engaged by distinct fungal organisms is an area of active inquiry (74, 133). Recent work demonstrates the importance of C-type lectin receptors in vaccine immunity as well as primary immune responses to *Histoplasma* and other related fungi (75). The contribution of Toll-like receptors in the primary *in vivo* host response to *Histoplasma*, however, has not been elucidated, and, by uncovering the role of MyD88, our work suggests that TLR signaling could play a key role in the corresponding host response. Importantly, MyD88 is also a mediator of IL-1 and IL-18 cytokine receptor signaling. Previous work has demonstrated that IL-1R deficiency resulted in an increase in host susceptibility to *Histoplasma*, as well as a deficiency in IFNγ production in the lungs of infected mice at later timepoints (54), similar to the MyD88$^{-/-}$ results presented here. However, we found that IL-1R$^{-/-}$ mice display no defects in overall host survival when infected with a dose of *Histoplasma* that was lethal to MyD88$^{-/-}$ mice. Although the IL-1R$^{-/-}$ mouse displayed a similar defect in cell recruitment to the lungs as the MyD88$^{-/-}$ mouse, IL-1 signaling was dispensable for early cytokine production in BMDCs. These results demonstrate that IL-1 signaling alone is not sufficient to explain the defect we observe during MyD88 deficiency, and thereby suggest that TLR recognition and/or IL-18 signaling are also important

for the host immune response. In several pathogen infection models, single TLR deficiencies are frequently not sufficient to produce a measurable difference in immune response, as the effects of recognition by individual TLRs are redundant. Future studies to determine the contribution of TLR recognition to *Histoplasma in vivo* may require the investigation of response in mice containing multiple TLR knockouts, such as mice lacking TLR2, 4, and 9 (134). Alternatively, since IL-18, in cooperation with IL-12, is known to induce a strong IFNγ response from T cells, it may be that the reduced ability of MyD88$^{-/-}$ mice to produce IFNγ reflects a failure of MyD88-dependent IL-18 signaling (135, 136).

A critical point to emerge from these experiments is that the requirement for MyD88 signaling in the response to *Histoplasma* was not confined to narrow immune cell subsets. Our work is in contrast to studies of infection responses to other pathogens, where the use of *in vivo* cell-specific deletion of MyD88 has elucidated the role of distinct cell-types in the host response. For example, during *Toxoplasma* infection, deletion of MyD88 only in the CD11c+ cell compartment (alveolar macrophages and dendritic cells) leads to a defect in IL-12 production and an increased susceptibility to infection (112). Since *Histoplasma* interacts primarily with phagocytic cell populations in the lung, we expected that deletion of MyD88 signaling in these specific cell-types would recapitulate the phenotype we observed in the global MyD88 knockout mouse. *In vitro*, this was indeed the case, with both alveolar macrophages and dendritic cells showing delayed cytokine production after infection with *Histoplasma*. Surprisingly, whereas deletion of MyD88 in the entire hematopoietic compartment caused a significant decrease in host survival, specific deletion of MyD88 in either (1) alveolar macrophages and dendritic cells or (2) macrophages and neutrophils failed to affect survival or fungal burden *in viv*o. These data suggest that intact MyD88 signaling in either one of these two subsets may be sufficient for host

protection. Alternatively, MyD88 may contribute to host protection through other immune cell types, such as T cells, and future studies will be required to elucidate the role of MyD88 signaling in additional hematopoietic cell types. Collectively, these results highlight the multifaceted role of MyD88 in the host response to *Histoplasma* infection and underscore the multiple strategies used by mammalian hosts to combat the ability of a primary fungal pathogen to cause disease.

Acknowledgements

Many thanks to Anthony DeFranco for the initial gift of the MyD88$^{fl/fl}$-Cre mouse strains used in these experiments. We are grateful to Anthony DeFranco, Richard Locksley, Joanne Engel, Kirk Jones, Linda Lee and members of the Sil laboratory for useful discussion as this work progressed. We thank Davina Hocking Murray and Johnny Tse for technical assistance. We thank Sinem Beyhan, Bevin English, Sarah Gilmore, and Nancy Van Prooyen for comments on the manuscript.

This work was supported by an NSF Graduate Research Fellowship to AC, NIH (PO1AI063302 and RO1AI093640) to AS, and an HHMI Early Career Scientist Award (http://www.hhmi.org/research/ecs/) to AS.

Figures

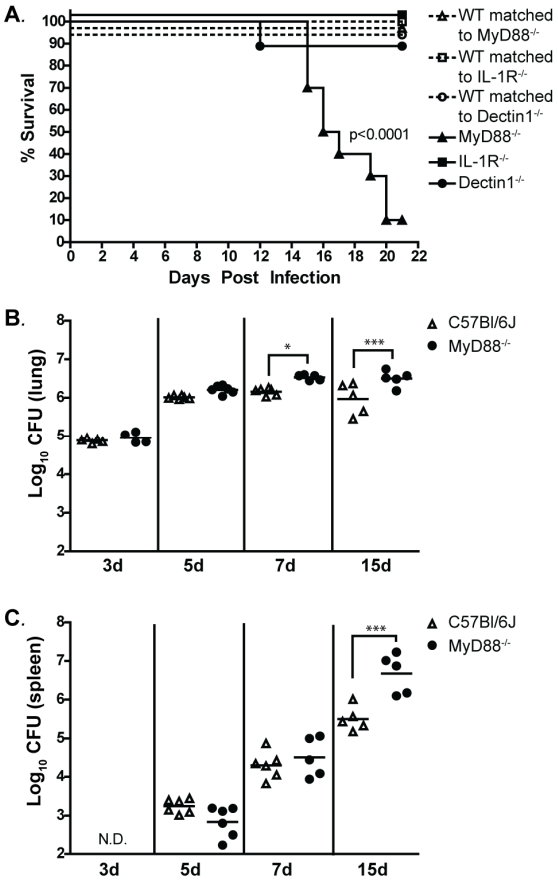


Figure 3.1. MyD88 is required for host survival after infection with *Histoplasma.*

A) Gender and age-matched MyD88$^{-/-}$, Dectin1$^{-/-}$, IL-1R$^{-/-}$ mice and their respective wild-type counterparts were infected intranasally with 1.8x10^4 *Histoplasma* cells and monitored for survival (n = 6-10). Differences in survival were determined using a log-rank test. B) Lungs and C) spleen of infected MyD88$^{-/-}$ and C57Bl/6J mice were harvested, homogenized and plated for CFUs at the indicated timepoints (n = 5-6 mice/ timepoint for each strain). All results are representative of at least three independent experiments. *p* values were determined using ANOVA analysis.

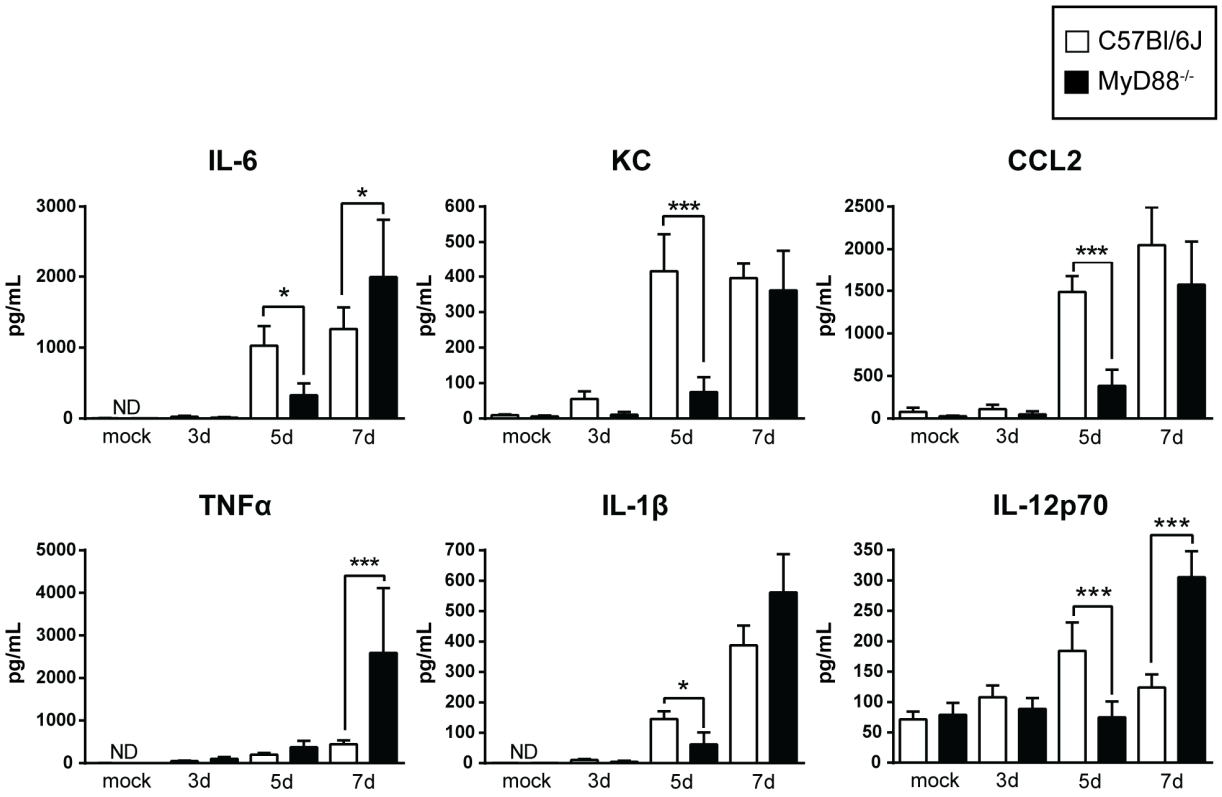


Figure 3.2. MyD88 is essential for the kinetics of normal cytokine production in the lungs of mice infected with *Histoplasma*

Lungs of MyD88$^{-/-}$ and C57Bl/6J infected mice were harvested, homogenized and evaluated for cytokine levels at the indicated timepoints. *p<0.05, **p<0.01, ***p<0.001, p values were determined by ANOVA analysis. All results are representative of at least three independent experiments. Bars represent the mean+SD for 5 mice.

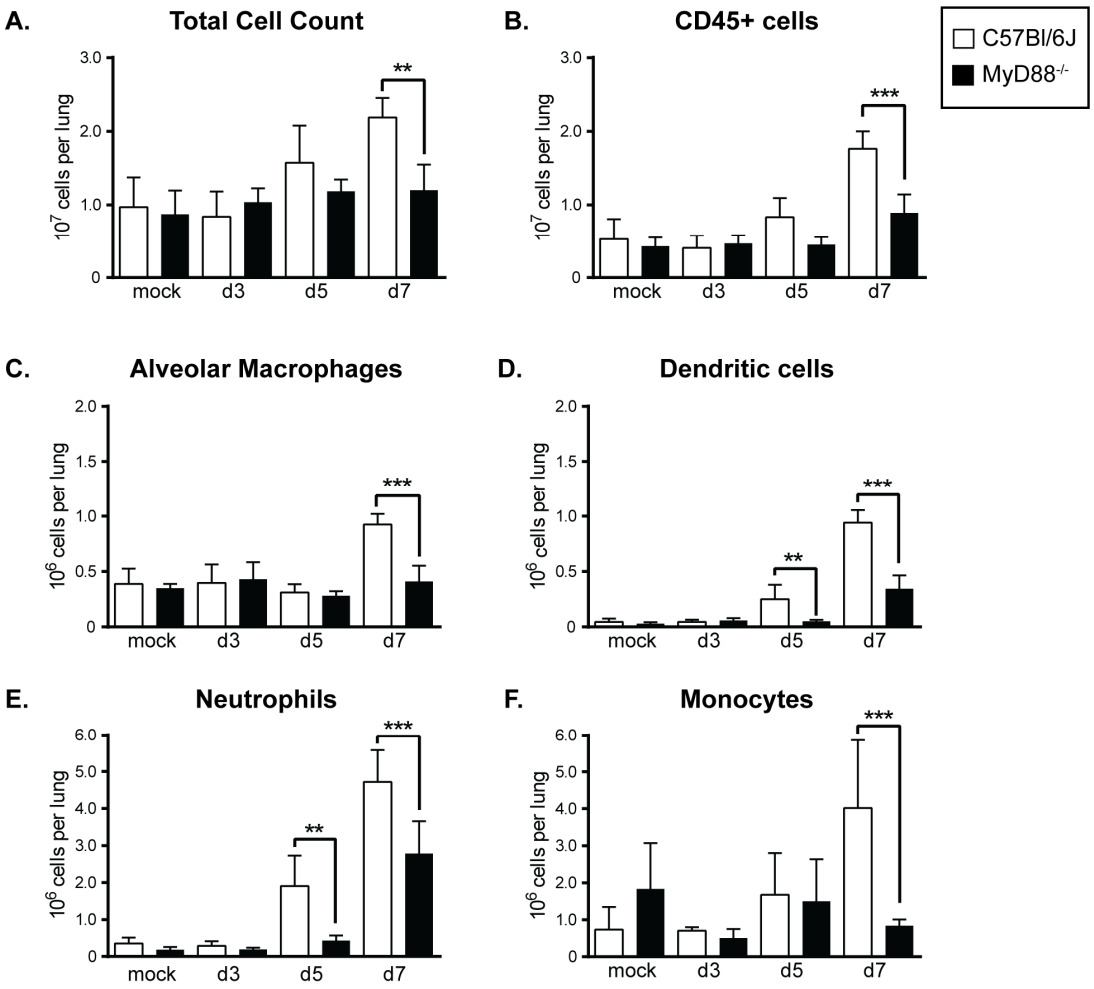


Figure 3.3. Inflammatory cell recruitment to the lung requires MyD88 signaling.
Infected lungs were dissociated into single cell suspensions and analyzed via flow cytometry to determine numbers of specific cell populations. A) Total lung cell counts. B) CD45+ cell counts. C) Alveolar macrophage cell count as defined by $CD11c^+$, $SiglecF^+$, autofluorescent cells. D) Myeloid dendritic cell count as defined by $CD11c^+$, $CD11b^+$, MHC Class II^{high} cells. E) Neutrophil cell count as defined by $CD11c^-$, $CD11b^+$, $Ly6C^+$, $Ly6G^+$ cells. F) Monocyte cell count as defined by $CD11c^-$, $CD11b^+$, $Ly6C^+$, $Ly6G^-$ cells. Bars represent the mean+SD of 5-6 mice. All results are representative of at least three independent experiments.**p<0.01, ***p<0.001, p values were determined using ANOVA analysis.

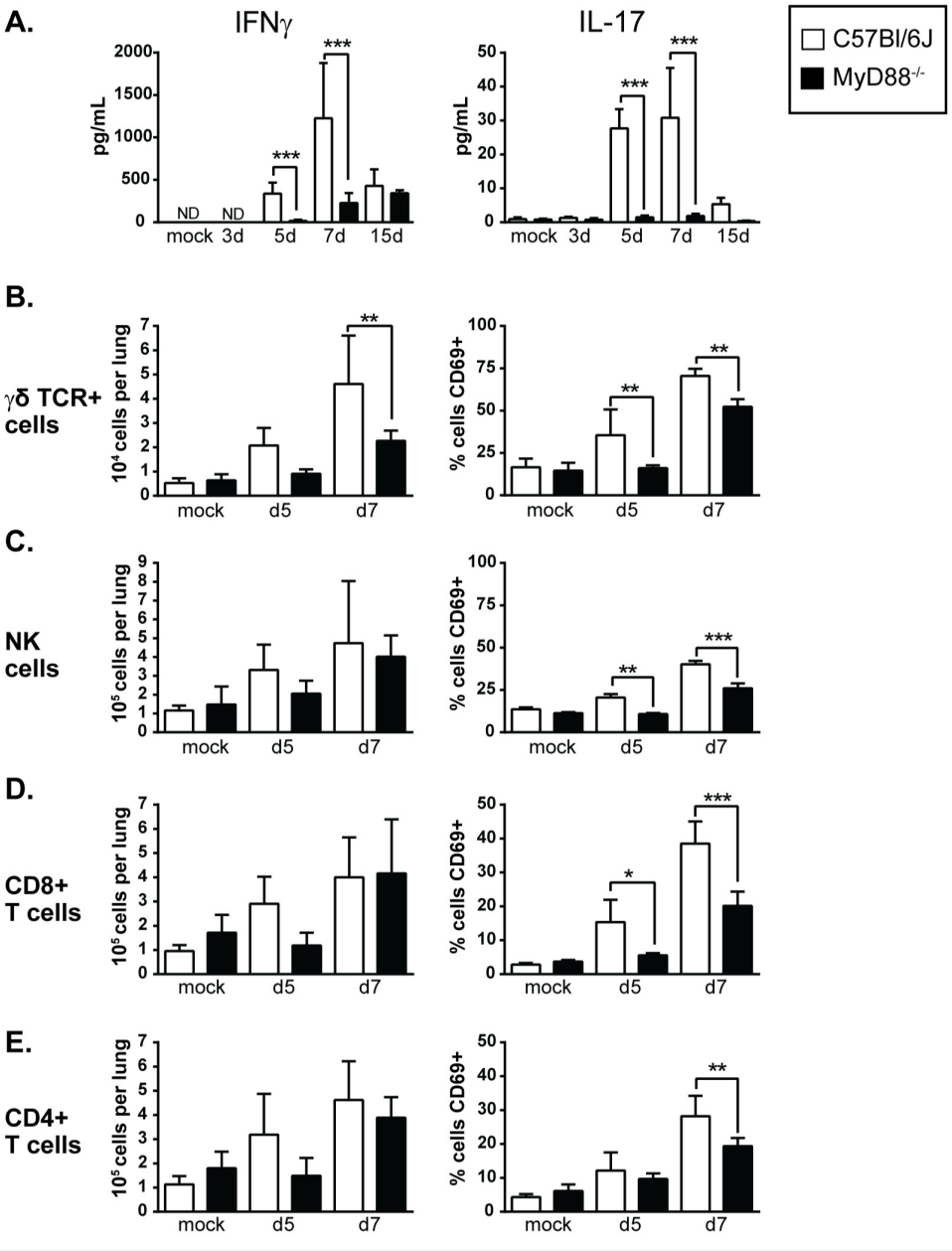


Figure 3.4. MyD88 is necessary for timely recruitment and activation of T and NK cells. ELISA analysis of global cytokine production in lungs of *Histoplasma* infected mice for A) total IFNy and Il-17A at 7 dpi. Analysis of 7 dpi lung cells counts and percent cells expressing CD69 for B) γδ TCR+ cells, C) NK cells, D) CD8+ T cells and E) CD4+ T cells. *p<0.05, **p<0.01, ***p<0.001, p values determined by ANOVA analysis.

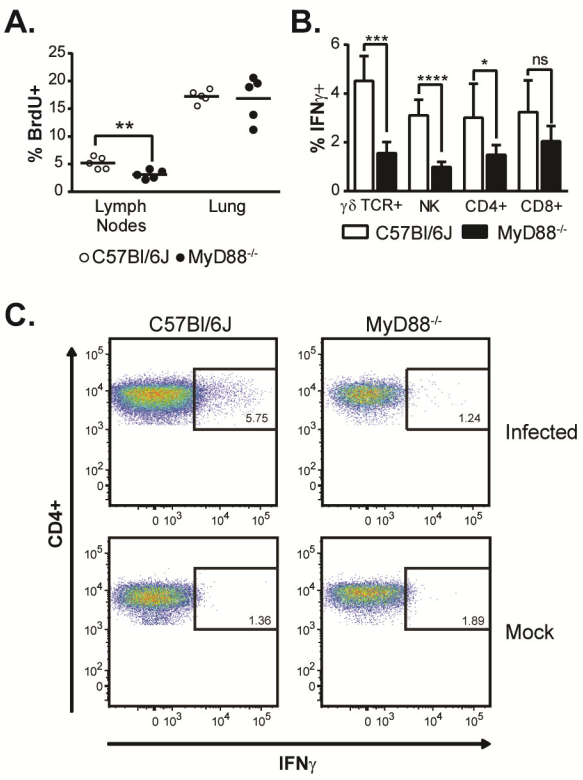


Figure 3.5. MyD88 is required for T cell proliferation and production of IFNγ by T and NK cells.

A) T cell proliferation in the lungs and mediastinal lymph nodes 7dpi as measured by *in vivo* BrdU incorporation. B) *Ex vivo* intracellular cytokine staining on lung cells 7 dpi to detect IFNγ production in γδ TCR+ cells, NK cells, CD4+ and CD8+ T cells isolated from the lung along with C) representative FACS plots for CD4+ gated populations. Bars represent the mean +SD of 5 mice. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, p values determined by ANOVA analysis.

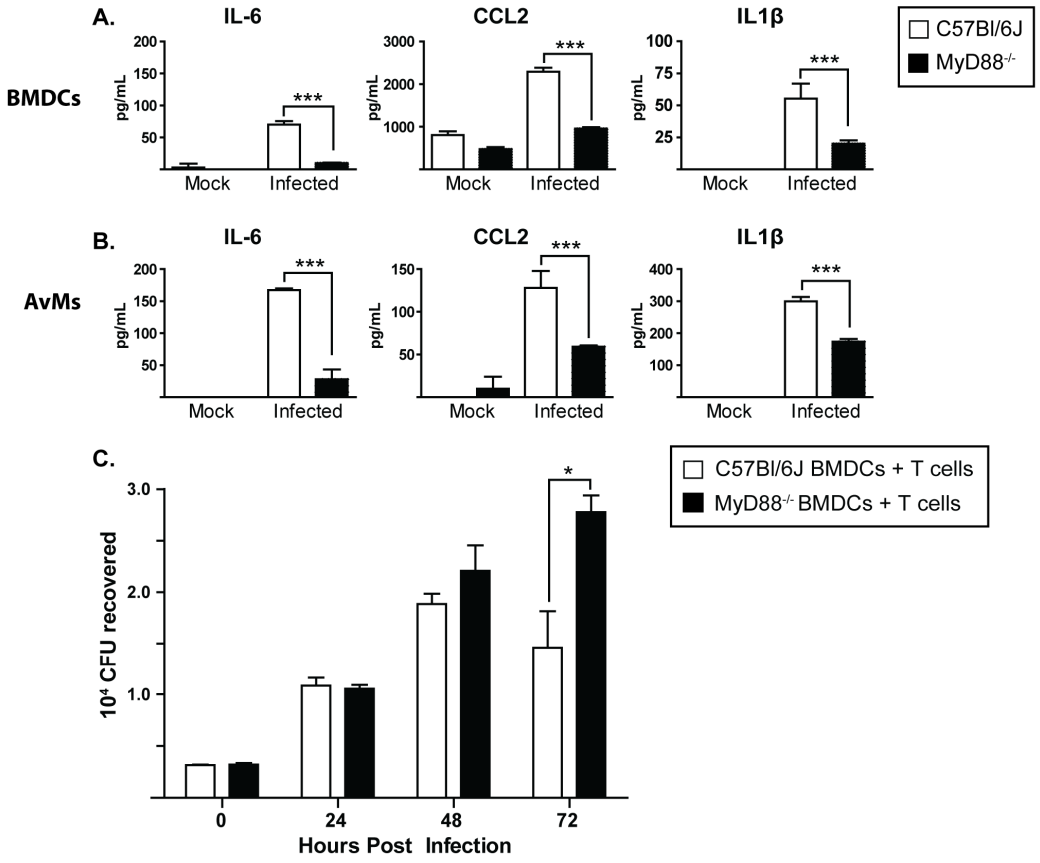


Figure 3.6. MyD88 is required for appropriate cytokine signaling in alveolar macrophages and bone-marrow derived dendritic cells *in vitro*.

A) Bone-marrow derived dendritic cells (BMDCs) and B) alveolar macrophages (AvMs) from WT and MyD88$^{-/-}$ mice were harvested and infected *in vitro* with *Histoplasma* at an MOI of 1. Supernatants from infected cells were collected in triplicate at 48 hours and evaluated for cytokine levels. C) BMDCs infected with wild-type *Histoplasma* (MOI=1) were co-cultured with purified splenic WT CD4+ T cells. Cells were lysed and CFUs counted at the indicated timepoints. *p<0.05, **p<0.01, ***p<0.001, p values determined by ANOVA analysis. Bars represent the mean +SD of 3-4 samples. All results are representative of at least three independent experiments.

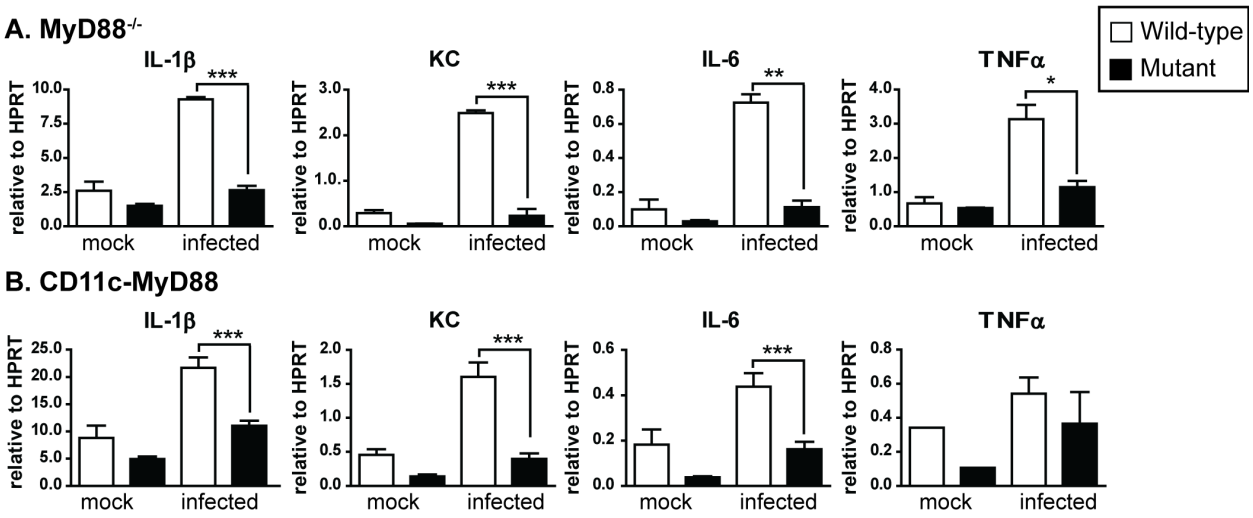


Figure 3.7. Alveolar macrophages and dendritic cells require MyD88 for cytokine gene expression *in vivo*.

Single cell suspensions were harvested from infected wild-type, MyD88$^{-/-}$ and CD11c-MyD88 mice at 3 dpi. CD11c+ (alveolar macrophages and dendritic cells) cells were purified from lung cells via magnetic bead separation, and subjected to RNA isolation followed by analysis of cytokine transcription. A) C57Bl/6J (Wild-type) and MyD88$^{-/-}$ (mutant) CD11c+ cells, B) MyD88$^{fl/fl}$ (Wild-type) and CD11c-MyD88 (mutant) CD11c+ cells. *p<0.05, **p<0.01, ***p<0.001, p values determined by ANOVA analysis. Bars represent the mean +SD of 3 samples harvested from 2-3 pooled mice. All results are representative of at least three independent experiments.

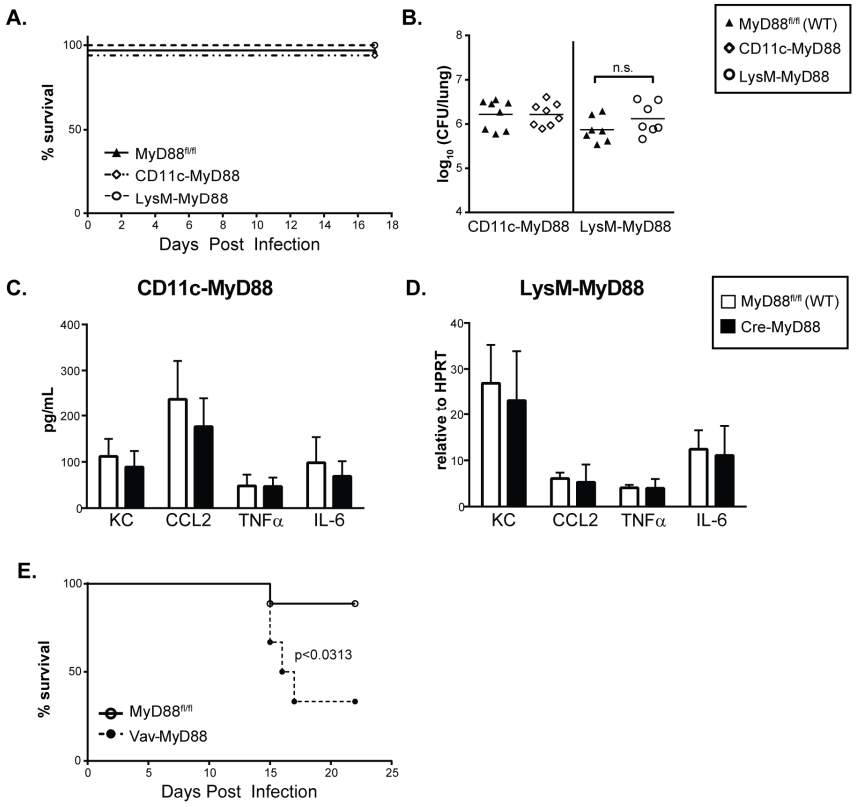
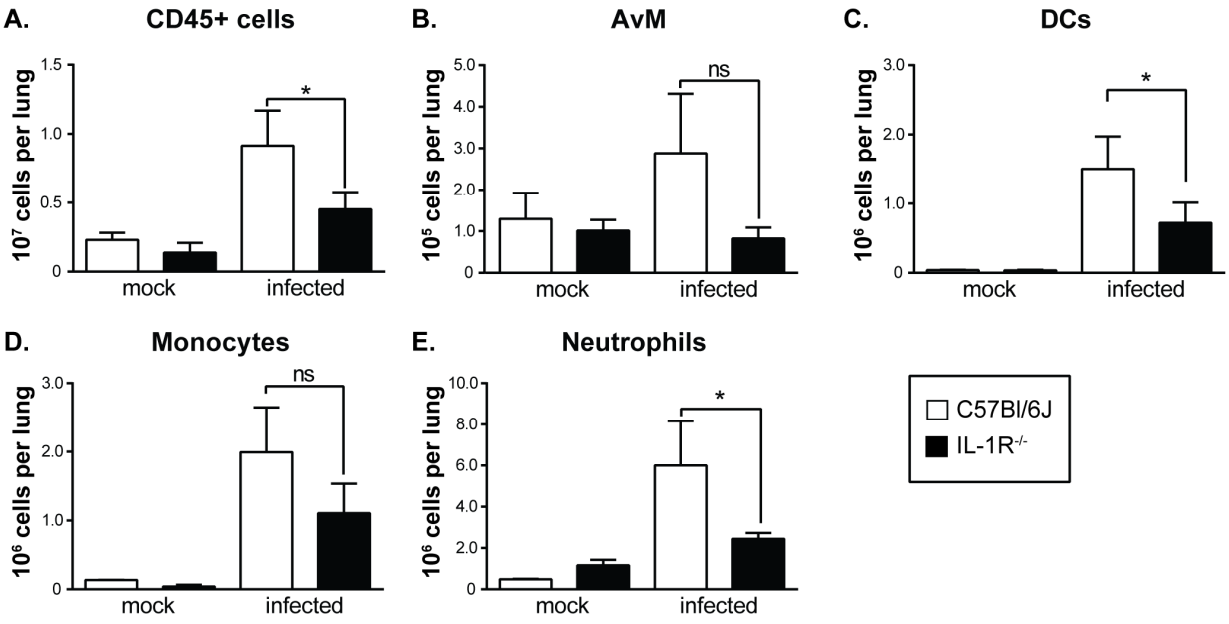


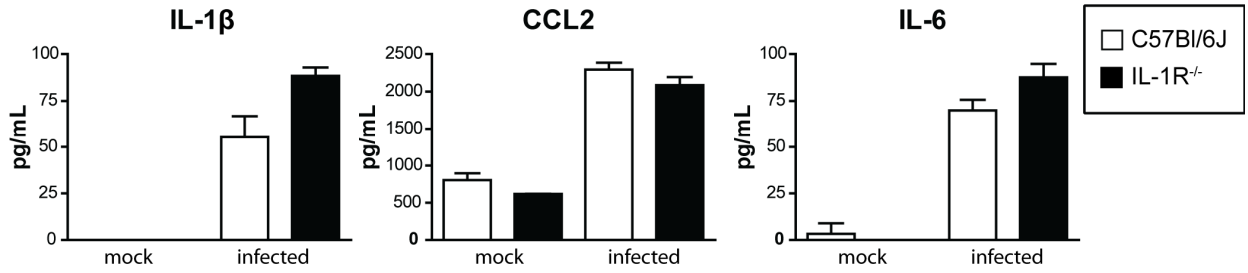
Figure 3.8: Loss of MyD88-dependent signaling in dendritic cells, alveolar macrophages or neutrophils is not sufficient to impair host survival, control of fungal growth, or global cytokine production in lungs.

MyD88$^{fl/fl}$ mice carrying the CD11c-Cre (CD11c-MyD88) or LysM-Cre (LysM-MyD88) transgene were infected with 1.6x10^4 *Histoplasma* and A) monitored for survival (n = 8-10 mice/strain) or B) fungal burden at 7 dpi. C) Whole lung homogenates of infected CD11c-MyD88 mice were analyzed for cytokine production at 5 dpi (n = 5-6 mice/strain). D) RNA harvested from whole lungs of infected LysM-MyD88 mice was analyzed via qRT-PCR for transcription of cytokines at 4 dpi (n = 3-5 mice/strain). E) MyD88$^{fl/fl}$ mice or MyD88$^{fl/fl}$ mice carrying the Vav-Cre transgene (Vav-MyD88$^{fl/fl}$) were infected with *Histoplasma* and monitored for survival (n = 6-9 mice/strain).



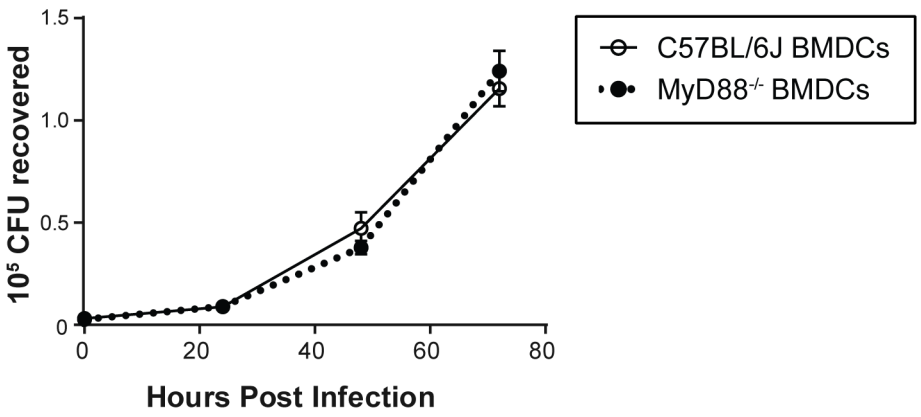
Supplementary Figure 3.1. IL-1R deficiency causes decreased inflammatory cell recruitment to the lung.

Infected lungs 5dpi were dissociated into single cell suspensions and analyzed via flow cytometry to determine numbers of specific cell populations. A) CD45+ cell counts. B) Alveolar macrophage cell count as defined by $CD11c^+$, $SiglecF^+$, autofluorescent cells. C) Dendritic cell count as defined by $CD11c^+$, $CD11b^+$, MHC Class II^{high} cells. D) Monocyte cell count as defined by $CD11c^-$, $CD11b^+$, side-scatter low, $Gr\text{-}1^+$ cells. E) Neutrophil cell count as defined by $CD11c^-$, $CD11b^+$, side-scatter high, $Gr\text{-}1^+$ cells. *$p<0.01$, p values were determined using ANOVA analysis.



Supplementary Figure 3.2. IL-1R deficiency does not affect cytokine signaling in bone marrow derived dendritic cells *in vitro*.

Bone-marrow derived dendritic cells from WT and IL-1R$^{-/-}$ mice were harvested and infected *in vitro* with *Histoplasma* at an MOI of 1. Supernatants from infected cells were collected in triplicate at 48 hours post-infection and evaluated for cytokine levels. $^{**}p<0.01$, $^{***}p<0.001$, *p* values were determined using ANOVA analysis.



Supplementary Figure 3.3. In the absence of T cells, *Histoplasma* grows similarly in wild-type and MyD88$^{-/-}$ BMDCs.

BMDCs were infected with wild-type *Histoplasma* (MOI=1). Cells were lysed and CFUs counted at the indicated timepoints. Timepoints are the mean +SD of three independent samples. All results are representative of at least three independent experiments.

Chapter 4: Conclusion

H. capsulatum is an intracellular fungal pathogen that colonizes the macrophage, disseminates throughout host organs, and causes significant disease if not checked by host immune responses. Understanding both *Histoplasma* survival inside host cells and host response to this unique eukaryotic and intracellular pathogen is an active area of research.

Identifying additional virulence factors in *Histoplasma*

In this work, we described an insertional mutagenesis screen for mutants of *Histoplasma* that fail to lyse macrophages. While we identified and characterized the *hcl1* mutant in this thesis, there remain additional lysis defective mutants that have yet to be characterized. We have identified and confirmed at least 26 lysis defective mutants, including a mutant in the previously identified virulence factor gene, *CBP1*. This leaves a rich and currently untapped source of *Histoplasma* virulence mechanisms to be identified. Future work includes confirming the involvement of the predicted gene affected by making complemented strains, and experiments to determine the role these genes play in *Histoplasma* virulence. Some predictions can be made based on homology to previously identified proteins and characterized domains. For example, some of the genes are predicted to be secreted proteins, suggesting that the proteins encoded by these genes potentially interact with and influence the host cell. Other identified mutants are defective in genes predicted to affect *Histoplasma* metabolism, like the *hcl1* mutant. While it remains a challenge to identify how these genes precisely contribute to the virulence of *Histoplasma*, the characterization of these remaining lysis defective mutants will help to inform and define our understanding of *Histoplasma* pathogenesis. Moreover, elucidating the cellular and molecular basis by which these genes contribute to *Histoplasma* survival within the

macrophage could have broader implications towards understanding survival mechanisms employed by other intracellular pathogens.

Future directions to define the role of Hcl1 in *Histoplasma* pathogenesis

Using an unbiased genetic approach, we identified an enzyme, HMG-CoA lyase, which is required for growth within macrophages, maintenance of a neutral phagosomal pH, and full virulence in the mouse. Our data suggests that the maintenance of a neutral phagosome during intracellular survival is critical for full pathogenesis of the fungus, as the HMG-CoA lyase mutant fails to maintain a neutral phagosomal pH. However, the relative contribution of phagosomal pH neutralization versus the ability to catabolize leucine to the survival *Histoplasma* within the macrophage is still unknown. In order to determine how an acidified phagosome contributes to the virulence defect observed in the *hcl1* mutant, it will be important to test if neutralization of the phagosome results in better survival of the mutant. Since the acidification of the *hcl1*-mutant containing phagosome is thought to be due to the build-up of acidic products produced by the mutant itself, neutralizing the phagosome cannot be done by blocking the intrinsic host mechanism for phagosome acidification, such as blocking the function of host vacuolar ATPase through the use of bafilomycin (137). Instead, infected macrophages could be treated with a weak base, such as chloroquine or ammonium chloride, to neutralize the phagosome and determine if, in a neutral environment, the *hcl1* mutant is virulent (18, 138).

Alternatively, it is possible that the inability to catabolize leucine may underlie the *hcl1* mutant defect in intracellular survival. In fact, research investigating *Candida* and *Samonella* macrophage interactions suggest that the macrophage phagosome is potentially a glucose low, amino-acid replete environment, which would make it advantageous for a pathogen to be able to utilize amino acids as carbon sources during intracellular growth (139, 140). Additionally, recent

work investigating the role of Stp2p, a *Candida* transcription factor that modulates the expression of amino acid permeases, suggests that amino acid catabolism contributes to the neutralization of acidic *Candida*-containing phagosomes (141). During the process of being utilized as a carbon source, amino acids are converted into tricarboxylic acid cycle intermediates via several methods, all of which remove the amine group. When *Candida* is grown in an amino acid rich environment, this amine group is predicted to be secreted as ammonia, subsequently neutralizing the surrounding environment. While the mechanism of *Histoplasma*-driven phagosomal neutralization is unknown, it is possible that catabolism of amino acids, similar to *C. albicans*, contributes to phagosomal pH neutralization in addition to being a potential carbon source for growth.

Future directions to further investigate the role of MyD88 during host response

In chapter 3, we identified an important role for the MyD88 signaling pathway in the host immune response to *Histoplasma*. We show that MyD88$^{-/-}$ mice are more susceptible to infection than Dectin1$^{-/-}$ and IL-1R$^{-/-}$ mice, and that MyD88 is required for control of fungal growth in the lungs and spleen. MyD88$^{-/-}$ mice fail to produce inflammatory cytokines, including CCL2 and IL-1β, that trigger critical events for controlling *Histoplasma* infection, such as the recruitment and activation of neutrophils and monocytes (53, 54). The production of cytokines appears to be partially mediated by a cell-intrinsic requirement for MyD88 in both alveolar macrophages and dendritic cells. In addition to the failure to enact key innate immune events, the Th1 response is also muted in the MyD88 deficient mouse, as IFNγ production by both innate (γδ TCR+ and NK cells) and adaptive (CD4+ T cells) cells is decreased. Ultimately, MyD88-dependent pathways

contribute to the appropriate activation of NK and T-cells to produce IFNγ, which leads to restriction of fungal growth by macrophages.

Interestingly, restriction of fungal growth by dendritic cells during incubation with primed T cells is also partly mediated by MyD88, as MyD88-deficient dendritic cells fail to restrict fungal growth similarly to wild-type dendritic cells. Our data indicates that cytokine production by dendritic cells is MyD88-dependent, and the production of cytokines could potentially influence the response of T cells to initiate fungal growth restriction by dendritic cells. However, the precise molecular mechanisms which drive this interaction remain unknown. Accordingly, future studies to clarify the role of MyD88 during the T cell response to *Histoplasma* may indicate that MyD88 signaling in dendritic cells is an important mediator of T cell activation. For example, while we showed that MyD88$^{-/-}$ dendritic cells fail to produce cytokines, we did not investigate the expression of co-stimulatory molecules, which may mediate the interaction between DC and T cells. It will also be important to determine if differences in T cell proliferation and IFNγ production can be observed in T cells co-cultured with MyD88$^{-/-}$ dendritic cells, which would indicate whether the activation of T cells is dependent on MyD88 signaling in dendritic cells.

An important question yet to be addressed is the relative contribution to host response by the pathways mediated by MyD88. MyD88 is a mediator of toll-like receptor signaling, as well as IL-1 and IL-18 cytokine receptor signaling. Previous work has demonstrated that IL-1R deficiency results in an increase in host susceptibility to *Histoplasma* (54). However, we found that IL-1R$^{-/-}$ mice display no defects in overall host survival when infected with a dose of *Histoplasma* that was lethal to MyD88$^{-/-}$ mice, although the IL-1R$^{-/-}$ mouse did display a similar defect in cell recruitment to the lungs as the MyD88$^{-/-}$ mouse. These results suggest that TLR

recognition and/or IL-18 signaling are also important for the host immune response. Studies to determine the contribution of TLR recognition to *Histoplasma in vivo* using multiple TLR knockouts, such as mice lacking TLR2, 4, and 9 or mice lacking TLR 7 and 9 are currently ongoing (134). In addition, since IL-18, in cooperation with IL-12, is known to induce a strong IFNγ response from T cells, it may be that the reduced ability of MyD88$^{-/-}$ mice to produce IFNγ reflects a failure of MyD88-dependent IL-18 signaling (135, 136).

Implications for treatment of histoplasmosis

In an immunocompromised host, invasive fungal infection can be lethal, especially in patients with HIV/AIDS, patients taking TNF inhibitors, or patients with primary immunodeficiencies that impact the IFNγ/IL-12 axis (5, 142). Although *Histoplasma* infection can be controlled by the administration of anti-fungals, the length of treatment is often significant, frequently results in nephro-toxicity, and can cause negative drug interactions that complicate patient care (143). Understanding the pathways involved in a protective immune response to *Histoplasma* unlocks the potential for immunomodulation that synergizes with anti-fungal treatment. In particular, current approaches that ameliorate host immune defects during immunodeficiency are particularly exciting. Successful examples of treatment of other fungal infections include the use of recombinant IFNγ and GM-CSF to treat refractory *Candida* and *Aspergillis* infections in leukemia patients (144, 145). These cytokines enhance the activity of neutrophils and macrophages in the host, allowing these cell types to better control fungal infection. This also boosts the immune function of immune deficient hosts to better fight infection, potentially allowing for the use of a less toxic or complicating anti-fungal treatment. Based on our work on MyD88 signaling in the host response, specific treatments that stimulate

MyD88 signaling, leading to the activation of effector cells and a Th1 response, may prove beneficial to the host during *Histoplasma* infection.

Further elucidation of the role of MyD88 signaling during *Histoplasma* infection may also help us more fully understand the mechanism of action for current treatment options. For example, polyenes such as amphotericin B, an antifungal drug most commonly used for severe cases of infection, trigger TLR2 signaling and result in the release of pro-inflammatory cytokines (146). While this has been associated with patient nephro-toxicity, it also has been shown to contribute to the anti-fungal response by inducing a protective Th1 response (147, 148). A greater understanding of how MyD88 signaling is modulated during amphotericin B treatment during *Histoplasma* infection could lead to the development of a more targeted and effective immuno-modulatory treatment. In fact, while the effects of an amphotericin B-mediated pro-inflammatory response have not been examined during *Histoplasma* infection, earlier studies have shown that *Histoplasma*-infected macrophages treated with both amphotericin B and recombinant-IFNγ demonstrate an increased oxidative burst compared to uninfected macrophages (149), suggesting a potential role for MyD88 signaling pathways in initiating the oxidative burst.

In conclusion, a more thorough understanding of how MyD88 signaling drives the immune response to *Histoplasma* is a crucial step in developing more effective treatment options for serious *Histoplasma* infection.

References

1. **Kauffman CA**. 2007. Histoplasmosis: a clinical and laboratory update. Clin. Microbiol. Rev. **20**:115–32.
2. **Assi MA, Sandid MS, Baddour LM, Roberts GD, Walker RC**. 2007. Systemic histoplasmosis: a 15-year retrospective institutional review of 111 patients. Medicine (Baltimore). **86**:162–9.
3. **Chu JH, Feudtner C, Heydon K, Walsh TJ, Zaoutis TE**. 2006. Hospitalizations for endemic mycoses: a population-based national study. Clin. Infect. Dis. **42**:822–5.
4. **Gutierrez ME, Canton A, Sosa N, Puga E, Talavera L**. 2005. Disseminated histoplasmosis in patients with AIDS in Panama: a review of 104 cases. Clin. Infect. Dis. **40**:1199–202.
5. **Smith JA, Kauffman CA**. 2009. Endemic fungal infections in patients receiving tumour necrosis factor-alpha inhibitor therapy. Drugs **69**:1403–1415.
6. **Russell D, Xu S, Chakraborty P**. 1992. Intracellular trafficking and the parasitophorous vacuole of *Leishmania mexicana*-infected macrophages. J. Cell Sci. **103**:1193–1210.
7. **Rohde K, Yates RM, Purdy GE, Russell DG**. 2007. *Mycobacterium tuberculosis* and the environment within the phagosome. Immunol. Rev. **219**:37–54.
8. **Tilney LG**. 1989. Actin filaments and the growth, movement, and spread of the intracellular bacterial parasite, *Listeria monocytogenes*. J. Cell Biol. **109**:1597–1608.
9. **Long KH, Gomez FJ, Morris RE, Newman SL**. 2003. Identification of Heat Shock Protein 60 as the Ligand on *Histoplasma capsulatum* That Mediates Binding to CD18 Receptors on Human Macrophages. J Immunol **170**:487–494.
10. **Ricardo-Carter C, Favila M, Polando RE, Cotton RN, Bogard Horner K, Condon D, Ballhorn W, Whitcomb JP, Yadav M, Geister RL, Schorey JS, McDowell MA**. 2013. *Leishmania major* inhibits IL-12 in macrophages by signalling through CR3 (CD11b/CD18) and down-regulation of ETS-mediated transcription. Parasite Immunol. **35**:409–20.
11. **Hajishengallis G, Wang M, Liang S, Shakhatreh M-AK, James D, Nishiyama S, Yoshimura F, Demuth DR**. 2008. Subversion of innate immunity by periodontopathic bacteria via exploitation of complement receptor-3. Adv. Exp. Med. Biol. **632**:203–19.
12. **Eissenberg LG, Schlesinger PH, Goldman WE**. 1988. Phagosome-lysosome fusion in P388D1 macrophages infected with *Histoplasma capsulatum*. J. Leukoc. Biol. **43**:483–91.
13. **Strasser JE, Newman SL, Ciraolo GM, Morris RE, Howell ML, Dean GE**. 1999. Regulation of the macrophage vacuolar ATPase and phagosome-lysosome fusion by *Histoplasma capsulatum*. J. Immunol. **162**:6148–54.
14. **Eissenberg LG, Goldman WE, Schlesinger PH**. 1993. *Histoplasma capsulatum* modulates the acidification of phagolysosomes. J. Exp. Med. **177**:1605–11.
15. **Hood MI, Skaar EP**. 2012. Nutritional immunity: transition metals at the pathogen-host interface. Nat. Rev. Microbiol. **10**:525–37.
16. **Winters MS, Chan Q, Caruso JA, Deepe GS**. 2010. Metallomic analysis of macrophages infected with *Histoplasma capsulatum* reveals a fundamental role for zinc in host defenses. J. Infect. Dis. **202**:1136–45.

17. **Nairz M, Fritsche G, Brunner P, Talasz H, Hantke K, Weiss G**. 2008. Interferon-gamma limits the availability of iron for intramacrophage *Salmonella typhimurium*. Eur. J. Immunol. **38**:1923–36.
18. **Newman SL, Gootee L, Brunner G, Deepe GS**. 1994. Chloroquine induces human macrophage killing of *Histoplasma capsulatum* by limiting the availability of intracellular iron and is therapeutic in a murine model of histoplasmosis. J. Clin. Invest. **93**:1422–9.
19. **Newman SL, Gootee L, Stroobant V, van der Goot H, Boelaert JR**. 1995. Inhibition of growth of *Histoplasma capsulatum* yeast cells in human macrophages by the iron chelator VUF 8514 and comparison of VUF 8514 with deferoxamine. Antimicrob. Agents Chemother. **39**:1824–9.
20. **Timmerman MM, Woods JP**. 1999. Ferric reduction is a potential iron acquisition mechanism for *Histoplasma capsulatum*. Infect. Immun. **67**:6403–8.
21. **Timmerman MM, Woods JP**. 2001. Potential role for extracellular glutathione-dependent ferric reductase in utilization of environmental and host ferric compounds by *Histoplasma capsulatum*. Infect. Immun. **69**:7671–8.
22. **Hilty J, George Smulian A, Newman SL**. 2011. *Histoplasma capsulatum* utilizes siderophores for intracellular iron acquisition in macrophages. Med. Mycol. **49**:633–42.
23. **Hwang LH, Mayfield JA, Rine J, Sil A**. 2008. *Histoplasma* requires SID1, a member of an iron-regulated siderophore gene cluster, for host colonization. PLoS Pathog. **4**:e1000044.
24. **Wolf JE, Kerchberger V, Kobayashi GS, Little JR**. 1987. Modulation of the macrophage oxidative burst by *Histoplasma capsulatum*. J. Immunol. **138**:582–6.
25. **Eissenberg LG, Goldman WE**. 1987. *Histoplasma capsulatum* fails to trigger release of superoxide from macrophages. Infect. Immun. **55**:29–34.
26. **Youseff BH, Holbrook ED, Smolnycki KA, Rappleye CA**. 2012. Extracellular superoxide dismutase protects *Histoplasma* yeast cells from host-derived oxidative stress. PLoS Pathog. **8**:e1002713.
27. **Holbrook ED, Smolnycki KA, Youseff BH, Rappleye CA**. 2013. Redundant catalases detoxify phagocyte reactive oxygen and facilitate *Histoplasma capsulatum* pathogenesis. Infect. Immun. **81**:2334–46.
28. **Aravalli RN, Hu S, Woods JP, Lokensgard JR**. 2008. *Histoplasma capsulatum* yeast phase-specific protein Yps3p induces Toll-like receptor 2 signaling. J. Neuroinflammation **5**:30.
29. **Bohse ML, Woods JP**. 2007. RNA interference-mediated silencing of the YPS3 gene of *Histoplasma capsulatum* reveals virulence defects. Infect. Immun. **75**:2811–7.
30. **Batanghari JW, Deepe GS, Di Cera E, Goldman WE**. 1998. *Histoplasma* acquisition of calcium and expression of CBP1 during intracellular parasitism. Mol. Microbiol. **27**:531–9.
31. **Sebghati TS, Engle JT, Goldman WE**. 2000. Intracellular parasitism by *Histoplasma capsulatum*: fungal virulence and calcium dependence. Science **290**:1368–72.
32. **Beck MR, Dekoster GT, Hambly DM, Gross ML, Cistola DP, Goldman WE**. 2008. Structural Features Responsible for the Biological Stability of *Histoplasma*'s Virulence Factor CBP. Biochemistry **47**:4427–38.
33. **Beck MR, Dekoster GT, Cistola DP, Goldman WE**. 2009. NMR structure of a fungal virulence factor reveals structural homology with mammalian saposin B. Mol. Microbiol. **72**:344–53.

34. **Lin J-S, Wu-Hsieh BA**. 2004. Functional T cells in primary immune response to histoplasmosis. Int. Immunol. **16**:1663–73.
35. **Gomez AM, Bullock WE, Taylor CL, Deepe GS**. 1988. Role of L3T4+ T cells in host defense against *Histoplasma capsulatum*. Infect. Immun. **56**:1685–91.
36. **Zhou P, Miller G, Seder RA**. 1998. Factors Involved in Regulating Primary and Secondary Immunity to Infection with *Histoplasma capsulatum*: TNF-{alpha} Plays a Critical Role in Maintaining Secondary Immunity in the Absence of IFN-{gamma}. J Immunol **160**:1359–1368.
37. **Kurita N, Brummer E, Yoshida S, Nishimura K, Miyaji M**. 1991. Antifungal activity of murine polymorphonuclear neutrophils against *Histoplasma capsulatum*. J. Med. Vet. Mycol. **29**:133–43.
38. **Gildea LA, Morris RE, Newman SL**. 2001. *Histoplasma capsulatum* yeasts are phagocytosed via very late antigen-5, killed, and processed for antigen presentation by human dendritic cells. J. Immunol. **166**:1049–56.
39. **Lin JS, Huang JH, Hung LY, Wu SY, Wu-Hsieh BA**. 2010. Distinct roles of complement receptor 3, Dectin-1, and sialic acids in murine macrophage interaction with *Histoplasma* yeast. J. Leukoc. Biol. **88**:95–106.
40. **Coady A, Sil A**. 2014. MyD88-dependent signaling drives host survival and early cytokine production during *Histoplasma* infection. Infect. Immun.
41. **Wu-Hsieh BA, Howard DH**. 1987. Inhibition of the intracellular growth of *Histoplasma capsulatum* by recombinant murine gamma interferon. Infect. Immun. **55**:1014–6.
42. **Wu S-Y, Yu J-S, Liu F-T, Miaw S-C, Wu-Hsieh BA**. 2013. Galectin-3 negatively regulates dendritic cell production of IL-23/IL-17-axis cytokines in infection by *Histoplasma capsulatum*. J. Immunol. **190**:3427–37.
43. **Newman SL, Gootee L**. 1992. Colony-stimulating factors activate human macrophages to inhibit intracellular growth of *Histoplasma capsulatum* yeasts. Infect. Immun. **60**:4593–7.
44. **Lane TE, Otero GC, Wu-Hsieh BA, Howard DH**. 1994. Expression of inducible nitric oxide synthase by stimulated macrophages correlates with their anti-*Histoplasma* activity. Infect. Immun. **62**:1478–1479.
45. **Lane TE, Wu-Hsieh BA, Howard DH**. 1991. Iron limitation and the gamma interferon-mediated anti-*Histoplasma* state of murine macrophages. Infect. Immun. **59**:2274–8.
46. **Lin JS, Yang CW, Wang DW, Wu-Hsieh BA**. 2005. Dendritic cells cross-present exogenous fungal antigens to stimulate a protective CD8 T cell response in infection by *Histoplasma capsulatum*. J. Immunol. **174**:6282–91.
47. **Gildea LA, Ciraolo GM, Morris RE, Newman SL**. 2005. Human dendritic cell activity against *Histoplasma capsulatum* is mediated via phagolysosomal fusion. Infect. Immun. **73**:6803–11.
48. **Schnur RA, Newman SL**. 1990. The respiratory burst response to *Histoplasma capsulatum* by human neutrophils. Evidence for intracellular trapping of superoxide anion. J. Immunol. **144**:4765–72.
49. **Newman SL, Gootee L, Gabay JE**. 1993. Human neutrophil-mediated fungistasis against *Histoplasma capsulatum*. Localization of fungistatic activity to the azurophil granules. J. Clin. Invest. **92**:624–31.
50. **Newman SL, Gootee L, Gabay JE, Selsted ME**. 2000. Identification of constituents of human neutrophil azurophil granules that mediate fungistasis against *Histoplasma capsulatum*. Infect. Immun. **68**:5668–72.

51. **Couto MA, Liu L, Lehrer RI, Ganz T**. 1994. Inhibition of intracellular *Histoplasma capsulatum* replication by murine macrophages that produce human defensin. Infect. Immun. **62**:2375–8.
52. **Serbina N V, Jia T, Hohl TM, Pamer EG**. 2008. Monocyte-mediated defense against microbial pathogens. Annu. Rev. Immunol. **26**:421–452.
53. **Szymczak WA, Deepe GS**. 2009. The CCL7-CCL2-CCR2 axis regulates IL-4 production in lungs and fungal immunity. J. Immunol. **183**:1964–74.
54. **Deepe GS, McGuinness M**. 2006. Interleukin-1 and host control of pulmonary histoplasmosis. J. Infect. Dis. **194**:855–64.
55. **Deepe GS, Gibbons R, Woodward E**. 1999. Neutralization of endogenous granulocyte-macrophage colony-stimulating factor subverts the protective immune response to *Histoplasma capsulatum*. J. Immunol. **163**:4985–93.
56. **Allendoerfer R, Deepe GS**. 1998. Blockade of Endogenous TNF-α Exacerbates Primary and Secondary Pulmonary Histoplasmosis by Differential Mechanisms. J Immunol **160**:6072–6082.
57. **Zhou P, Sieve MC, Bennett J, Kwon-Chung KJ, Tewari RP, Gazzinelli RT, Sher A, Seder RA**. 1995. IL-12 prevents mortality in mice infected with *Histoplasma capsulatum* through induction of IFN-gamma. J. Immunol. **155**:785–95.
58. **Sampaio EP, Hsu AP, Pechacek J, Bax HI, Dias DL, Paulson ML, Chandrasekaran P, Rosen LB, Carvalho DS, Ding L, Vinh DC, Browne SK, Datta S, Milner JD, Kuhns DB, Long Priel DA, Sadat MA, Shiloh M, De Marco B, Alvares M, Gillman JW, Ramarathnam V, de la Morena M, Bezrodnik L, Moreira I, Uzel G, Johnson D, Spalding C, Zerbe CS, Wiley H, Greenberg DE, Hoover SE, Rosenzweig SD, Galgiani JN, Holland SM**. 2013. Signal transducer and activator of transcription 1 (STAT1) gain-of-function mutations and disseminated coccidioidomycosis and histoplasmosis. J. Allergy Clin. Immunol. **131**:1624–34.
59. **Zerbe CS, Holland SM**. 2005. Disseminated histoplasmosis in persons with interferon-gamma receptor 1 deficiency. Clin. Infect. Dis. **41**:e38–41.
60. **Lionakis MS**. 2012. Genetic Susceptibility to Fungal Infections in Humans. Curr. Fungal Infect. Rep. **6**:11–22.
61. **Hage CA, Bowyer S, Tarvin SE, Helper D, Kleiman MB, Wheat LJ**. 2010. Recognition, diagnosis, and treatment of histoplasmosis complicating tumor necrosis factor blocker therapy. Clin. Infect. Dis. **50**:85–92.
62. **Deepe GS, Gibbons RS**. 2009. Interleukins 17 and 23 influence the host response to *Histoplasma capsulatum*. J. Infect. Dis. **200**:142–51.
63. **Deepe GS**. 2000. Immune response to early and late *Histoplasma capsulatum* infections. Curr. Opin. Microbiol. **3**:359–62.
64. **Kroetz DN, Deepe GS**. 2011. The role of cytokines and chemokines in *Histoplasma capsulatum* infection. Cytokine.
65. **Allendoerfer R, Biovin GP, Deepe GS**. 1997. Modulation of immune responses in murine pulmonary histoplasmosis. J. Infect. Dis. **175**:905–14.
66. **Allendoerfer R, Magee DM, Deepe GS, Graybill JR**. 1993. Transfer of protective immunity in murine histoplasmosis by a CD4+ T-cell clone. Infect. Immun. **61**:714–8.
67. **Wheat LJ, Connolly-Stringfield PA, Baker RL, Curfman MF, Eads ME, Israel KS, Norris SA, Webb DH, Zeckel ML**. 1990. Disseminated histoplasmosis in the acquired

immune deficiency syndrome: clinical findings, diagnosis and treatment, and review of the literature. Medicine (Baltimore). **69**:361–74.
68. **Deepe GS**. 1994. Role of CD8+ T cells in host resistance to systemic infection with *Histoplasma capsulatum* in mice. J. Immunol. **152**:3491–500.
69. **Clemons K, Darbonne W, Curnutte J, Sobel R, Stevens D**. 2000. Experimental histoplasmosis in mice treated with anti-murine interferon-gamma antibody and in interferon-gamma gene knockout mice. Microbes Infect. **2**:997–1001.
70. **Allendörfer R, Brunner GD, Deepe GS**. 1999. Complex requirements for nascent and memory immunity in pulmonary histoplasmosis. J. Immunol. **162**:7389–96.
71. **Okamura H, Kashiwamura S, Tsutsui H, Yoshimoto T, Nakanishi K**. 1998. Regulation of interferon-gamma production by IL-12 and IL-18. Curr. Opin. Immunol. **10**:259–64.
72. **Marth T, Kelsall BL**. 1997. Regulation of Interleukin-12 by Complement Receptor 3 Signaling. J. Exp. Med. **185**:1987–1995.
73. **Romani L**. 2011. Immunity to fungal infections. Nat. Rev. Immunol. **11**:275–288.
74. **Hardison SE, Brown GD**. 2012. C-type lectin receptors orchestrate antifungal immunity. Nat. Immunol. **13**:817–22.
75. **Wang H, LeBert V, Hung CY, Galles K, Saijo S, Lin X, Cole GT, Klein BS, Wüthrich M**. 2014. C-type lectin receptors differentially induce th17 cells and vaccine immunity to the endemic mycosis of North America. J. Immunol. **192**:1107–19.
76. **Geijtenbeek TBH, Gringhuis SI**. 2009. Signalling through C-type lectin receptors: shaping immune responses. Nat. Rev. Immunol. **9**:465–79.
77. **Edwards JA, Alore E, Rappleye CA**. 2011. The yeast-phase virulence requirement for α-glucan synthase differs among *Histoplasma capsulatum* chemotypes. Eukaryot. Cell **10**:87–97.
78. **Rappleye CA, Eissenberg LG, Goldman WE**. 2007. *Histoplasma capsulatum* α-(1,3)-glucan blocks innate immune recognition by the β-glucan receptor. Proc. Natl. Acad. Sci. **104**:1366–1370.
79. **Netea MG, Ferwerda G, van der Graaf CAA, Van der Meer JWM, Kullberg BJ**. 2006. Recognition of fungal pathogens by toll-like receptors. Curr. Pharm. Des. **12**:4195–201.
80. **Levitz SM**. 2004. Interactions of Toll-like receptors with fungi. Microbes Infect. **6**:1351–5.
81. **Adachi O, Kawai T, Takeda K, Matsumoto M, Tsutsui H, Sakagami M, Nakanishi K, Akira S**. 1998. Targeted disruption of the MyD88 gene results in loss of IL-1-and IL-18-mediated function. Immunity **9**:143–150.
82. **Medzhitov R, Preston-Hurlburt P, Kopp E, Stadlen A, Chen C, Ghosh S, Janeway CA**. 1998. MyD88 Is an Adaptor Protein in the hToll/IL-1 Receptor Family Signaling Pathways. Mol. Cell **2**:253–258.
83. **Bellocchio S, Montagnoli C, Bozza S, Gaziano R, Rossi G, Mambula SS, Vecchi A, Mantovani A, Levitz SM, Romani L**. 2004. The contribution of the Toll-like/IL-1 receptor superfamily to innate and adaptive immunity to fungal pathogens in vivo. J. Immunol. **172**:3059–69.
84. **Bretz C, Gersuk G, Knoblaugh S, Chaudhary N, Randolph-Habecker J, Hackman RC, Staab J, Marr KA**. 2008. MyD88 signaling contributes to early pulmonary responses to *Aspergillus fumigatus*. Infect. Immun. **76**:952–958.

85. **Russell DG**. 2011. *Mycobacterium tuberculosis* and the intimate discourse of a chronic infection. Immunol. Rev. **240**:252–268.
86. **Cano M V, Hajjeh RA**. 2001. The epidemiology of histoplasmosis: a review. Semin. Respir. Infect. **16**:109–118.
87. **Worsham PL, Goldman WE**. 1988. Quantitative plating of *Histoplasma capsulatum* without addition of conditioned medium or siderophores. J. Med. Vet. Mycol. **26**:137–43.
88. **Nguyen VQ, Sil A**. 2008. Temperature-induced switch to the pathogenic yeast form of *Histoplasma capsulatum* requires Ryp1, a conserved transcriptional regulator. Proc. Natl. Acad. Sci. U. S. A. **105**:4880–4885.
89. **Hwang L, Hocking-Murray D, Bahrami AK, Andersson M, Rine J, Sil A**. 2003. Identifying phase-specific genes in the fungal pathogen *Histoplasma capsulatum* using a genomic shotgun microarray. Mol. Biol. Cell **14**:2314–2326.
90. **Webster RH, Sil A**. 2008. Conserved factors Ryp2 and Ryp3 control cell morphology and infectious spore formation in the fungal pathogen *Histoplasma capsulatum*. Proc. Natl. Acad. Sci. U. S. A. **105**:14573–14578.
91. **Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ**. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25**:3389–3402.
92. **Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG**. 2007. Clustal W and Clustal X version 2.0. Bioinformatics **23**:2947–2948.
93. **Sullivan TD, Rooney PJ, Klein BS**. 2002. *Agrobacterium tumefaciens* integrates transfer DNA into single chromosomal sites of dimorphic fungi and yields homokaryotic progeny from multinucleate yeast. Eukaryot. Cell **1**:895–905.
94. **Roberts JR, Mitchell GA, Miziorko HM**. 1996. Modeling of a Mutation Responsible for Human 3-Hydroxy-3-methylglutaryl-CoA Lyase Deficiency Implicates Histidine 233as an Active Site Residue. J. Biol. Chem. **271**:24604–24609.
95. **Roberts JR, Narasimhan C, Miziorko HM**. 1995. Evaluation of cysteine 266 of human 3-hydroxy-3-methylglutaryl-CoA lyase as a catalytic residue. J. Biol. Chem. **270**:17311–17316.
96. **Tuinstra RL, Miziorko HM**. 2003. Investigation of conserved acidic residues in 3-hydroxy-3-methylglutaryl-CoA lyase: implications for human disease and for functional roles in a family of related proteins. J. Biol. Chem. **278**:37092–37098.
97. **Tuinstra RL, Wang C-Z, Mitchell GA, Miziorko HM**. 2004. Evaluation of 3-hydroxy-3-methylglutaryl-coenzyme A lyase arginine-41 as a catalytic residue: use of acetyldithio-coenzyme A to monitor product enolization. Biochemistry **43**:5287–5295.
98. **Aguilar J, Zavala A, Diaz-Perez C, Cervantes C, Diaz-Perez A, Campos-Garcia J**. 2006. The atu and liu clusters are involved in the catabolic pathways for acyclic monoterpenes and leucine in *Pseudomonas aeruginosa*. Appl. Environ. Microbiol. **72**:2070.
99. **Chavez-Aviles M, Diaz-Perez AL, Cruz H, Campos-Garcia J, FEMS**. 2009. The *Pseudomonas aeruginosa* liuE gene encodes the 3-hydroxy-3-methylglutaryl coenzyme A lyase, involved in leucine and acyclic terpene catabolism. Lett **296**:117–123.
100. **Pié J, López-Viñas E, Puisac B, Menao S, Pié A, Casale C, Ramos FJ, Hegardt FG, Gomez-Puertas P, Casals N, Pie J, Lopez-Vinas E, Pie A**. 2007. Molecular genetics of HMG-CoA lyase deficiency. Mol. Genet. Metab. **92**:198–209.

101. **Rodriguez JM, Ruiz-Sala P, Ugarte M, Peñalva MA**. 2004. Fungal Metabolic Model for Type I 3-Methylglutaconic Aciduria. J. Biol. Chem. **279**:32385–32392.
102. **Berliner MD**. 1973. *Histoplasma capsulatum*: effects of pH on the yeast and mycelial phases in vitro. Sabouraudia **11**:267–270.
103. **Youseff BH, Holbrook ED, Smolnycki KA, Rappleye CA, S ,**. 2012. Extracellular superoxide dismutase protects *Histoplasma* yeast cells from host-derived oxidative stress.
104. **Naderer T, Ellis MA, Sernee MF, De Souza DP, Curtis J, Handman E, McConville MJ**. 2006. Virulence of *Leishmania major* in macrophages and mice requires the gluconeogenic enzyme fructose-1,6-bisphosphatase. Proc. Natl. Acad. Sci. U. S. A. **103**:5502–5507.
105. **McKinney JD, zu Bentrup K, Muñoz-Elías EJ, Miczak A, Chen B, Chan WT, Swenson D, Sacchettini JC, Jacobs WR, Russell DG**. 2000. Persistence of Mycobacterium tuberculosis in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. Nature **406**:735–738.
106. **Lorenz MC, Fink GR**. 2001. The glyoxylate cycle is required for fungal virulence. Nature **412**:83–86.
107. **Mitchell GA, Wang SP, Ashmarina L, Robert MF, Bouchard G, Laurin N, Kassovska-Bratinova S, Boukaftane Y**. 1998. Inborn errors of ketogenesis. Biochem. Soc. Trans. **26**:136–140.
108. **Hoebe K, Janssen E, Beutler B**. 2004. The interface between innate and adaptive immunity. Nat. Immunol. **5**:971–4.
109. **Martin TR, Frevert CW**. 2005. Innate immunity in the lungs. Proc. Am. Thorac. Soc. **2**:403–11.
110. **Allendoerfer R, Deepe GS**. 1997. Intrapulmonary response to *Histoplasma capsulatum* in gamma interferon knockout mice. Infect. Immun. **65**:2564–9.
111. **Hou B, Reizis B, Defranco AL**. 2008. Toll-like receptors activate innate and adaptive immunity by using dendritic cell-intrinsic and -extrinsic mechanisms. Immunity **29**:272–282.
112. **Hou B, Benson A, Kuzmich L, DeFranco AL, Yarovinsky F**. 2010. Critical coordination of innate immune defense against *Toxoplasma gondii* by dendritic cells responding via their Toll-like receptors. Proc. Natl. Acad. Sci. 2–7.
113. **Wang X, Seed B**. 2003. A PCR primer bank for quantitative gene expression analysis. Nucleic Acids Res. **31**:e154.
114. **Spandidos A, Wang X, Wang H, Dragnev S, Thurber T, Seed B**. 2008. A comprehensive collection of experimentally validated primers for Polymerase Chain Reaction quantitation of murine transcript abundance. BMC Genomics **9**:633.
115. **Spandidos A, Wang X, Wang H, Seed B**. 2010. PrimerBank: a resource of human and mouse PCR primer pairs for gene expression detection and quantification. Nucleic Acids Res. **38**:D792–9.
116. **Adachi Y, Ishii T, Ikeda Y, Hoshino A, Tamura H, Aketagawa J, Tanaka S, Ohno N**. 2004. Characterization of beta-glucan recognition site on C-type lectin, Dectin 1. Infect. Immun. **72**:4159–71.
117. **Vantourout P, Hayday A**. 2013. Six-of-the-best: unique contributions of γδ T cells to immunology. Nat. Rev. Immunol. **13**:88–100.
118. **Cain JA, Deepe GS. J**. 1998. Evolution of the Primary Immune Response to *Histoplasma capsulatum* in Murine Lung. Infect. Immun. **66**:1473–1481.

119. **Sancho D, Gómez M, Sánchez-Madrid F**. 2005. CD69 is an immunoregulatory molecule induced following activation. Trends Immunol. **26**:136–40.
120. **Chen K, Kolls JK**. 2013. T cell-mediated host immune defenses in the lung. Annu. Rev. Immunol. **31**:605–33.
121. **Deepe GS, Gibbons RS, Smulian AG, Deep GS**. 2008. *Histoplasma capsulatum* manifests preferential invasion of phagocytic subpopulations in murine lungs. J. Leukoc. Biol. **84**:669–78.
122. **Fermin Lee A, Chen HY, Wan L, Wu SY, Yu JS, Huang AC, Miaw SC, Hsu DK, Wu-Hsieh BA, Liu FT**. 2013. Galectin-3 Modulates Th17 Responses by Regulating Dendritic Cell Cytokines. Am. J. Pathol. **183**:1209–22.
123. **Villamón E, Gozalbo D, Roig P, Murciano C, O'Connor JE, Fradelizi D, Gil ML**. Myeloid differentiation factor 88 (MyD88) is required for murine resistance to *Candida albicans* and is critically involved in *Candida* -induced production of cytokines. Eur. Cytokine Netw. **15**:263–71.
124. **Loures F V, Pina A, Felonato M, Feriotti C, de Araújo EF, Calich VLG**. 2011. MyD88 signaling is required for efficient innate and adaptive immune responses to *Paracoccidioides brasiliensis* infection. Infect. Immun. **79**:2470–80.
125. **Marr K, Arunmozhi Balajee S, Hawn TR, Ozinsky A, Pham U, Akira S, Aderem A, Conrad Liles W, Balajee S, Hawn T**. 2003. Differential role of MyD88 in macrophage-mediated responses to opportunistic fungal pathogens. Infect. … **71**:5280–5286.
126. **Yauch LE, Mansour MK, Shoham S, Rottman JB, Levitz SM**. 2004. Involvement of CD14, toll-like receptors 2 and 4, and MyD88 in the host response to the fungal pathogen *Cryptococcus neoformans in vivo*. Infect. Immun. **72**:5373–82.
127. **Rivera A, Ro G, Van Epps HL, Simpson T, Leiner I, Sant'Angelo DB, Pamer EG**. 2006. Innate immune activation and CD4+ T cell priming during respiratory fungal infection. Immunity **25**:665–75.
128. **Bellocchio S, Montagnoli C, Bozza S, Gaziano R, Rossi G, Mambula SS, Vecchi A, Mantovani A, Levitz SM, Romani L**. 2004. The Contribution of the Toll-Like/IL-1 Receptor Superfamily to Innate and Adaptive Immunity to Fungal Pathogens In Vivo. J. Immunol. **172**:3059–3069.
129. **Spörri R, Reis e Sousa C**. 2005. Inflammatory mediators are insufficient for full dendritic cell activation and promote expansion of CD4+ T cell populations lacking helper function. Nat. Immunol. **6**:163–70.
130. **Gallucci S, Lolkema M, Matzinger P**. 1999. Natural adjuvants: endogenous activators of dendritic cells. Nat. Med. **5**:1249–55.
131. **Trevejo JM, Marino MW, Philpott N, Josien R, Richards EC, Elkon KB, Falck-Pedersen E**. 2001. TNF-alpha -dependent maturation of local dendritic cells is critical for activating the adaptive immune response to virus infection. Proc. Natl. Acad. Sci. U. S. A. **98**:12162–7.
132. **Aline F, Bout D, Dimier-Poisson I**. 2002. Dendritic Cells as Effector Cells: Gamma Interferon Activation of Murine Dendritic Cells Triggers Oxygen-Dependent Inhibition of Toxoplasma gondii Replication. Infect. Immun. **70**:2368–2374.
133. **Brown GD**. 2011. Innate antifungal immunity: the key role of phagocytes. Annu. Rev. Immunol. **29**:1–21.

134. **Arpaia N, Godec J, Lau L, Sivick KE, McLaughlin LM, Jones MB, Dracheva T, Peterson SN, Monack DM, Barton GM**. 2011. TLR signaling is required for *Salmonella typhimurium* virulence. Cell **144**:675–88.
135. **Nakanishi K, Yoshimoto T, Tsutsui H, Okamura H**. 2001. Interleukin-18 regulates both Th1 and Th2 responses. Annu. Rev. Immunol. **19**:423–74.
136. **Akira S**. 2000. The role of IL-18 in innate immunity. Curr. Opin. Immunol. **12**:59–63.
137. **Bowman EJ, Siebers A, Altendorf K**. 1988. Bafilomycins: a class of inhibitors of membrane ATPases from microorganisms, animal cells, and plant cells. Proc. Natl. Acad. Sci. U. S. A. **85**:7972–6.
138. **Hart PD, Young MR**. 1991. Ammonium chloride, an inhibitor of phagosome-lysosome fusion in macrophages, concurrently induces phagosome-endosome fusion, and opens a novel pathway: studies of a pathogenic mycobacterium and a nonpathogenic yeast. J. Exp. Med. **174**:881–9.
139. **Rubin-Bejerano I, Fraser I, Grisafi P, Fink GR**. 2003. Phagocytosis by neutrophils induces an amino acid deprivation response in *Saccharomyces cerevisiae* and *Candida albicans*. Proc. Natl. Acad. Sci. U. S. A. **100**:11007–12.
140. **Eriksson S, Lucchini S, Thompson A, Rhen M, Hinton JCD**. 2003. Unravelling the biology of macrophage infection by gene expression profiling of intracellular *Salmonella enterica*. Mol. Microbiol. **47**:103–18.
141. **Vylkova S, Lorenz MC**. 2014. Modulation of phagosomal pH by *Candida albicans* promotes hyphal morphogenesis and requires Stp2p, a regulator of amino acid transport. PLoS Pathog. **10**:e1003995.
142. **Antachopoulos C, Walsh TJ, Roilides E**. 2007. Fungal infections in primary immunodeficiencies. Eur. J. Pediatr. **166**:1099–117.
143. **Adenis AA, Aznar C, Couppié P**. 2014. Histoplasmosis in HIV-Infected Patients: A Review of New Developments and Remaining Gaps. Curr. Trop. Med. reports **1**:119–128.
144. **Dignani MC, Rex JH, Chan K-W, Dow G, deMagalhaes-Silverman M, Maddox A, Walsh T, Anaissie E**. 2005. Immunomodulation with interferon-gamma and colony-stimulating factors for refractory fungal infections in patients with leukemia. Cancer **104**:199–204.
145. **Delsing CE, Gresnigt MS, Leentjens J, Preijers F, Frager FA, Kox M, Monneret G, Venet F, Bleeker-Rovers CP, van de Veerdonk FL, Pickkers P, Pachot A, Kullberg BJ, Netea MG**. 2014. Interferon-gamma as adjunctive immunotherapy for invasive fungal infections: a case series. BMC Infect. Dis. **14**:166.
146. **Sau K, Mambula SS, Latz E, Henneke P, Golenbock DT, Levitz SM**. 2003. The antifungal drug amphotericin B promotes inflammatory cytokine release by a Toll-like receptor- and CD14-dependent mechanism. J. Biol. Chem. **278**:37561–8.
147. **Mesa-Arango AC, Scorzoni L, Zaragoza O**. 2012. It only takes one to do many jobs: Amphotericin B as antifungal and immunomodulatory drug. Front. Microbiol. **3**:286.
148. **Cenci E, Mencacci A, Del Sero G, Bistoni F, Romani L**. 1997. Induction of protective Th1 responses to *Candida albicans* by antifungal therapy alone or in combination with an interleukin-4 antagonist. J. Infect. Dis. **176**:217–26.
149. **Wolf JE, Massof SE**. 1990. *In vivo* activation of macrophage oxidative burst activity by cytokines and amphotericin B. Infect. Immun. **58**:1296–1300.
150. **Life-Worldwide.org**. Histoplasmosis. Lead. Int. Fungal Educ.

Publishing Agreement

It is the policy of the University to encourage the distribution of all theses, dissertations, and manuscripts. Copies of all UCSF theses, dissertations, and manuscripts will be routed to the library via the Graduate Division. The library will make all theses, dissertations, and manuscripts accessible to the public and will preserve these to the best of their abilities, in perpetuity.

I hereby grant permission to the Graduate Division of the University of California, San Francisco to release copies of my thesis, dissertation, or manuscript to the Campus Library to provide access and preservation, in whole or in part, in perpetuity.

Author Signature Alison M. Cooley Date 1/6/14