I, Roger A Fecher, hereby submit this original work as part of the requirements for the degree of Doctor of Philosophy in Immunology.

It is entitled:
Inverse correlation between IL-10 and HIF-1α in macrophages infected with Histoplasma capsulatum

Student’s name: Roger A Fecher

This work and its defense approved by:

Committee chair: George Deepe, M.D.
Committee member: James Bridges, Ph.D.
Committee member: Charles Caldwell, Ph.D.
Committee member: Gurjit Hershey, M.D., Ph.D.
Committee member: David Hideman, Ph.D.
Committee member: Joseph Qualls, Ph.D.
Inverse correlation between IL-10 and HIF-1α in macrophages infected with *Histoplasma capsulatum*

Dissertation

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By

Roger Alan Fecher, B.S.

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The University of Cincinnati

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Dissertation Committee:

George Deepe, Advisor

Gurjit Khurana Hershey

David Hildeman

Charles Caldwell

James Bridges

Joseph Qualls
ABSTRACT

Hypoxia inducible factor (HIF)-1α is a transcription factor that regulates metabolic and immune response genes in the setting of low oxygen tension and inflammation. We investigated the function of HIF-1α in the host response to *Histoplasma capsulatum* since both mouse and human granulomas induced by this pathogenic fungus develop hypoxic microenvironments during the early adaptive immune response. Here we demonstrated that myeloid HIF-1α-deficient mice exhibited elevated fungal burden during the innate immune response (prior to seven days post-infection) as well as decreased survival in response to a sublethal inoculum of *H. capsulatum*. Deletion of HIF-1α in neutrophils or alveolar macrophages and DCs did not alter fungal burden thus implicating Mφs as the pivotal cell in host resistance. HIF-1α was stabilized in Mφs following infection. The absence of myeloid HIF-1α did not alter immune cell recruitment to the lungs of infected animals but was associated with an elevation of the anti-inflammatory cytokine IL-10. IL-10 blockade restored protective immunity to the mutant mice. Macrophages (Mφ) constituted the majority of IL-10 producing cells. Increased activity of the transcription factor CREB in HIF-1α-deficient Mφs drove IL-10 production in response to *H. capsulatum*. IL-10 inhibited Mφ control of fungal growth in response to the activating cytokine IFN-γ. Thus, we identified a critical function for Mφ HIF-1α in tempering IL-10 production following infection. We established that transcriptional regulation of IL-10 by HIF-1α and CREB is critical for activation of Mφ by IFN-γ and effective handling of *H. capsulatum*.

Our data provide insight into the mechanism of HIF-1α elevation in the host response to *H. capsulatum*. In addition, we provide gene expression data that may help elucidate the immune mechanisms that lead to fungal growth restriction. In summary, this investigation provides novel insights into HIF-1α and IL-10 regulation in innate immune defense against *H. capsulatum*.
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<th>Full Form</th>
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<tr>
<td>BMDMφ</td>
<td>Bone marrow derived macrophage</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CCR or CXCR</td>
<td>Chemokine receptor</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>CR3</td>
<td>Complement receptor 3</td>
</tr>
<tr>
<td>CREB</td>
<td>CAMP response element binding protein</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>FAC</td>
<td>Ferric ammonium citrate</td>
</tr>
<tr>
<td>FDR</td>
<td>False discovery rate</td>
</tr>
<tr>
<td>Fe</td>
<td>Iron</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage colony stimulating factor</td>
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<tr>
<td>h</td>
<td>Hour</td>
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<tr>
<td>HIF-1α</td>
<td>Hypoxia inducible factor-1α</td>
</tr>
<tr>
<td>HPRT</td>
<td>Hypoxanthine guanine phosphoribosyltransferase</td>
</tr>
<tr>
<td>HRE</td>
<td>Hypoxia response element</td>
</tr>
<tr>
<td>Hsp60</td>
<td>Heat shock protein 60</td>
</tr>
<tr>
<td>i.n.</td>
<td>Intranasal</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>KEGG</td>
<td>Kyoto encyclopedia of genes and genomes</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>LNA</td>
<td>Locked nucleic acid</td>
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<tr>
<td>Mφ</td>
<td>Macrophage</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
</tr>
<tr>
<td>miRNA</td>
<td>Micro RNA</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecule pattern</td>
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<tr>
<td>PHD</td>
<td>Prolyl hydroxylase domain-containing protein</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear neutrophil</td>
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<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
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<tr>
<td>QC</td>
<td>Quality control</td>
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<tr>
<td>RNA-seq</td>
<td>RNA sequencing</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Real time polymerase chain reaction</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
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<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cells</td>
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<td>WT</td>
<td>Wild type</td>
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CHAPTER 1

Introduction
Histoplasmosis

i. *H. capsulatum* disease

Infection with *H. capsulatum* causes significant morbidity and mortality worldwide. This fungus is a dimorphic ascomycete that grows in its hyphal form in soil and bird and bat guano. Upon inhalation of spores, *H. capsulatum* transforms into the pathogenic yeast phase. This form replicates within Mφs that carry the yeast from the lungs to virtually any organ (1, 2). Induction of adaptive immunity, particularly the type 1 helper T cell response (Th1), is required for activation of Mφs and efficient clearance of the yeast. Exposure to *H. capsulatum* usually results in asymptomatic clearance; however, histoplasmosis can manifest as an acute flu-like pulmonary illness, a chronic cavitary lung disease, or a progressive disseminated form depending upon the immune status of the host (1). For example, immunocompromised individuals are especially at risk for disseminated infection; the HIV pandemic resulted in a dramatic increase in lethal histoplasmosis. Today, highly-active retroviral therapy (HAART) reduces this risk likely due to the preservation of patient CD4 T cells (2). Other susceptible immunocompromised populations include those with a recent organ transplant as well as individuals taking immunosuppressive drugs such as TNF-α inhibitors or corticosteroids (3). Clinical disease can also develop in immunocompetent individuals. Although the impact of a large inoculum on clinical disease development is unknown, outbreaks in an immunocompetent population have occurred when there is a localized exposure to a large infectious dose (4).

Cases of histoplasmosis occur worldwide, but are concentrated in endemic regions. The North American area is centered on the Ohio and Mississippi river valleys. Large surveys of skin testing in the 1960s revealed that 80% of young adult men from this region were positive for *H.*
capsulatum exposure (1, 5). Disease burden is regional; an analysis of United States hospital records estimated 3370 inpatient stays and 254 deaths associated with histoplasmosis in 2002, with almost 90% of hospitalizations occurring in Midwest and Southern states (6). Central and South America contain large endemic areas, and reviews have brought attention to the under-diagnosed burden of HIV-associated histoplasmosis in these regions (7). Additional endemic foci are in China, Southeast Asia, the Indian subcontinent, Australia, and Africa (8).

ii. H. capsulatum phenotypic diversity

Isolates of H. capsulatum exhibit considerable genotypic and phenotypic variability between and within these regions. Genetic analysis has revealed that this fungus is not monophyletic and can be classified into seven or eight distinct clades (9). Genetic differences between clades lead to differences in phenotype and virulence. Most isolates have both α- and β-glucan in their cell walls, but isolates from the North American 2 clade lack α-glucan (10). While α-glucan strains account for the majority of infections in immunocompetent individuals in North America, α-glucan infections are associated with HIV positive individuals (11). In mouse models, α-glucan strains cause more severe disease than α-glucan+, but only at high infectious inocula (12). Thus, H. capsulatum expression of α-glucan may limit the pathogenic potential except in the setting of an elevated infectious dose or a defective adaptive immune response.

H. capsulatum is not contagious. Despite infection usually being a "dead end" for replication, the pathogen appears specifically adapted to mammalian hosts. The transformation from mycelial to yeast phase at 37°C is crucial for infection; strains lacking this ability are avirulent (11). The yeast is equipped for evading intracellular killing by phagocytes, with
mechanisms to degrade reactive oxygen species (ROS), regulate lysosomal pH, and capture essential nutrients that might otherwise be deprived (13–16). Many mammalian species are infected with *H. capsulatum* and are accidental hosts that must cope with the yeast's capability for survival within Mφs. In human and mouse infection, Mφs provide a niche for fungal proliferation that cannot be halted without adaptive immunity.

iii. Fungal recognition

Immune cells use a variety of surface receptors to recognize and ingest *H. capsulatum*. Pattern recognition receptors (PRRs) such as C-type lectin receptors (CLRs) and toll like receptors (TLRs) are necessary for innate immune cytokine production and/or phagocytosis of pathogenic fungi (17, 18). Dectin-1 recognition of β-(1,3)-glucan is required for optimal proinflammatory cytokine production, but not phagocytosis of yeast cells; α-glucan on *H. capsulatum* masks β-glucan recognition (19, 20). In humans, a rare mutation that decreases surface expression of dectin-1 is not associated with a higher incidence of histoplasmosis. While this seems to suggest that dectin-1 is not needed to combat infection, the unknown exposure rate of these individuals to *H. capsulatum* makes it difficult to determine the role of dectin-1 in human disease (21). Other CLRs such as dectin-2 and mincle bind this fungal pathogen, but their role in immunity has not been elucidated (22).

While Mφs and dendritic cells (DCs) exhibit overlapping expression of many surface receptors, those utilized for phagocytosis are cell specific. Macrophages bind and ingest yeasts via CD11/CD18 integrins while DCs utilize very late activation antigen 5 (VLA-5) to recognize ligands heat shock protein 60 (hsp60) and cyclophilin A, respectively (23–25). CD11/CD18
blockade reduces, but does not prevent, *H. capsulatum* uptake by both human and mouse Mφs; this finding suggests that other receptors are capable of driving phagocytosis – at least in the absence of CD11/CD18. In contrast to Mφs, human DCs rely on VLA-5 for fungal recognition (26). Differential pathogen recognition by Mφs and DCs may trigger unique signaling cascades. CD11b/CD18 triggers activation of the tyrosine kinase Syk and downstream production of proinflammatory cytokines in Mφs (20). VLA-5, on the other hand, activates kinases that regulate proliferation and survival including ERK/MAPK and PI3K/Akt. This leads to production of anti-apoptotic Bcl-2, which may prevent fungal driven apoptosis (27). Thus, engagement of different receptors on these phagocytes may account for the contrasting intracellular fate of the organism.

**Figure 1.1 Macrophage and dendritic cell recognition of *H. capsulatum***

Macrophages recognize *H. capsulatum* via CR3 (CD11b/CD18) and dectin-1. While CR3 is required for phagocytosis, CR3 and dectin-1 are both required for cytokine production, which occurs via Syk/JNK/AP-1 signaling pathway. Dendritic cells recognize *H. capsulatum* via VLA-5, which can signal through ERK/MAPK as well as PI3K/Akt pathways.
Innate immune response

Following *H. capsulatum* recognition, appropriate cell mobilization is required for an effective immune response. Neutrophils (PMNs) and Mφs are recruited early to the site of infection and their roles will be covered below.

i. PMNs

Human PMNs are fungistatic, not fungicidal, against *H. capsulatum* (28). This activity relies on cathepsin G, defensins, and bactericidal-permeability-increasing protein (BPI) within azurophilic granules (28). Neutrophil depletion studies in the mouse model have addressed the influence of these cells in host defense. These studies demonstrated decreased survival and elevated fungal burden associated with decreased PMNs (29, 30). However, the antibody used in these studies is now known to recognize both PMNs and inflammatory monocytes; therefore the role of PMNs is still unsettled (30). Although neutropenic patients are at risk for some fungal pathologies, histoplasmosis is not among them (31). We will show that PMNs do not contribute to the mouse control of fungal burden in chapter 2 of this thesis. Thus, PMNs are not critical in the immune response to *H. capsulatum*.

ii. Macrophages

Macrophage activation is critical for control of intracellular *H. capsulatum* growth (32). IFN-γ and GM-CSF are utilized for mouse Mφ activation while GM-CSF, M-CSF, and IL-3 are essential in human cells. The mechanisms of growth restriction are distinct in mouse and human cells. While phagosome acidification is used by mouse Mφs for growth restriction, it is
dispensable within human M\(\phi\)s (33). \textit{H. capsulatum} phagocytosis stimulates a respiratory burst within human M\(\phi\)s; mouse M\(\phi\) production of ROS requires opsonized yeast or activation with GM-CSF (34, 35). Studies utilizing NADPH oxidase deficient mice suggest that inhibition of fungal replication \textit{in vivo} may require ROS production (36). However, ROS do not directly inhibit fungal growth; therefore, its importance is likely as a signaling molecule (13, 36, 37). Additional mechanisms of inhibition suggested by \textit{in vitro} studies include reactive nitrogen species and metal deprivation (35, 38, 39). Even with activation, M\(\phi\)s cannot efficiently sterilize tissues and thus harbor \textit{H. capsulatum} (32).

**Adaptive immune response**

i. DCs

As the most potent antigen-presenting cells, DCs provide a link between innate and adaptive immunity. Human DCs are capable of killing \textit{H. capsulatum} (26). This fungicidal activity was dependent on lysosomal hydrolases, but not the respiratory burst or nitric oxide production. DCs drive CD4\(^+\) or CD8\(^+\) T cell proliferation by presenting fungal antigen directly or from apoptotic M\(\phi\)s, respectively (26, 40). Adoptively transferred \textit{H. capsulatum}-loaded DCs are able to suppress maladaptive IL-4 production and improve survival following CD4\(^+\) depletion (41). The DC ability to kill yeast and present antigen indicates that they are capable of driving a T cell response even prior even prior to an activating signal.

ii. T cells
Early studies demonstrated that CD4$^+$ and CD8$^+$ T cells are necessary for an effective immune response to *H. capsulatum* infection; nude mice that lack T cells exhibit high mortality following a low dose infection with this pathogen (42). CD4$^+$ T cell depletion during primary infection of mice led to increased morbidity while loss of CD8$^+$ T cells decreased clearance efficiency but did not lead to mouse death (43). Following vaccination, CD8$^+$ T cells confer protection in the absence of CD4$^+$ T cells; conversely, CD4$^+$ T cells confer protection in the absence of CD8$^+$ T cells (44). Increased mortality and fungal burden in the absence of T cells is caused by a lack of protective cytokines such as IFN-γ and TNF-α (43, 45). An increased incidence of histoplasmosis in AIDS patients supports the protective role of CD4$^+$ T cells in humans.

Reactivation of disease can occur when latent *H. capsulatum* sequestered in granulomas in the lung replicate. CD4$^+$ and CD8$^+$ T cells influence mouse reactivation (46). Elimination of both of these T cell populations six weeks after infection elevates fungal burden (46). AIDS patients living in non-endemic areas exhibit reactivation disease concomitant with low CD4$^+$ T cell counts (47).

**CD4$^+$ T cells in clinical histoplasmosis**

Our understanding of the importance of CD4$^+$ cells in histoplasmosis has emerged from observations in both mice and humans. By 1971, physicians noted that patients with histoplasmosis usually exhibited an increase in T or B lymphocytes, while patients with progressive disseminated disease tended to lack this response (42). Early studies in athymic mice confirmed the importance of T cells for control of histoplasmosis (42). In the 1980s, the HIV epidemic brought a dramatic increase in histoplasmosis incidence, severity, and awareness. An impaired CD4$^+$ T cell response is the major risk factor. A recent study found that the majority of
HIV-infected individuals with a CD4+ T cell count below 200 cells/mm³ will develop histoplasmosis (48). Progressive disseminated histoplasmosis typically presents in those with low CD4+ T cell counts (<100 cells/mm³). Today most instances occur in patients not receiving HAART. Mortality in this group may approach 50% (2). Work in Central and South America have demonstrated that it continues to be a leading cause of death in AIDS patients although it is likely under-diagnosed and untreated (49).

Patients taking medications that suppress CD4+ T cell number or function are another major population with increased risk for histoplasmosis, particularly disseminated disease. Immunosuppressive agents that influence T cell function and/or number include steroids, folic acid analogues, calcineurin inhibitors, and targeted monoclonal antibodies. Glucocorticoids effect all immune cell types, but have an especially dramatic influence on T cell number and function (50). High dose glucocorticoids, either alone or in combination with other immunosuppressive agents, have been linked to histoplasmosis (51–53). Immunosuppressants that target T cells are associated with cases of histoplasmosis (54–56). Exact assessment of risk is difficult because many patients take multiple medications, have an underlying condition that may influence immunity, or both.

A. Th1

During infection, CD4+ T cells polarize into several helper phenotypes such as Th1, Th2, Th17, and T regulatory cells (Treg) with unique cytokine profiles (57). Following pathogen exposure, antigen presenting cells produce IL-12 that promotes Th1 differentiation and IFN-γ production (58). Mutations in the IL-12 pathway are associated with increased risk of fungal infections (59, 60). In mouse histoplasmosis, IL-12 blockade accelerates mouse mortality in an
IFN-γ-dependent manner (61). While the source of IL-12 is not clear, activated Mφs and dendritic cells are known producers following activation (62).

In histoplasmosis, IFN-γ is produced primarily by Th1 cells, activates intracellular killing, and is necessary for control of primary infection (63). The role of IFN-γ in secondary infection is not clear since some groups have found that it contributes to survival while others have neutralized it with no effect (29, 63). The importance of IFN-γ signaling in humans has been established with reports linking genetic deficiency in the receptor to disseminated disease (64). The impact of this cytokine will be discussed in the cytokine section below.

B. Th17

Th17 cells produce inflammatory cytokines such as IL-17, IL-6, and GM-CSF. The Th17 response is important for controlling fungal infections including *Candida albicans*, *Paracoccidioides brasiliensis*, *Blastomyces dermatitidis*, and *Coccidioides posadasii* and may be beneficial but not essential for control of *H. capsulatum* (65). In wild-type mice, IL-17 neutralization results in a larger fungal burden starting at day 7 post-infection and continuing through resolution, but does not alter mouse survival (66). Elevated IL-17 improves fungal clearance in the CCR5 KO mouse (67). CCR5 KO mice exhibit a shift toward Th17 which leads to decreased Treg numbers (67). These mice have accelerated clearance of *H. capsulatum*; both the elevation in Th17 and depression of Treg response may improve intracellular killing. In humans, Hyper-IgE syndrome (HIES) is defined by a mutation in STAT3 leading to decreased Th17 cells. Even though HIES is a rare disorder, there are several case reports of HIES patients that developed disseminated or gastrointestinal histoplasmosis (68). The fact that the gastrointestinal tissue is a target in most of these patients suggests that, as with mucosal
candidiasis, Th17 cells may be more important in the regulation of mucosal rather than systemic immunity for *H. capsulatum*.

**C. Th2**

In contrast to Th1 and Th17, cytokines representative of Th2 responses, including IL-4, IL-5, and IL-13, exacerbate histoplasmosis. IL-4 is a central type 2 cytokine, and transgenic mice over-expressing IL-4 exhibit delayed fungal clearance (69). CCR2 KO mice also exhibit increased IL-4 production and impaired clearance (70). In these mice IL-4 triggers production of another Th2-associated cytokine, IL-33, by infected Mφs (71). Neutralization of either IL-4 or IL-33 improves *H. capsulatum* clearance in this model.

There are multiple mechanisms by which Th2 cytokines dampen host immunity. IL-4 and IL-13 promote alternative activation of Mφs. *H. capsulatum* proliferates in Mφs primed with IL-4 as opposed to those activated with GM-CSF or IFN-γ Mφs (72). IL-4 also enhances intracellular survival by increasing cellular zinc content (72). In addition to providing zinc for the pathogen, prevention of zinc sequestration limits the Mφ oxidative burst (35).

**D. Treg**

Tregs mediate immune suppression in several ways, including cell-cell interactions and production of cytokines such as IL-10 and TGF-β (57). A CD4+ CD25+ population, which was presumed to be Tregs, is increased in infected mice treated with anti-TNF-α and infected with *H. capsulatum* (73). This population is able to suppress antigen-specific T cell proliferation via IL-10 production. While the importance of Tregs during *H. capsulatum* is largely unknown, IL-10 impedes clearance of this pathogen (74). These data suggest that Treg production of IL-10
inhibits immunity, but more studies are necessary to determine the impact of this population in vivo.

iii. B cells

The protective effect of antibodies that develop after *H. capsulatum* infection is currently unclear. Early adoptive transfer studies of mouse serum from immunized mice did not protect mice subsequently infected with *H. capsulatum* (75). However, more recent studies have demonstrated that polyclonal antibody preparations may generate divergent results as a consequence of the presence of protective, non-protective, and inhibitory antibodies (76). Immunoglobulins generated by the host in response to fungal pathogens typically target cell wall components including β-glucan and heat shock protein 60 (hsp60) (77). Monoclonal antibodies to *H. capsulatum* surface ligands histone 2B and hsp60 are protective (78). Nevertheless, B cell knockout mice exhibit no change in fungal burden during primary infection but exhibit elevated fungal burden and a prolonged resolution in secondary disease (43). These results suggest that humoral immunity is not as important as cellular immunity, but that it may contribute to the immune response primarily during secondary infection. Given the high rate of infection in endemic regions, this may be a critical factor in patients that develop histoplasmosis after living for a prolonged period in one of these areas.

**Metals in immunity**

Deprivation of trace metals is an effective immune strategy to slow or stop an infection. In mouse histoplasmosis, activation of Mφs involves mechanisms that limit at least two essential
minerals: iron and zinc. *H. capsulatum* must obtain iron bound to ferritin or transferrin in the phagosome/lysosome compartment in order to replicate. It possesses several means to accomplish this including siderophores, ferric reductases, and maintenance of neutral pH (14–16, 79). Mouse Mφs activated with IFN-γ and lipopolysaccharide counteract fungal iron acquisition, possibly via NO production; conversely, increasing iron availability increases yeast survival (38, 39). *H. capsulatum* control by NO has not been reported in human Mφs.

Zinc must be obtained by an intracellular invader for survival and growth. Both human and mouse Mφs activated with GM-CSF upregulate metallothioneins, small metal-binding proteins which reduce intracellular free zinc (35, 72). Metallothionein-driven reduction of zinc supports production of ROS by increasing activity of the phagosomal H⁺ channel (35). Thus, GM-CSF’s ability to inhibit yeast growth in Mφs may be due to zinc deprivation and enhanced ROS. In contrast to GM-CSF, IL-4 increases zinc availability to intracellular yeast (72).

**Cytokines in *H. capsulatum***

Following differentiation into several helper phenotypes such as Th1 and T regulatory cells (Treg), CD4⁺ T cells produce cytokines that shape the immune response. Loss of IFN-γ, TNF-α, or GM-CSF can each independently increase mouse mortality in histoplasmosis while loss of IL-10 promotes fungal clearance.

i. IFN-γ

In histoplasmosis, IFN-γ is produced primarily by Th1 cells with additional contribution from CD8⁺ and γδ T cells as well as NK cells (80). While there are both IL-12-dependent and independent pathways that drive IFN-γ synthesis, *H. capsulatum* largely if not exclusively
triggers IL-12-dependent production (61, 81). IL-12 blockade in mouse histoplasmosis accelerates mortality in an IFN-γ-dependent manner (61). IFN-γ activates mouse Mφs to inhibit *H. capsulatum* growth through stimulation of nitric oxide generation (82, 83). The importance of IFN-γ signaling in humans has been established with reports linking genetic deficiency in the receptor to disseminated disease (64).

**ii. TNF-α**

TNF-α neutralization impairs survival of mice (84). TNF-α exerts multiple effects including activation of phagocytic cells, induction of apoptosis, and control of the CD4+ phenotype. TNF-α is critical for host defenses to *H. capsulatum* in both primary and secondary infection. In primary infection, TNF-α blockade reduces nitric oxide production by Mφs. In contrast, the major defect in secondary infection is overproduction of detrimental IL-4 and IL-10 (84). In both primary and secondary infection, TNF-α neutralization abolishes the ability of mouse T cells to mediate protection from *H. capsulatum* (85). This suggests that TNF-α is an important signal to generate protective capacity in T cells in addition to effects on infected phagocytes. Additionally TNF-α is essential for the induction of caspase activation and the promotion of additional TNF-α production and apoptosis in infected Mφs (86). This suggests a protective mechanism in which permissive Mφs release a burst of TNF-α to neighboring cells during the process of apoptosis. While TNF-α does not activate Mφ antifungal activity, this release of cytokine may help prime the cell for infection.
TNF-α and Human Disease

Our understanding of the mechanism of TNF-α in histoplasmosis has evolved through both mouse studies and clinical observations. The requirement of TNF-α for optimal control of histoplasmosis was first observed in mice, and the ability of TNF-α to suppress detrimental cytokines such as IL-4 and IL-10 was elucidated in this model (84). These findings predicted the relevance for human disease, which was eventually revealed by the introduction of TNF-α blocking therapeutics such as etanercept, adalimumab, and infliximab. These medications increase patient susceptibility to *H. capsulatum*, and patients are especially at risk for severe disseminated infection (87). Histoplasmosis is the most commonly reported fungal infection associated with TNF-blockers (87, 88). Clinical studies revealed the ability of TNF-α to antagonize Tregs. Reduced Treg number and function in rheumatoid arthritis patients is restored by TNF-α blockers (89). This observation prompted further studies in mouse histoplasmosis; TNF-α blockade in mice was found to induce an expanded pool of *H. capsulatum*-specific suppressive T cells (73). Unexpectedly, these T cells did not have typical Treg markers; therefore TNF-α blockade may act by promoting suppressor function in non-Treg CD4+ populations (73). Recent human studies have reflected this finding, showing that TNF-α blockers can induce immunosuppressive features in Th17 cells (90). These data indicate that TNF-α is an important mediator of protection against *H. capsulatum* in both mice and humans likely via its role in Mφ activation.

iii. GM-CSF
GM-CSF is an important pro-inflammatory cytokine that promotes activation of myeloid cells such as Mφs and PMNs. Neutralization leads to mouse death following *H. capsulatum* infection (91). The elevated fungal burden in these mice is a consequence of enhanced IL-4 and IL-10 as well as diminished IFN-γ and TNF-α (91). Recombinant GM-CSF promotes fungal clearance in a dose dependent manner in the presence or absence of an antifungal drug (92). GM-CSF acts at least in part by promoting fungistatic activity in Mφs through sequestration of zinc, a novel mechanism for pathogen control (35). The direct action of GM-CSF is likely responsible for this phenomenon since cellular zinc redistribution does not occur in response to stimulation with TNF-α (35). However, the overall contribution of cytokine production and zinc sequestration to *H. capsulatum* growth restriction has not been elucidated.

**iv. IL-10**

IL-10 production and regulation are important in controlling the immune response to a wide range of pathogenic microorganisms. IL-10 is secreted by immune cells including Mφs, dendritic cells, NK cells, PMNs, and CD4⁺ and CD8⁺ T cells. Engagement of TLR2, TLR3, TLR4, TLR9, or CLRs can drive IL-10 expression; recognition of fungal β-glucan by the CLR dectin-1 can induce myeloid IL-10 production (93). Downstream Syk signaling requires mitogen and stress-activated protein kinases 1 and 2 (MSK1/2) and the transcription factor cAMP response element-binding protein (CREB) (93). Among its diverse effects, this cytokine can inhibit Mφ antimicrobial activity by limiting production of inflammatory cytokines, chemokines, and reactive oxygen and nitrogen intermediates (94–96).
During infection with *H. capsulatum*, T cells contribute to IL-10 release, but myeloid cells are the predominant producing population (97). In the innate response to *H. capsulatum*, IL-10 dampens immunity by limiting IFN-γ production; IL-10⁻/⁻ mice exhibit elevated IFN-γ in association with accelerated *H. capsulatum* clearance (74). Modulation of IFN-γ by IL-10 attenuates the activation of Mφs which need IFN-γ to kill *H. capsulatum* (98). Thus, IL-10 inhibits IFN-γ signaling in *H. capsulatum* infection, which impedes fungal clearance.

**Granuloma and hypoxia development**

Granulomas are an organized collection of immune cells that form in response to a stimulus including a variety of infectious agents such as *H. capsulatum* (99–101). The spatial organization of cells within the granuloma is thought to create a microenvironment primed to isolate inflammation, limit pathogen survival, and prevent further dissemination of infectious agents (100). Extracellular and intracellular pathogens and infected Mφs are found in the center, which may be caseous and/or hypoxic. Here Mφs can fuse to form giant cells although the consequences of this change are not understood (102). Around this core there is a surrounding sphere of uninfected activated Mφs. An outer ring of T cells are important in directing granuloma function through cytokine secretion. TNF-α and IL-10 are both critical for granuloma formation and maintenance. In addition to its roles in Mφ trafficking, the absence of TNF-α is associated with disorganized granuloma development (103). Maintenance of well-formed granulomas also requires TNF-α (104). IL-10 acts by restricting TNF-α production, inhibiting chemokine production, and down-regulating Mφ activation (105, 106). Biological modeling of this complex system suggests that the balance between these pro- and anti-inflammatory cytokines is necessary for control of pathogen growth without host damage (107).
The importance of TNF-α in both limiting organism growth and granuloma formation and maintenance seems to support the notion that granulomas are a form of host protection. However, the organism may benefit from this isolated environment as well. Recent evidence suggests that *Mycobacterium tuberculosis*-induced granulomas serve as a repository for pathogenic organisms (101). Clinical cases of reactivation histoplasmosis, particularly in individuals that have left endemic areas into regions that are not known to contain the fungus, suggest that *H. capsulatum* granulomas may also contain viable organisms – although studies in the 1950s indicated that healed granulomas only contained dead organisms (47, 108, 109). Since *H. capsulatum*-infection leads to granuloma formation in the lungs and disseminated organs (such as the liver and kidney), there is the potential for an infectious repository in several anatomical locations (100, 110, 111).

Over time the microenvironment of the granuloma can exhibit decreased oxygen availability; the contribution of increased metabolic demand of accumulating cells and decreased oxygen delivery are unknown. Caseous necrosis, which is associated with tissue hypoxia, is found in response to *Mycobacteria tuberculosis* as well as *H. capsulatum* granulomas (100, 112). By day 7 post infection, *H. capsulatum*-induced granulomas exhibit hypoxia as assessed by hypoxyprobe staining (113). While this limits fungal growth, it does not kill the organism. Hypoxia within immune cells can also increase hypoxia-inducible factor-1α (HIF-1α), the primary transcription factor in the response to low oxygen.

**HIF-1α**

i. *Essential transcription factor in the response to hypoxia*
HIF-1 was initially described as a mediator of the cellular response to hypoxia by Gregg Semenza (114). This transcription factor was responsible for induction of erythropoietin expression via binding to the hypoxia response element (HRE). The HRE was identified by first generating plasmids that express a portion of the EPO gene enhancer 5’ to a reporter gene. The relative amount of the reporter gene was determined in transfected cells exposed to hypoxia. They then performed mutation analysis followed by an electrophoretic mobility shift assay (EMSA) with a competitive nucleotide sequence to determine the pivotal nucleotides. They thus identified the DNA binding region required for association of the protein complex that they called HIF-1, which was necessary for hypoxia-induced expression of EPO.

It has since been demonstrated that HIF-1α regulates over 100 genes involved in oxygen sensing, cell survival, cellular metabolism, cell proliferation, and cell migration pathways (115, 116). The essential role of this transcription factor is demonstrated by the embryonic lethal nature of the whole body knockout. Defective vascular development, neural fold defects, and increased tissue hypoxia within these mice lead to mid-gestation loss (117, 118). The pathways important in myeloid cells will be examined below.

**ii. Oxygen-dependent regulation**

HIF-1 is a multi-subunit transcription factor composed of a constitutively expressed β subunit, HIF-1β/ARNT, and an oxygen labile α subunit, HIF-1α (119). The oxygen-dependent degradation of HIF-1α is controlled via hydroxylation of the oxygen-dependent degradation (ODD) domain (119). Prolyl hydroxylase domain-containing (PHD) enzymes are responsible for hydroxylation of the ODD in the setting of low oxygen (120). There are three PHD isoforms
PHD1, PHD2, and PHD3 with varying subcellular localization; PHD1 is expressed in the nucleus while PHD2 is localized to the cytoplasm and PHD3 is found in both (121). PHD2 is primarily responsible for HIF-1α hydroxylation. These enzymes require 3 co-factors for function: O₂, iron, and 2-oxoglutarate (121). The requirement for O₂ as an essential co-factor is what drives decreased activity in the setting of hypoxia, which is responsible for elevated HIF-1α protein.

Following addition of hydroxyl groups, HIF-1α recognition by the von Hippel Lindau tumor suppressor protein (VHL) allows for ubiquitination and subsequent degradation by the 26S proteasome (122, 123). In the setting of normoxia, the efficiency of this degradation machinery reduces the half-life of HIF-1α to 5 minutes (124). This regulation is illustrated in Figure 1.2.
Figure 1.2 HIF-1α regulation in response to hypoxia and pathogens

In elevated O$_2$, HIF is hydroxylated, recognized by PHD, ubiquitinated and targeted for proteosomal degradation. In low O$_2$, PHDs do not hydroxylate HIF, which permits binding to ARNT. Subsequent translocation to the nucleus leads to HRE recognition and induction of downstream target genes. Pathogens elevate HIF-1α protein through enhanced transcription or decreased degradation.

iii. Activation domains

HIF-1α contains N-terminal and C-terminal transactivation domains, N-TAD and C-TAD, respectively. Hydroxylation of these domains modulates transcription of HIF-1α targets.
Full transactivation requires hydroxylation of the C-TAD, which is necessary for binding of the CREB-binding protein (CBP)/p300 complex (125, 126). This complex is important for DNA binding and recruitment of transcriptional machinery. Factor inhibiting HIF-1 (FIH-1), an oxygen-dependent hydroxylase, can modify an asparagine residue within the C-TAD to prevent CBP/p300 association (127–129). These modifications work in concert to modulate HIF-1α activity in various states of oxygenation.

While some data suggest that MAPK-mediated phosphorylation of HIF-1α is associated with increased HIF-1α transcriptional activity, data from other groups have shown that MAPK-mediated phosphorylation prevents nuclear translocation (130, 131). Thus, further work needs to be done to determine the contribution of MAPK-dependent phosphorylation in HIF-1α activation.

iv. Drivers of HIF-1α expression

HIF-1α protein can be elevated via multiple mechanisms of stabilization or transcriptional induction. Many of these methods occur in response to infection, which presents an opportunity for the host and/or the pathogen to utilize HIF-1α for their own purpose.

Stabilization

Reduced activity or expression of PHDs (most importantly PHD2) leads to decreased HIF-1α protein degradation and increased transcriptional activation (132). While this occurs in the setting of low oxygen, PHDs also require the co-factors iron and 2-oxoglutarate (2-OG) for activity. Since HIF-1α is constitutively expressed, even a transient decrease in PHD-dependent hydroxylation can substantially increase protein accrual in the absence of transcription (132).
Long utilized to simulate hypoxia, the iron chelator desferrioxamine (DFO) limits iron availability, which increases HIF-1α by limiting PHD activity (133). Pathogens may create a low intracellular iron environment by releasing siderophores that bind and sequester this metal. *H. capsulatum* and several members of the *Enterobacteriaceae* family utilize siderophores to acquire iron from host cells (15, 79, 134, 135). Bacterial siderophores have been shown to induce HIF-1α expression through iron depletion while fungal siderophores have not been investigated (135).

NO promotes HIF-1α accumulation via inhibition of PHD2 possibly via s-nitrosylation of this protein (136). Indeed NO donors were able to enhance HIF-1α protein accumulation (137). Overexpression of inducible NO synthase (iNOS) was able to drive HIF-1α accumulation in these cells. As an example, respiratory syncytial virus (RSV), which was previously known to elicit VEGF production, enhances HIF-1α by driving NO production in epithelial cells (138).

**Transcriptional induction**

LPS can induce HIF-1α expression through increased transcription rather than protein stabilization in the setting of normoxia (131, 139–141). Toll like receptor 4 (TLR4) is required for maximal expression of Mφ HIF-1α and downstream cytokines TNF-α, IL-1β, and IL-12 in response to LPS or the gram-negative bacteria *Salmonella typhimurium* and *Pseudomonas aeruginosa*; however, HIF-1α is increased in the setting of infection even in the absence of this recognition receptor (139). These data demonstrate that recognition and signaling other than TLR4 are capable of driving HIF-1α elevation in Mφ.

Phosphatidyl inositol-4,5-bisphosphate-3-kinase (PI3K) signaling regulates translation of HIF-1α via its downstream targets protein kinase B (Akt) and mammalian target of rapamycin
(mTOR) as shown in Fig. 1.3 (142–144). mTOR-dependent translational regulation occurs via 2 mechanisms. First, it phosphorylates the eukaryotic translation initiation factor 4E (eIF4E) binding protein (4E-BP), which leads to the dissociation of these components (145). This allows formation of the eIF4F complex, which is necessary for ribosomal binding of mRNA and translation initiation. Second, mTOR phosphorylates p70S6K, which promotes translation of 5’ terminal oligopyrimidine tract (TOP) mRNAs including HIF-1α (145).

Figure 1.3 HIF-1α regulation in response to PI3K signaling

PI3K signaling occurs in response to cytokines including TNF-α and IL-1β. This drives activation of Akt and mTOR. mTOR phosphorylates the eukaryotic translation initiation factor 4E (eIF4E) binding protein (4E-BP), which leads to the dissociation of these components (145). This allows formation of the eIF4F complex, which is
necessary for ribosomal binding of mRNA and translation initiation. mTOR also phosphorylates p70S6K, which promotes translation of 5’ TOP mRNAs. These both act to enhance translation of HIF-1α.

Pathogen-associated HIF-1α increase via stabilization and/or transcription

Two of the first reports to implicate HIF-1α in the response to infection were in the setting of Bartonella henselae and human herpesvirus-8 (146, 147). Both of these pathogens drive aberrant proliferation of endothelial vasculature typically observed in HIV-infected patients. Since vascular endothelial growth factor (VEGF) is a robust target of HIF-1α, the contribution of this transcription factor was investigated in the response to these pathogens. While both pathogens drove HIF-1α stabilization, the mechanism of induction was unclear. However, stimulation with B. henselae-derived LPS was unable to increase HIF-1α (146). Although many recent pathogen studies have ascribed elevated HIF-1α to an LPS response, this suggests that not all LPS elicits the same reaction.

While the mechanism of HIF-1α protein stabilization and/or transcriptional induction is unknown, it accumulates in the setting of infection with Chlamydia pneumoniae, Vesicular Stomatitis virus, Hepatitis B and C, Human Papilloma Virus, Toxoplasma gondii, Leishmania amazonensis, and the fungal pathogens Aspergillus fumigatus and Candida albicans (148–156). These studies suggest that, while HIF-1α plays an undeniable role in the cellular response to hypoxia, it may have been co-opted as a transcription factor in the response to pathogens as well. In most of these studies HIF-1α is important for host protection and pathogen control, but Chlamydia pneumoniae, Toxoplasma gondii, and Leishmania amazonensis have utilized this protein for their own purposes. This dichotomy will be explored below.
v. HIF regulated genes

Following protein accumulation, HIF-1α can translocate to the nucleus and bind to functional components of the HIF-1 factor complex including HIF-1β and p300 as demonstrated in Figure 1.2. The HIF-1 complex then binds to HREs to increase transcription.

HIF target genes fall into a variety of functional categories including cell proliferation/survival, apoptosis, angiogenesis, adhesion, motility, glucose metabolism, and extracellular matrix metabolism (157). The genes predominantly involved in host/pathogen interactions would include those involved in immune cell recruitment, migration, and activation, as well as those that play a key role in metabolism. Functional HREs have been described in the promoters for MT2-MMP, β1 integrin, and β2 integrin, which are important for cell migration (158–160). HIF-1α target genes that are known to play a role in the innate immune response include CD11b, iNOS, cathelicidins, CRAMP, Mφ migration inhibitor factor (MIF), the pro-inflammatory molecules TNF-α, IL-12, and CCL2 as well as the anti-inflammatory cytokines IL-10 (161–164). Many of the glycolytic enzymes as well as the glucose transporters GLUT1 and GLUT3 are chiefly dependent on HIF-1α (157). These regulators of metabolism may be important since metabolic intermediates can alter immune cell activation or have direct antimicrobial effects (165).

vi. Other isoforms
Two additional isoforms of HIF-1α exist: HIF-2α and HIF-3α (157). HIF-2α shares 48% amino acid identity with HIF-1α (166). High sequence homology within the ODD leads to similar oxygen-dependent protein stability of HIF-2α compared to HIF-1α. However, it is clear that these genes are not entirely functionally redundant since knockout of either one is embryonic lethal (117, 118, 167).

HIF-2α can also bind to ARNT/ HIF-1β and drive gene transcription through the same HRE. The list of target genes for HIF-1α and HIF-2α is overlapping, but not identical (168). Paradoxically in some situations HIF-1α and HIF-2α can have antagonistic functions (169). For instance, in the setting of Mφ polarization HIF-1α enhances NO synthesis while HIF-2α suppresses it. HIF-1α and HIF-2α both induce VEGF production; HIF-2α is able to limit angiogenesis by also driving production of the soluble VEGF receptor (170). The factors that mediate differences in the HIF-1α and HIF-2α response are unclear, but one recent report suggests that alterations in transcription factor complexes may be responsible (171).

HIF-3α, a more distantly related isoform, can also act as an oxygen-dependent transcription factor (172). However, multiple splice variants and transcription initiation sites have led to unique forms that are expressed in different tissues at different times. The impact of these factors is complex since different variants can have opposite functions. Indeed a conventional knockout mouse was generated, but the absence of several HIF-3α splice variants has made it difficult to interpret the contribution of these variants to the phenotype of mutant mice that exhibit enlarged right ventricle and pulmonary hyperplasia (173). HRE-reporter assays suggest that HIF-3α can inhibit HIF-1α and HIF-2α driven gene expression, but other studies have demonstrated HIF-3α-dependent induction of HIF-1α target genes EPO and GLUT1 (172).
Several splice variants of HIF-1α also exist in both human and mouse (157, 174–176). In mice HIF-1α i.2 is the predominant isoform; it is constitutively and ubiquitously expressed (176). HIF-1α i.1 is tissue specific and transcriptionally regulated (175). It regulates expression of proinflammatory molecules MIP-1α, TNF-α, IL-6, and IL-12. Unfortunately few studies have examined the expression of HIF-1α i.1 and i.2 so it is difficult to draw broad conclusions on their impact. Although their existence has been recognized, the human splice variants have received very little attention.

vii. HIF-1α in innate response to infection

HIF-1α accumulates following infection with a wide variety of pathogens. As mentioned previously, this accrual may occur as a result of protein stabilization, transcriptional induction, or both. The contribution of these mechanisms may alter the timing of induction as well as the duration of the response. HIF-1α protein is elevated in response to group A Streptococcus (GAS), Pseudomonas aeruginosa, Salmonella typhimurium, Chlamydia pneumoniae, Toxoplasma gondii, Leishmania amazonensis and donovani, Aspergillus fumigatus, Yersinia enterocolitica, and Helicobacter pylori (135, 139, 148, 153, 155, 177–183). Transcriptional induction occurred in response to Helicobacter pylori, Aspergillus fumigatus, and Yersinia enterocolitica while HIF-1α protein was stabilized in response to Yersinia enterocolitica (135, 155, 182, 183). The role of transcription and/or protein stabilization was not elucidated following infection with the other listed pathogens. Although LPS-mediated transcriptional induction may occur, the absence of HIF-1α induction in gram-negative Legionella pneumophila infection demonstrates that this mechanism is not implicated in the immune response to all pathogens.
Critically, this and the absence of HIF-1α induction in response to LPS from *B. henselae* as presented above suggests that differences in LPS content and/or structure may lead different HIF-1α responses.

In response to most of these pathogens, HIF-1α is required for eradication and host survival; however, at least three of these agents have co-opted HIF-1α to promote their replication. Early *H. capsulatum* infection responses are predominated by immune cells of the myeloid lineage. As such, observations of the involvement of HIF-1α within these cells are described in detail below.

**viii. HIF-1α in myeloid cells**

In order to investigate the role of mouse HIF-1α, it was necessary to generate a conditional knockout. Myeloid conditional knockout mice were generated utilizing the Cre-loxP system. In this system, HIF-1α floxed mice were crossed with LysMcre mice. The resultant *Lyz2cre Hif1α<sup>fl/fl</sup>* mice exhibit HIF-1α deletion within alveolar MΦs, inflammatory monocytes, monocyte-derived MΦs, monocyte-derived DCs, PMNs and likely within alveolar type II cells (178, 184). In all of the studies presented in the following chapters, *Lyz2cre/cre Hif1α<sup>fl/fl</sup>* mice were utilized. These mice exhibit nearly complete deletion of HIF-1α, but also lack lysozyme 2 (178). While *Lyz2cre/cre Hif1α<sup>fl/fl</sup>* mice did not exhibit any phenotypic abnormalities under normal conditions, they did exhibit profound changes in pathogen killing, cytokine production, and cell motility following infection (178).

These mice allow the examination of HIF-1α function in the innate immune response to infection. An effective innate response requires a variety of myeloid-specific cellular processes
including phagocytic uptake and production of antimicrobial mediators – including nitric oxide as well as antimicrobial peptides and granule proteases (185). The impact of HIF-1α on phagocytosis is unclear. Neutrophils exhibit elevated phagocytosis of E. coli when HIF-1α is elevated (186). However, phagocyte uptake is not altered in response to Aspergillus fumigatus when HIF-1α is deleted (155). While alterations in phagocytosis of other infectious pathogens have not been examined, phagocytosis of opsonized red blood cells is enhanced in hypoxia (187).

Following pathogen uptake, HIF-1α is important in phagocyte activation in response to Streptococcus pyogenes, Streptococcus agalactiae, Pseudomonas aeruginosa, and Salmonella typhimurium (139, 178, 179). HIF-1α evokes direct killing of many pathogenic microbes by primary mediators such as ROS, NO, and antimicrobial peptides (185, 188–190). Decreased iNOS transcription and subsequently low NO have been implicated in diminished clearance in myeloid HIF-1α knockouts. Following infection with GAS Lyz2cre Hif1α^{fl/fl} mice exhibit larger necrotic ulcers and elevated bacterial loads compared to controls (185). During infection these mice exhibit decreased transcription of iNOS and decreased nitrite release. Inhibition of iNOS in bone marrow derived Mφs was able to elevate intracellular GAS burden to a similar level as the conditional knockout. iNOS was diminished in Lyz2cre Hif1α^{fl/fl} mice relative to controls following infection with Salmonella typhimurium and Helicobacter pylori as well, but the impact it had on pathogen restriction was not assessed (179, 183). Although NO may act as an antimicrobial agent, its function in these infections is difficult to interpret since it may act as a signaling molecule (191). It may even enhance HIF-1α protein accumulation (192). If this is the case even small differences in NO generation may lead to large changes in cellular activation downstream of HIF-1α or other signaling pathways.
Recruitment and migration of immune cells to the site of infection are critical for pathogen control. HIF-1α regulates this process through upregulation of adhesion molecules and chemokines (185, 193). Following hypoxic stimulation, myelocytes exhibit enhanced binding to activated endothelium dependent on β2 integrin (160). Following infection with *Aspergillus fumigatus*, Lyz2cre Hif1αfl/fl mice have decreased PMN migration to the lungs, which leads to an elevated burden and decreased survival (155). However, the story may be more complicated since *Helicobacter pylori*-infected Lyz2cre Hif1αfl/fl mice exhibit elevated Mφ migration to the gastric mucosa relative to controls (183).

HIF-1α within myeloid cells is thus important in control of pathogen replication through direct mechanisms as well as cell recruitment/migration to the site of infection. Phagocytic cell types including Mφs and PMNs play a key role in these innate immune responses to pathogens. Some of the molecular targets of HIF-1α have been more extensively studied in these cells in the setting of infection. Since our data will show that HIF-1α in PMNs is not essential for an efficient immune response to *H. capsulatum* (see Chapter 2 page 72 for more detail), I have elected to focus on the function of HIF-1α in Mφs.

**ix. HIF-1α in Mφs**

The function of HIF-1α in Mφs has been studied extensively (141, 154, 165, 169, 170, 176, 178, 194–203). This work largely focuses on the response to hypoxia, but some recent work examines this transcription factor in the response to pathogen associated molecular patterns such as LPS or live pathogens. It is not currently clear if hypoxia and pathogen driven HIF-1α induction evoke a differential response by the cell.
**HIF-1α in hypoxic Mφs**

As early responders to infected tissues, circulating monocytes must be prepared for a variety of microenvironments. The HIF-1α pathway allows for a rapid and robust response to low oxygen levels through regulation of cellular glycolysis. In hypoxia, HIF-1α directly induces expression of GLUT1 and glycolytic enzymes including 6-Phosphofructo-2-kinase, phosphoglycerate kinase 1, and pyruvate kinase M2 (204–206). These function to enhance glucose uptake and glycolysis, which does not require oxygen for ATP production. Following anaerobic glycolysis, the end product, pyruvate, is metabolized into lactate by lactate dehydrogenase, another HIF-1α target (118, 207). Indeed HIF-1α-deficient Mφs in hypoxia have reduced ATP pools and lactate compared to control cells (178). As a result, energy-demanding processes such as aggregation, migration, and invasion are impaired in the HIF-1α knockout Mφs (178).

In addition to a direct impact on energy availability, HIF-1α may be required for functional maturation of Mφs (195, 208). Classically activated M1 Mφs are dependent on glycolysis for ATP production while M2 Mφs utilize oxidative phosphorylation (165). Recent work has shown that HIF-1α and HIF-2α work in concert to drive Mφ polarization by modulating NO homeostasis and arginase 1 expression in response to Th1 and Th2 cytokines, respectively (169). IFN-γ and/or LPS trigger M1 Mφ differentiation; they are microbicidal in part due to production of ROS and NO. They produce Th1 associated cytokines TNF-α and IL-12 (209). In contrast, M2 Mφs are associated with a Th2 response and produce IL-10, low levels of IL-12, and reduced iNOS transcript (209). However, M1/M2 polarization is highly dependent on *in vitro* stimulation conditions, and Mφ *in vivo* display a continuum of functional states and a high
degree of plasticity (210, 211). Control of Mφ polarization represents an additional avenue by which HIF-1α directs the innate immune response.

HIF-1α is required for Mφ migration, chemokine-driven recruitment, and Mφ aggregation in the setting of hypoxia (178). HIF-1α regulates the hypoxia-driven expression of the chemokine receptor CXCR4, which is important for myeloid cell recruitment as well as the recruiting chemokine and cytokines CXCL8, IL-1β, and VEGF (208, 212, 213). Additional targets include the integrins CD18, CD11b, and β2, which are necessary for diapedesis (160, 214, 215).

Once to the site of inflammation, HIF-1α is essential for phagocytosis as well as production of proteins associated with microbicidal activity. Hypoxia induced HIF-1α was critical for uptake of opsonized sheep red blood cells as well as *E. coli* (200). Following phagocytosis, production of iNOS and TNF-α is important for an effective phagocyte response to infection; hypoxia-driven Mφ manufacturing of these proteins is also dependent on this transcription factor (185). Control of these processes allows the cell to persist in an “off” state prior to migration to the site of infection while low oxygen levels can rapidly activate a phagocytic response.

**Effects of pathogen-induced HIF-1α**

Since HIF-1α positively regulates Mφ polarization, migration, and phagocytosis in hypoxia, it could be hypothesized that it is critical for an effective host response to infection. Although it is important for bacterial killing and inflammatory cytokine production in response to some infectious agents, other pathogens have evolved to exploit this transcription factor.
HIF-1α deficient Mφs have an impaired capacity to kill gram positive and gram negative bacteria compared to wild-type cells (178, 185). In response to GAS, this defective killing is due to decreased iNOS expression and NO release (185). Although the mechanism was not elucidated, HIF-1α knockout Mφs exhibited elevated burden following in vitro infection with Group B Streptococcus (GBS) compared to controls (178). While most studies did not examine phagocytosis, a defect in uptake was not noted between HIF-1α sufficient and deficient Mφs in response to GBS or the fungal pathogen Aspergillus fumigatus (155, 178).

Intracellular pathogens Toxoplasma gondii, Leishmania species, and Chlamydia pneumoniae require Mφ HIF-1α for replication. Toxoplasma inhibits PHD2 expression and activity to elevate HIF-1α protein (153, 180). Leishmania depletes cellular iron to inhibit PHD activity (182). The mechanism these agents utilize to subvert the immune response has not been thoroughly elucidated. Although the mechanism by which Chlamydia enhances HIF-1α was not studied, HIF-1α-dependent cellular glucose elevation during early infection, which it presumably acquires for its own metabolism, was critical for pathogen growth (148). Paradoxically, Chlamydia secretes the proteolytic protein CPAF during prolonged infection to degrade HIF-1α (148). While the effect of maintained HIF-1α was not examined, this suggests that the downstream targets of HIF-1α are ultimately detrimental to pathogen growth.

In most of the infectious models to studied, Mφ pro-inflammatory cytokine production is largely dependent on HIF-1α. This transcription factor is necessary for LPS-induced Mφ production of TNF-α, IL-12, IL-1α, and IL-1β although it is not required for IL-10 or IL-4 (139). GAS-induced TNF-α also requires HIF-1α (185). Even though HIF-1α is not essential for H. pylori eradication, it is needed for production of IL-1β and IL-6 during infection (183). While
indirect effects cannot be ruled out, HREs exist in the promoters for many of these targets including TNF-α, IL-1β, IL-6, IL-10 (139, 216, 217).

Taken together, these data demonstrate that Mϕ HIF-1α is an important regulator of the innate response to infectious agents. HIF-1α controls production of the antimicrobial molecule NO and Th1 associated inflammatory cytokines. While activation of the HIF-1α pathway is generally beneficial for the host, some pathogens are able to exploit this by skewing Mϕs toward a suboptimal immune response.
Synopsis

The function of the transcription factor HIF-1α in response to hypoxia has been extensively studied since its discovery over 20 years ago. However, research focusing on the mechanisms of normoxic HIF-1α induction as well as those that attempt to elucidate the role by which the host and pathogens utilize HIF-1α has only just begun. Many important questions about the mechanism of induction and the function of this transcription factor in the immune response remain.

The work presented in the following chapters investigates three main hypotheses: (i) myeloid HIF-1α is required for mouse control of the fungal pathogen *H. capsulatum*, (ii) HIF-1α regulates immune cell recruitment and cytokine production following infection, and (iii) HIF-1α elevation in response to this fungal pathogen is the result of protein stabilization and transcriptional induction.

In Chapter 2, we investigate the first two hypotheses by infecting myeloid specific HIF-1α knockout mice (*Lyz2cre Hif1α<sup>−/−</sup>*) with *H. capsulatum*. The absence of myeloid HIF-1α decreased survival and increased fungal burden. Contrary to our hypothesis, the collapse of immunity was not associated with a reduction in lung pro-inflammatory protective cytokines, but rather with an elevation in IL-10. We go on to examine the mechanism of IL-10 induction and the role of this cytokine in the elevated fungal burden and decreased survival seen in HIF-1α deficiency.

In Chapter 3, we lay the ground work to assess the role of transcriptional induction in HIF-1α elevation. We demonstrate that a soluble mediator released by infected Mφs is able to induce HIF-1α and its downstream target genes. We provide evidence that TNF-α is one such
soluble mediator. In the discussion we build on this knowledge and provide future studies that will help to elucidate the mechanism(s) of HIF-1α elevation following infection.

In Chapter 4, we provide RNA-seq data obtained from both control and HIF-1α deficient Mφs prior to and after infection with *H. capsulatum*. This data suggested that expression of miR27a may be involved in regulating IL-10 expression. We expect that the information obtained in this chapter will provide additional insight into the immune response to *H. capsulatum* both in the setting of HIF-1α deficiency as well as when endogenous HIF-1α is present.

Taken together, these studies uncover novel insight into the function of HIF-1α in Mφs following infection and suggest an important role in regulation of IL-10 production. Our working model incorporates the facets that we have noted as well as the hypotheses we present in the discussion of this thesis (Fig. 1.4).
Macrophage recognition of *H. capsulatum* via CR3 (CD11b/CD18) and dectin-1 may enhance HIF-1α either directly via NO/ROS mediated stabilization or via Syk signaling driven transcriptional induction. Transcriptional induction occurs via a soluble mediator, such as TNF-α or IL-1β. These cytokines can then act in an autocrine or paracrine manner to enhance HIF-1α transcription. In the absence of HIF-1α, CREB is able to bind to CBP to enhance IL-10 production, which reduces Mφ activation and ultimately leads to elevated fungal burden and mouse death.
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CHAPTER 2

Inverse correlation between IL-10 and HIF-1α in macrophages infected with *Histoplasma capsulatum*
Full title: Inverse correlation between IL-10 and HIF-1α in macrophages infected with *Histoplasma capsulatum*

Running title: Mφ HIF-1α negatively influences IL-10 in fungal infection

Authors:

Roger A. Fecher*, †

Michael C. Horwath*, †

Dirk Friedrich‡

Jan Rupp‡

George S. Deepe, Jr.*, §

*Division of Infectious Diseases, University of Cincinnati College of Medicine, 3230 Eden Ave, Cincinnati, OH 45267, USA

†Division of Immunobiology, Cincinnati Children’s Hospital Medical Center, University of Cincinnati, 2600 Clifton Ave, Cincinnati, OH 45220, USA

‡Department of Infectious Diseases and Microbiology, University of Lübeck, Ratzeburger Allee 160, 23538 Lübeck, Germany

§Medical Service, Veterans Affairs Hospital, Cincinnati, OH 45220, USA
Address correspondence to Dr. George Deepe, Phone: 513-558-4706; e-mail address: george.deepe@uc.edu; and fax 513-558-2089.
Abstract

Hypoxia inducible factor (HIF)-1α is a transcription factor that regulates metabolic and immune response genes in the setting of low oxygen tension and inflammation. We investigated the function of HIF-1α in the host response to *Histoplasma capsulatum* since granulomas induced by this pathogenic fungus develop hypoxic microenvironments during the early adaptive immune response. Here we demonstrated that myeloid HIF-1α-deficient mice exhibited elevated fungal burden during the innate immune response (prior to seven days post-infection) as well as decreased survival in response to a sublethal inoculum of *H. capsulatum*. The absence of myeloid HIF-1α did not alter immune cell recruitment to the lungs of infected animals but was associated with an elevation of the anti-inflammatory cytokine IL-10. Treatment with mAb to IL-10 restored protective immunity to the mutant mice. Macrophages (Mφ) constituted the majority of IL-10 producing cells. Deletion of HIF-1α in neutrophils or DCs did not alter fungal burden thus implicating Mφs as the pivotal cell in host resistance. HIF-1α was stabilized in Mφs following infection. Increased activity of the transcription factor CREB in HIF-1α-deficient Mφs drove IL-10 production in response to *H. capsulatum*. IL-10 inhibited Mφ control of fungal growth in response to the activating cytokine IFN-γ. Thus, we identified a critical function for Mφ HIF-1α in tempering IL-10 production following infection. We established that transcriptional regulation of IL-10 by HIF-1α and CREB is critical for activation of Mφ by IFN-γ and effective handling of *H. capsulatum*. 
Introduction

*H. capsulatum* is the most common endemic pulmonary mycosis in the United States (1). While immunocompetent hosts typically resolve infection with minimal symptoms, severe infections can develop in immunocompromised individuals. Coordinated activity of the innate and adaptive immune systems is required for fungal growth restriction. Early innate recognition is required for phagocytosis, cytokine production, and recruitment of additional innate cells and adaptive cells. This accumulation and signaling drive development of granulomas. During the initiation of adaptive immunity, granulomas become hypoxic; one of the central transcription factors in the response to hypoxia is HIF-1α (2).

HIF-1 is a multi-subunit transcription factor composed of a constitutively expressed β subunit, HIF-1β/ARNT, and an oxygen labile α subunit, HIF-1α (3). The oxygen-dependent degradation of HIF-1α is controlled via hydroxylation of the oxygen-dependent degradation (ODD) domain (3). Oxygen-dependent prolyl hydroxylase domain-containing (PHD) enzymes are responsible for hydroxylation of the ODD in the setting of low oxygen (4). Subsequent polyubiquitination leads to recognition by the 26S proteasome and degradation (5, 6). The requirement for O₂ as an essential co-factor drives decreased activity in the setting of hypoxia, which is responsible for elevated HIF-1α protein.

Infection with a variety of pathogens has been associated with increased HIF-1α protein and/or expression of downstream targets. LPS can induce HIF-1α expression through increased transcription rather than protein stabilization in the setting of normoxia (7–10). While the mechanism of HIF-1α protein stabilization and/or transcriptional induction is unknown, it accumulates in the setting of infection with *Chlamydia pneumoniae*, Vesicular Stomatitis virus, Hepatitis B and C, Human Papilloma Virus, *Toxoplasma gondii*, *Leishmania amazonensis*, and
the fungal pathogens *Aspergillus fumigatus* and *Candida albicans* (11–19). These studies suggest that, while HIF-1α plays an undeniable role in the cellular response to hypoxia, it may have been co-opted as a transcription factor in the response to pathogens as well.

HIF-1α regulates numerous genes involved in both innate and adaptive immune responses. This transcription factor has been implicated as a key element in phagocyte and T cell function in response to a wide variety of pathogens (20). In infectious diseases, HIF-1α targets within phagocytes include microbicidal genes as well as soluble mediators that recruit and activate immune cells (21). HIF-1α evokes direct killing of many pathogenic microbes by primary mediators such as reactive oxygen species, nitric oxide (NO), and antimicrobial peptides (22–25). Direct targets of HIF-1α in myeloid cells include the pro-inflammatory molecules TNF-α, IL-12, and CCL2 as well as the anti-inflammatory IL-10 (26–29).

IL-10 inhibits antimicrobial activity of Mφ by limiting production of inflammatory cytokines, chemokines, and reactive oxygen and nitrogen intermediates (30–32). In the innate response to *H. capsulatum*, IL-10 dampens immunity by limiting IFN-γ production; IL-10−/− mice exhibit elevated IFN-γ in association with accelerated *H. capsulatum* clearance (33). Modulation of IFN-γ by IL-10 attenuates the activation of Mφ, which need IFN-γ to kill *H. capsulatum* (34). Although IL-10 is produced by multiple cell populations, myeloid cells are the predominant producer during *H. capsulatum* infection (35).

The presence of hypoxia and the prominence of HIF-1α in dictating antimicrobial activity, metabolism, and cytokine generation led us to consider the contribution of this transcription factor in the myeloid response to *H. capsulatum* infection. To this end, we infected myeloid specific HIF-1α knockout mice (Lyz2cre *Hif1α*fl/fl) via the pulmonary route with *H. capsulatum*. The absence of myeloid HIF-1α decreased survival and increased fungal burden as
early as day 3 post-infection. The collapse of immunity was not associated with a reduction in lung pro-inflammatory protective cytokines, but rather with an elevation in IL-10. Mφs, the principal source of this cytokine, were the dominant myeloid cell population required for the phenotype of the mutant mice. Mφ production of IL-10 was tempered by HIF-1α and depended on enhanced CREB-binding protein (CBP)-driven transcriptional induction. Elevated IL-10 from *Lyz2cre Hif1α*/*β* Mφ prevented IFN-γ driven activation and, thus, enhanced fungal burden relative to control Mφ. These results demonstrate that HIF-1α is critical for controlling the progression of infection with the fungal pathogen *H. capsulatum* by limiting IL-10.
Materials and Methods

Mice

Male C57BL/6 and breeding pairs of Itgax-cre (C57BL/6 background) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). We thank Dr. Timothy Eubank, Ohio State University, for the Hif1α^fl/fl, Lyz2cre, and Lyz2cre Hif1α^fl/fl mice. Itgaxcre and Hif1α^fl/fl mice were crossed to generate Itgaxcre Hif1α^fl/fl mice. We thank Gang Huang, Cincinnati Children’s Hospital Medical Center, for Lyz2cre Hif1α^fl/fl Hif2α^fl/fl. Animals were housed in isolator cages and maintained by the Department of Laboratory Animal Medicine, University of Cincinnati, accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. All animal experiments were performed in accordance with the Animal Welfare Act guidelines of the National Institutes of Health, and all protocols were approved by the Institutional Animal Care and Use Committee of the University of Cincinnati.

Preparation of H. capsulatum and infection of mice

H. capsulatum strain G217B and yeast cells of the same strain that express GFP were grown for 72 h at 37°C as described (36, 37). To infect mice, 6–8 week-old animals were inoculated intranasally with 2x10^6 yeasts or 2x10^7 yeasts (indicated as high dose) in ~30 µl of Hank’s Balanced Salt Solution (HyClone, Logan, UT). For in vitro infection, cells were allowed to adhere to plates for 3 h. Cells were infected with 1-5 yeast per Mφ for the indicated times.

Generation of bone marrow-derived Mφs (BMDMφs) and in vitro inhibition

Bone marrow was isolated from tibiae and femurs of 6–10-week-old mice by flushing with HBSS. Cells were dispensed into tissue culture flasks at a density of 1x10^6 cells/ml of RPMI-
1640 supplemented with 10% fetal bovine serum, 0.1% gentamicin sulfate, 5 µM 2-mercaptoethanol, and 10 ng/ml of mouse GM-CSF (Peprotech, Rocky Hill, NJ). Flasks were incubated at 37 °C in 5% CO₂. Mφs were harvested at day 7. Non-adherent cells were removed, ice-cold PBS was added, and cells were scraped from the flask. Cells were collected, washed with PBS, and dispensed into culture dishes. For inhibition studies, piceatannol (Tocris, Bristol, UK) was added to Mφs 90 minutes before infection. For CBP interaction inhibition studies, chetomin (Santa Cruz, Dallas, TX) was added to Mφs 30 minutes prior to infection and KG501 (Calbiochem, San Diego, CA) was added 90 minutes before infection.

**RNA Isolation, cDNA synthesis, and quantitative real-time reverse transcription PCR**

Total RNA from whole lungs of mice was isolated using TRIzol (Invitrogen, Carlsbad, CA) and from in vitro Mφ cultures using the RNeasy Kit (Qiagen, Chatsworth, CA). Oligo(dT)-primed cDNA was prepared by using the reverse transcriptase system (Promega, Madison, WI).

Quantitative real-time reverse transcription PCR analysis was performed using TaqMan master mixture and primers (Applied Biosystems, Foster City, CA). Samples were analyzed with ABI Prism 7500. The hypoxanthine phosphoribosyl transferase housekeeping gene was used as an internal control. The conditions for amplification were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min.

**Isolation of lung leukocytes**

Lungs were homogenized with the gentleMACS dissociator (Miltenyi Biotec, Auburn, CA) in 5ml of HBSS with 2 mg/ml of collagenase D (Roche, Mannheim, Germany) and 40U of DNase I (Roche) for 30 min at 37°C. The homogenate was percolated through a 40 mm nylon mesh.
(Spectrum Laboratories, Rancho Dominguez, CA) and washed three times with HBSS.
Leukocytes were isolated by separation on Lympholyte M (Cedarlane, Burlington, ON).

**Western blot**
Following 24 h of infection, non-adherent cells were washed off with ice cold PBS. Cells were scraped off in ice cold PBS and spun down. Cells were lysed in RIPA buffer. Proteins were separated by electrophoresis in SDS/PAGE and transferred to PVDF membranes. The membranes were used for immunodetection of HIF-1α (Novus Biologicals, Littleton, CO) and β actin (Santa Cruz).

**Confocal microscopy**
Following 24 h of infection, BMDMφs were stained with HIF-1α antibody and DAPI nuclear stain for 30 min. Images were acquired on a Zeiss LSM710 confocal and analyzed with ZEN 2011 software.

**Organ culture for H. capsulatum**
Organs were homogenized in sterile HBSS, serially diluted, and plated onto mycosel-agar plates containing 5% sheep blood and 5% glucose. Plates were incubated at 30 °C for 7 days. The limit of detection was \(10^2\) CFU.

**Flow cytometry, cell sorting, and gating strategy**
Cells from mouse lungs were incubated with CD16/32 to limit nonspecific binding. Leukocytes were then stained with the indicated antibodies at 4°C for 15-30 min in phosphate-buffered saline
containing 1% bovine serum albumin and 0.01% sodium azide. Cells were stained with combinations of the following antibodies: FITC-conjugated Ly6G; FITC-conjugated Ly6C; FITC-conjugated CD11c; PerCP-conjugated CD11b; APC-conjugated CD3; PE-conjugated CD4; FITC-conjugated CD8; and APC-conjugated F4/80 (BD Biosciences, San Jose, CA). For intracellular IL-10 staining, cells were incubated with Cytofix/ Cytoperm (BD Biosciences), washed in Permeabilization Buffer (BD Biosciences), and stained for 45 min with PE-conjugated IL-10 (R&D systems, Minneapolis, MN). Cells were washed and resuspended in 1% paraformaldehyde. Isotype controls were used. Data were acquired using BD Accuri C6 cytometer and analyzed using the FCS Express 4.0 Software (DeNovo Software, Los Angeles, CA). For cell sorting experiments, leukocytes from the lungs of Lyz2cre and Lyz2cre Hif1αfl/fl mice were isolated at day 3 or day 7 post-infection using 5 laser FACS Aria II (BD Biosciences) following cell surface staining. Cells were identified using side (SSC-A) and forward scatter (FSC-A), followed by doublet exclusion using forward scatter height (FSC-H) against FSC-A. Cells were subsequently phenotypically characterized by the following surface markers: neutrophils (PMNs) were Ly-6Ghi, CD11b+, F4/80−; dendritic cells (DCs) were F4/80−, CD11b−/+, CD11c+; Mφ were F4/80+, CD11c+, CD11b+; CD4 T cells were CD3+, CD4+, CD8−; CD8 T cells were CD3+, CD4−, CD8+. As gated here, the Mφ population does not include interstitial Mφ or alveolar Mφ. Gating strategy depicted in Supplemental Fig. 1.

**PMN depletion and IL-4 and IL-10 neutralization**

PMNs were depleted by i.p. injection of anti-Ly6G mAb (1A8), containing 0.1 mg protein, 24 h prior to infection, and at days 1, 3, and 5 post-infection. PMN depletion was confirmed by flow cytometry staining for Ly6G/C (Nimp14), CD11b, and CD11c in experimental animals. IL-4 was
neutralized by i.p. injections of anti-IL-4 (11B11), containing 1 mg protein, at the time of infection and at day 3 post-infection. IL-10 was neutralized by i.p. injections of anti-IL-10 (JES5-2A5), containing 0.25 mg protein, at the time of infection, and at days 3, 5, and 7 post-infection. Control mice were treated with an equivalent quantity of IgG antibody.

**Histology**

Lungs were inflated, excised, fixed in 10% formalin, and embedded in paraffin blocks. Sections (5 µm) were stained with H&E. Serial pictures were obtained on an Olympus BX51 microscope and Olympus DP71 camera. Lung sections were reconstructed using Photoshop Photomerge CS5 (Adobe Systems, San Jose, CA). Area of inflammation was measured using the measure function in ImageJ (National Institutes of Health, Bethesda, MD) in a blinded fashion.

**Measurement of cytokines**

Cytokines were quantified in lung homogenates and cell-free Mφ culture supernatants using a Milliplex MAP immunoassay (Millipore, Billerica, MA) following an overnight incubation with the assay beads according to the manufacturer protocol. A minimum of 100 beads were counted for each analyte per well. The beads were analyzed on a Luminex Magpix instrument (Luminex Corporation, Austin, TX) using Luminex xPONENT software. Additional analysis was performed utilizing the Milliplex Analyst (Millipore) software.

**Griess assay**
The quantity of NO in the supernatant of cultured bone marrow derived macrophages was measured using the Griess Reagent System. Briefly, sample aliquots were mixed with an equal volume of Griess reagent (1% sulfanilamide/0.1% naphthylethylenediamine dihydrochloride/2% H$_3$PO$_4$). A microplate reader was used to measure the absorbance at 540 nm (BioTek Instruments, Inc., Burlington, VT). NO$_2^-$ was determined using NaNO$_2$ as a standard. Media background was determined and subtracted from the experimental values. Nitrate was converted into nitrite prior to the reaction with Griess reagent, since nitrite is rapidly oxidized to nitrate.

**Statistics**

Statistical p values were calculated with one-way ANOVA for multiple comparisons and adjusted with Bonferroni’s or Holm Sidak correction and nonpaired Student’s t test where two groups were compared; *p < 0.05; **p < 0.01; ***p < 0.001; NS, not significant
Results

Myeloid HIF-1α is required for fungal clearance and survival following infection with *H. capsulatum*.

Since HIF-1α regulates production of inflammatory cytokines and chemokines known to be essential in response to *H. capsulatum*, we sought to determine the necessity of this transcription factor within the myeloid cell compartment (38, 39). *Lyz2cre Hif1α*^fl/fl^ mice exhibit HIF-1α deletion within alveolar Mφs, inflammatory monocytes, monocyte-derived DCs, and PMNs (38). We infected control and *Lyz2cre Hif1α*^fl/fl^ mice with a sublethal number of *H. capsulatum* yeast cells. As early as 10 days post-infection, *Lyz2cre Hif1α*^fl/fl^ mice began to succumb to infection; by 30 days, over 90% of them died while all control *Lyz2cre* mice and *Hif1α*^fl/fl^ mice survived (Fig. 1A). To assess if the decreased survival in *Lyz2cre Hif1α*^fl/fl^ mice was associated with an enhanced fungal burden, we measured the number of organisms in lungs at 1, 3, and 7 days post-infection. *Lyz2cre Hif1α*^fl/fl^ mice exhibited a striking increase in fungal burden relative to controls as early as 3 days and through 7 days post-infection (Fig. 1B).

Cell recruitment and expansion are unaltered in HIF-1α knockout mice.

To elucidate any alterations in the organization of the inflammatory response, we evaluated the amount of inflammation in histopathology sections of lung tissue from infected control and *Lyz2cre Hif1α*^fl/fl^ mice at 3 and 7 days post-infection (Fig. 1C). At day 3, both control and *Lyz2cre Hif1α*^fl/fl^ mice exhibited acute peribronchiolar inflammation; the area of involvement in *Lyz2cre Hif1α*^fl/fl^ mice was increased relative to control mice (Fig. 1D). By 7 days the inflammation had coalesced into consolidation in both groups; there was no difference in the area between control and *Lyz2cre Hif1α*^fl/fl^ mice.
While we saw no change in the inflamed area at day 7, we speculated that the lack of HIF-1α might alter specific leukocyte populations since it is required for optimal immune cell recruitment (18, 38, 40). To examine the composition of inflammatory cell populations, we stained lungs at days 3 and 7 post-infection for Mφs, PMNs, DCs, and CD4+ and CD8+ T cells. Absolute cell numbers in each of these populations were unaltered relative to control at 3 days post-infection (Fig. 1E). PMNs were elevated at day 7 post-infection, but the numbers of other populations were similar between the two groups of mice (Fig. 1F). These results suggested that cell recruitment and expansion following infection do not explain the elevated fungal burden and decreased survival of Lyz2cre Hif1αfl/fl mice.

**Lack of HIF-1α in myeloid cells enhances production of IL-10.**

Since inflammatory cell recruitment was not deficient in Lyz2cre Hif1αfl/fl mice, we surveyed cytokines that are known to be important in the murine response to *H. capsulatum* in lung homogenates at 3 and 7 days post-infection. Relative to controls, Lyz2cre Hif1αfl/fl murine lungs exhibited an elevation in the anti-inflammatory cytokine IL-10 as well as prototypically protective cytokines IFN-γ and IL-1β as early as day 3 post-infection (Fig. 2A) (36, 41, 42). No change in TNF-α or GM-CSF between the two groups was observed on day 3 post-infection or in any cytokines prior to infection (Fig. 2A, data not shown). At 7 days of infection, the lungs of Lyz2cre Hif1αfl/fl mice manifested elevated production of IL-10, IFN-γ, IL-1β, GM-CSF and IL-4 (Fig. 2B). The IL-10 concentration at day 7 post-infection in lungs of the Itgaxcre mice (202 ± 13 pg/mL, n=4) did not differ (p>0.05) from that of infected controls (197 ± 6 pg/mL, n=8).

While IL-4 is known to inhibit the immune response to *H. capsulatum*, elevation of this cytokine subsequent to the rise in fungal burden suggested that it was a consequence rather than
a driver of the altered immune response in Lyz2cre Hif1αβ/β mice (43, 44). To determine the contribution of IL-4 to the phenotype of Lyz2cre Hif1αβ/β mice we administered mAb to this cytokine both prior to and during the course of infection. Since murine survival was unaltered in these mice, we concluded that IL-4 elevation was not responsible for the phenotype of Lyz2cre Hif1αβ/β mice (Supplemental Fig. 2A).

One concern in interpreting the elevation in cytokines in Lyz2cre Hif1αβ/β mice is that the higher fungal burden may have enhanced production. To test this assertion, we infected control animals with a one log higher inoculum of H. capsulatum; this number of yeasts replicated the fungal burden in Lyz2cre Hif1αβ/β mice (Fig. 2C). We measured cytokines in lung homogenates following instillation of the lower and higher number of yeast cells at 7 days post-infection. The exaggerated fungal burden in control mice increased IFN-γ and IL-1β cytokine production comparable to the amount in Lyz2cre Hif1αβ/β mice while TNF-α was unaltered (Fig. 2D). However, the quantity of IL-10 in the heavily infected controls did not differ from that of mice that received the lower inoculum (Fig. 2D). Thus, the high fungal burden was not the driving force in amplified IL-10 in Lyz2cre Hif1αβ/β mice. This finding demonstrates that the enhanced IL-10 production in Lyz2cre Hif1αβ/β mice is a consequence of HIF-1α deficiency while the heightened pathogen burden in these mice caused the increase in proinflammatory cytokines.

Since IL-10 was augmented early during infection, we examined its influence on the fungal burden and survival of Lyz2cre Hif1αβ/β mice. Anti-IL-10 mAb given to Lyz2cre Hif1αβ/β and control mice reduced fungal burden compared to IgG control in Lyz2cre Hif1αβ/β mice and enhanced survival (Fig. 2E, 2F, and Supplemental Fig. 4).
Mφs are the primary IL-10 producing cells from *Lyz2cre Hif1α*^ΔΔ^ mice following infection with *H. capsulatum*.

We sought to determine the primary IL-10 producing cell populations within the lungs of infected mice. We inoculated control and *Lyz2cre Hif1α*^ΔΔ^ mice with *H. capsulatum* and performed intracellular IL-10 staining within several immune cell populations including Mφs at days 3 and 7. Representative flow cytometry plots demonstrate IL-10 staining in myeloid cells at day 7 post-infection (Fig. 3A). Prior to infection, intracellular IL-10 is not detectable in control or *Lyz2cre Hif1α*^ΔΔ^ mice (data not shown). At day 3, multiple myeloid populations produced IL-10, but Mφs constituted the majority of IL-10^+^ cells in both control and *Lyz2cre Hif1α*^ΔΔ^ mice (Fig. 3B). Likewise, at day 7, Mφs still constituted the most numerous IL-10 producers in both groups (Fig. 3D). Relative to controls, the number of IL-10^+^ PMNs, DCs, and Mφs isolated from *Lyz2cre Hif1α*^ΔΔ^ mice at day 3 were increased, whereas at day 7 post-infection more IL-10^+^ lung Mφs and T cells were present in the mutant mice (Fig. 3B and 3D). There was no change between the two groups in the IL-10 MFI in any of the cell populations at either day 3 or day 7 post-infection (Fig. 3C and 3E). Thus, Mφ are the most numerous IL-10 producing cell population in response to *H. capsulatum*. The increase in IL-10 found in the lungs was most likely a consequence of more cells rather than inflated production at the single cell level since MFIs did not vary between identical cell populations from the two groups of mice.

**Loss of Mφ HIF-1α is responsible for the survival defect in *Lyz2cre Hif1α*^ΔΔ^ mice.**

*Lyz2cre Hif1α*^ΔΔ^ mice exhibit HIF-1α deletion in all myeloid cells. In order to hone in on the cell population(s) responsible for the survival defect within mutant mice, we generated *Itgaxcre Hif1α*^ΔΔ^ mice. In these mice HIF-1α is eliminated in alveolar Mφs and tissue resident
and monocyte-derived DCs (45). We infected control, Lyz2cre Hif1α¬/¬, and Itgaxcre Hif1α¬/¬ mice with a sublethal number of H. capsulatum yeast cells. As observed previously the vast majority of Lyz2cre Hif1α¬/¬ mice succumbed to infection while all control and Itgaxcre Hif1α¬/¬ mice survived (Fig. 4A). These results suggested that HIF-1α in alveolar Mϕs and DCs was dispensable for survival following H. capsulatum infection. Thus, we can infer that the presence of this transcription factor in PMNs or Mϕs was required to confer protective immunity.

We sought to directly address the impact of PMN HIF-1α. Studies that examine the necessity of PMNs in H. capsulatum by depleting them suggest that they are essential for protective immunity (46, 47). However, these reports utilize an antibody (clone RB6) that recognizes both Ly6G and Ly6C. Since the latter is expressed on PMNs and inflammatory monocytes, it is difficult to discriminate which of these populations is responsible for the aggressive infection in mice. We therefore sought to clarify the contribution of PMNs by depleting these cells in both wild type and Lyz2cre Hif1α¬/¬ mice with a Ly6G specific antibody. Following administration of 1A8 mAb or isotype control mAb, Lyz2cre Hif1α¬/¬ and wild type mice were infected for 7 days. The loss of PMNs was confirmed by differential lung leukocyte count (Fig. 4B). The lack of PMNs did not elevate the fungal burden in either control mice or Lyz2cre Hif1α¬/¬ mice (Fig. 4C). Since PMNs were dispensable for fungal clearance, we concluded that these cells did not contribute to the phenotype observed in Lyz2cre Hif1α¬/¬ mice via either HIF-1α-dependent or independent mechanisms. These results demonstrate that Mϕ HIF-1α was required for murine survival. The importance of this population is consistent with our prior data that IL-10 contributes to the phenotype of Lyz2cre Hif1α¬/¬ mice and that Mϕ are the primary producer of IL-10 in these mice.
Infection with *H. capsulatum* drives HIF-1α transcription and protein nuclear localization within Mφs.

The accumulated data strongly implicated HIF-1α deficiency within Mφs as the critical node for the elevated fungal burden and decreased survival in *Lyz2cre Hif1α<sup>ββ</sup>* mice. To assess transcriptional induction of HIF-1α as well as several downstream targets including vascular endothelial growth factor (*Vegf-a*) and phosphoglycerate kinase 1 (*Pgk-1*) *in vivo*, we infected animals with *H. capsulatum* expressing GFP and sorted infected pulmonary Mφs (F4/80<sup>+</sup>CD11b<sup>+</sup>CD11c<sup>−</sup>GFP<sup>+</sup> or GFP<sup>−</sup>) at 1, 3, and 7 days post-infection. Infected cells exhibited transcriptional upregulation of *Hif-1α, Vegf-a, and Pgk-1* (Fig. 5A).

Evidence of other microbes driving HIF-1α led us to inquire whether *H. capsulatum* infection induces HIF-1α in the absence of hypoxia (8, 48–50). Accordingly, we infected control and *Lyz2cre Hif1α<sup>ββ</sup>* BMDMφs for 24 h and assessed the transcription of *Hif-1α* and its downstream targets; *Hif-1α* and *Pgk-1* were induced over 5 fold while *Vegf-a* was induced over 10 fold in a HIF-1α dependent manner (Fig. 5B). We assessed expression of CD11b on Mφ because it is the principal phagocytic receptor and it is regulated by HIF-1α: this integrin was not altered in *Lyz2cre Hif1α<sup>ββ</sup>* cells *in vitro or in vivo* (51, 52) (Supplemental Fig. 3A and 3B).

To directly assess HIF-1α protein stabilization and cellular localization, we utilized uninfected cells or cells infected for 24 h from both control and HIF-1α-deficient BMDMφs and analyzed protein expression and localization via western blot and confocal microscopy, respectively. Western blot analysis demonstrated HIF-1α protein in infected *Lyz2cre* cells, but no HIF-1α was detected in infected cells from *Lyz2cre Hif1α<sup>ββ</sup>* mice (Fig. 5C). Confocal microscopic analysis demonstrated that HIF-1α was located within the nucleus following infection of wild type but not *Lyz2cre Hif1α<sup>ββ</sup>* BMDMφs with *H. capsulatum* (Fig. 5D). We
concluded that *H. capsulatum* infection causes HIF-1α upregulation and translocation to the nucleus where it can modulate downstream targets.

**The transcription factor CREB, in the absence of HIF-1α, drives elevated IL-10 production in response to *H. capsulatum* infection.**

Since HIF-1α has been shown to positively regulate IL-10 production, the increase in this cytokine found in the lungs of Lyz2cre Hif1αβ/β mice was unexpected (53). To investigate the mechanism of IL-10 production, we infected control and Lyz2cre Hif1αβ/β BMDMφs for 24 and 48 h. Fungal burdens at 48 h were not different between control and Lyz2cre Hif1αβ/β BMDMφs (Fig. 6A). We quantified IL-10 in the cell culture supernatants; Lyz2cre Hif1αβ/β derived Mφs secreted nearly twice as much IL-10 only at 48 h (Fig. 6B). This elevation was associated with a concomitant enhancement in IL-10 transcription in Lyz2cre Hif1αβ/β BMDMφs compared to control (Fig. 6C).

Competition between transcription factors for a limiting supply of CBP has been shown to regulate IL-10 production (54). Therefore we hypothesized that the absence of HIF-1α resulted in increased HIF-2α binding to CBP and subsequent elevation in IL-10 protein (55, 56). To test this postulate we infected BMDMφs from controls, Lyz2cre Hif1αβ/β mice, and Lyz2cre Hif1αβ/β Hif2αβ/β double knockout mice for 24 h and examined IL-10 protein in supernatants. The quantity of IL-10 in the absence of both HIF-1α and HIF-2α exceeded that of cells from control and Lyz2cre Hif1αβ/β mice (Fig. 6D).

We then examined the function of CREB, another transcription factor known to bind CBP and drive IL-10 transcription (57). To test the contribution of CREB and HIF-1α on IL-10 production, we treated wild type BMDMφs with small molecule inhibitors that prevent CREB
(KG-501) or HIF-1α (chetomin) association with CBP. KG-501, but not chetomin, inhibited IL-10 transcription in response to *H. capsulatum* infection (Fig. 6E). KG-501 also decreased IL-10 protein during infection with *H. capsulatum* (Fig. 6F).

To address the concern of off-target effects of the inhibitor, we utilized siRNA to reduce CREB in both control and *Lyz2cre Hif1α*<sup>fl/fl</sup> BMDM<sup>ϕ</sup>s. CREB knockdown reduced transcription of IL-10 in both control and *Lyz2cre Hif1α*<sup>fl/fl</sup> BMDM<sup>ϕ</sup>s (Fig. 6G). These results demonstrate that CREB is important for IL-10 production in both control and *Lyz2cre Hif1α*<sup>fl/fl</sup> BMDM<sup>ϕ</sup>s, but the presence of HIF-1α tempers IL-10 levels.

**Elevated IL-10 production by *Lyz2cre Hif1α*<sup>fl/fl</sup> BMDM<sup>ϕ</sup>s inhibits IFN-γ induced fungal control.**

Since M<sup>ϕ</sup>s are the primary IL-10 producing cell and one of the primary sites of phagocytosis, we queried the number of these cells infected following administration of GFP<sup>+</sup> *H. capsulatum*. There was an increase in infected cells from *Lyz2cre Hif1α*<sup>fl/fl</sup> mice relative to controls with no alteration in GFP MFI within the infected cells (Figure 7A and 7B; data not shown). This finding implied that M<sup>ϕ</sup> from *Lyz2cre Hif1α*<sup>fl/fl</sup> mice were more permissive for intracellular invasion that was not a consequence of altered expression of CD11b. Hence we asked if the M<sup>ϕ</sup> from the mutant mice were less responsive to an exogenous activating signal such as IFN-γ. IL-10 is known to inhibit the capacity of M<sup>ϕ</sup>s to respond to IFN-γ (58). To address responsiveness of M<sup>ϕ</sup>s to activating cytokine we infected wild type and *Lyz2cre Hif1α*<sup>fl/fl</sup> BMDM<sup>ϕ</sup>s with GFP<sup>+</sup> *H. capsulatum* for up to 72 h with or without the addition of IFN-γ. While cytokine activation decreased the percentage of infected control BMDM<sup>ϕ</sup>s, this signal did not
alter the infection of *Lyz2cre Hif1α* BMDMφs (Fig. 7C). Administration of anti-IL-10 at the time of infection enabled IFN-γ mediated activation of *Lyz2cre Hif1α* BMDMφs (Fig. 7D). Taken together our results suggest that elevated IL-10 from HIF-1α deficient-Mφs inhibits IFN-γ responsiveness that is required for effective fungal clearance.

**Profiles of inducible nitric oxide synthase (iNOS) and NO in *Lyz2cre Hif1α* mice.**

HIF-1α evokes direct killing of many pathogenic microbes by primary mediators such as nitric oxide (22, 24). Decreased iNOS transcription and subsequently low nitric oxide levels have been implicated in diminished clearance of group A *Streptococcus* and *Mycobacterium marinum* in the setting of HIF-1α deficiency (22). Inhibition of iNOS in both of these models was able to elevate pathogen burden. To determine if NO was critically altered in HIF-1α deficiency, we first assessed the ability of *H. capsulatum* to evoke iNOS in bone marrow derived macrophages. Although infection led to iNOS induction in both control and *Lyz2cre Hif1α* cells, it was significantly reduced in the absence of HIF-1α (Fig. 8A). However, NO production was unaltered in the absence of HIF-1α (Fig. 8B).

Since IFN-γ activates murine macrophages to inhibit *H. capsulatum* growth through stimulation of nitric oxide generation, we activated and infected bone marrow derived macrophages (59, 60). *Lyz2cre Hif1α* and control cells secreted similar quantities of nitrite (Fig. 8C).

Finally we examined iNOS induction in *vivo*. During early infection, iNOS was not detectable in whole lung homogenates (Fig. 8D). iNOS is elevated at day 3 post-infection in both *Lyz2cre Hif1α* and control mice (Fig. 8D). By day 7 post-infection, *Lyz2cre Hif1α* mice express more iNOS than controls (Fig. 8D). These results suggest that diminished NO production
in the absence of HIF-1α is not responsible for the elevated fungal burden and diminished survival of Lyz2cre Hif1α<sup>fl/fl</sup> mice.
Discussion

In this study, we demonstrated that the transcription factor HIF-1α in the myeloid cell compartment was a central molecular regulator of the protective immune response to \textit{H. capsulatum}. Its absence in these cells produced a progressive infection that led to the death of the vast majority of animals. The principal defect was not an alteration in the inflammatory response but rather an early and striking elevation in IL-10. Despite an abundance of cytokines known to enhance immunity, IL-10 overrode their impact on host resistance. Immunity was restored when this cytokine was neutralized by mAb to this cytokine. Since the exaggerated production of IL-10 was detected prior to the onset of an adaptive immune response to \textit{H. capsulatum}, we surmised that its impact influenced innate immunity (61). The principal source of IL-10 was Mϕ thus localizing impaired immunity to this specific population. Two findings provided additional evidence that Mϕs were the cause of the collapse of immunity rather than another myeloid cell population. First, elimination of PMNs in both control and \textit{Lyz2cre Hif1α}\textsuperscript{fl/fl} mice did not alter fungal burden. Second, \textit{Itgaxcre Hif1α}\textsuperscript{fl/fl} mice which exhibit selective deletion in tissue and resident DCs and alveolar Mϕ survived the sublethal challenge. The unexpected elevation in IL-10 by HIF-1α-deficient Mϕ is not caused by HIF-2α but is largely driven by CREB. \textit{In vitro} studies revealed that anti-IL-10 neutralization reversed the failure of Mϕ from \textit{Lyz2cre Hif1α}\textsuperscript{fl/fl} mice to respond to IFN-γ-mediated activation.

While HIF-1α is classically described as a key element in the cell response to hypoxia, mounting evidence over the past decade has described its requirement in the immune response to several pathogens; often, HIF-1α is required for phagocyte activation and killing, but occasionally it is co-opted by the pathogen to promote intracellular survival (18, 20, 25, 62, 63). As a requisite factor for protective immunity exerted by Mϕ, HIF-1α has been shown to regulate
production of cytokines, chemokines, and antimicrobial molecules (22, 24, 28, 39, 64, 65). Thus, in the context of infection, HIF-1α target genes within phagocytes and Mφ in particular can be organized into two categories: one is production of soluble mediators that recruit and activate other populations of immune cells and two, regulation of microbicidal activity by cells exposed to pathogens.

Multiple pathogens are known to induce the transcription or stabilization of HIF-1α protein even in the absence of hypoxia (11, 16, 18, 19, 25, 66, 67). Accordingly, *H. capsulatum*-infected Mφ promoted expression of HIF-1α protein as demonstrated by western blot. The robust transcriptional induction of HIF-1α protein leads to an elevation in both the nucleus and the cytoplasm, which likely represents a massive accumulation with a delay prior to nuclear translocation. We confirmed the *in vitro* induction results with *in vivo* studies in which sorted lung Mφ from wild-type mice transcribed the gene at day 7 post-infection, but not earlier. This finding does not correspond to the temporal identification of poor control of fungal burden in the mutant mice which begins on day 3. Although we could not detect by transcription a change in HIF-1α prior to day 7, clearly its impact precedes transcriptional upregulation. The likely explanation is that *H. capsulatum* stimulates protein stabilization prior to transcriptional induction.

We anticipated a role for myeloid HIF-1α in shaping the adaptive immune response to murine histoplasmosis since hypoxia is detected within liver granulomas induced by i.p. injection of yeast cells (2). However, protective immunity was subverted in the *Lyz2cre Hif1αΔ/Δ* mice prior to the onset of adaptive immunity. These data indicated that a maladaptive innate response led to the phenotype of *Lyz2cre Hif1αΔ/Δ* mice.
To hone in on the innate immune cell population responsible for the defective immune response in Lyz2cre Hif1αβ/β mice, we evaluated the contribution of various myeloid lineage cells. Mφs, alveolar Mφs, PMNs, and resident DCs are important for phagocytosis of yeast cells following infection (51, 68, 69). These cells release cytokines and chemokines that facilitate mobilization of the inflammatory response to combat invasion by H. capsulatum. Since Itgaxcre Hif1αβ/β mice did not manifest impaired survival we conclude that HIF-1α within DCs and alveolar Mφs is dispensable following H. capsulatum infection. The fact that elimination of PMNs from Lyz2cre Hif1αβ/β mice did not alter fungal burden signifies that HIF-1α deficiency in this population was not a contributor to the failed protective immune response. Our data with PMN depletion contradicts prior studies whose results strongly suggest that PMNs are essential for H. capsulatum clearance (46, 47). However, those studies employed the antibody RB6 which is now known to recognize Ly-6G and Ly-6C. The latter is borne by inflammatory monocytes (70). Therefore it is quite likely that in those experiments both PMNs and inflammatory monocytes were depleted. The mAb that we employed, 1A8, is considered to be more selective for Ly-6G (70).

The pivotal contribution of HIF-1α in response to H. capsulatum was not to drive inflammatory cytokine production, but rather to temper IL-10. This finding is quite unexpected since HIF-1α binds to hypoxia response elements (HREs) that exist in the promoters for several cytokines and chemokines important in the myeloid response to H. capsulatum including TNF-α, CCL2, and IL-10 (71–73). The loss of HIF-1α did not reduce pro-inflammatory cytokine production or cell recruitment to the lungs following infection. In fact, there was an increase in several of these prototypically protective cytokines in Lyz2cre Hif1αβ/β lungs relative to control. These data contrast with infectious models in which decreased myeloid pro-inflammatory
cytokine production is noted in the absence of HIF-1α; however, our data are congruent with a study of *Pseudomonas aeruginosa* infection in mice where the lack of HIF-1α in myeloid cells does not diminish the generation of inflammatory cytokines (18, 7, 25). In our model of histoplasmosis, the fungal burden and not the absence of the transcription factor was responsible for the enhanced pro-inflammatory cytokine production observed in the *Lyz2cre Hif1α<sup>fl/fl</sup>* mice. The exception was IL-10; this cytokine was elevated in the lungs of the conditional knockouts independent of the fungal load. This result establishes that the loss of HIF-1α results in an early increase in IL-10, and this heightened response undermined the integrity of the innate immune response. A previous study by our group documented that the loss of IL-10 enhanced clearance of the fungus (33). However, the influence of this loss was not observed until adaptive immunity was operative. The current information clearly documents that early, exaggerated IL-10 can alter the function of Mφ.

While several studies demonstrate that HIF-1α induces IL-10 transcription, here we established that deficiency of this transcription factor alone or in conjunction with HIF-2α deficiency actually elevates IL-10 transcript and protein production during *H. capsulatum* infection (53, 74). Although counterintuitive, there are several lines of evidence that support the notion that HIFs may moderate IL-10. First, inhibition of *Hif-1α* transcriptional induction in BMDMφs enhances IL-10 transcript and secreted protein following infection with *Mycobacterium tuberculosis* (75). Second, LPS-challenged mice deficient in myeloid HIF-2α exhibit an elevation in circulating IL-10 (76). Taken together, these results indicate that the influence of HIFs on IL-10 may be context dependent.

One of the principal issues raised by our findings is how IL-10 dampened innate immunity. Mφs must be activated by exogenous signals to exert anti-*Histoplasma* activity, and
IFN-γ is central to the activation of Mϕ (77, 60). One known effect of IL-10 is that it blunts IFN-γ-induced activation of Mϕs thus thwarting the arming of these phagocytes to limit intracellular infection (78, 58). We asked if the heightened IL-10 in Mϕs from Lyz2cre Hif1α^fl/fl mice altered responsiveness to IFN-γ. Indeed, IFN-γ did not restrict fungal growth in these cells in vitro unless IL-10 was neutralized. In vivo, anti-IL-10 restored immunity as evidenced by a reduction in CFUs and an increase in survival. Although the bulk of IFN-γ is produced after day 5 largely by CD4^+ T cells in an IL-12-dependent manner, there is an early production of this cytokine by unidentified sources (79). This initial generation appears to be important for early activation of Mϕs to limit intracellular growth.

In several infectious models, HIF-1α has been found to exert protection via induction of iNOS and subsequent generation of NO (22, 24). In fact IFN-γ-mediated macrophage activation leads to *H. capsulatum* growth inhibition through stimulation of nitric oxide generation (59, 60). However, we found that infection of Lyz2cre Hif1α^fl/fl mice is associated with comparable iNOS transcript during early infection followed by an elevation during late infection. While these results were not anticipated, they are in agreement with previous studies that have demonstrated that IL-10 enhances macrophage NO generation in the presence of an activation signal (80, 81).

CREB was responsible for elevated IL-10 in the absence of HIF-1α. In order to drive gene transcription, HIF-1α directly interacts with the CBP/p300 complex which can then bind to target gene promoters (62). In addition to HIF-1α, CBP/p300 can interact with a wide variety of transcription factors; competition for a limiting quantity of CBP/p300 regulates transcriptional induction of HIF-1α targets (82, 83). We hypothesized that the lack of HIF-1α allowed other transcription factors to bind to CBP thus enhancing transcriptional induction of IL-10. One such factor is CREB, which is required for β-glucan-stimulated IL-10 in murine BMDMϕs (84).
data indicate that CREB drives elevated IL-10 transcription in Mφ in response to *H. capsulatum*; HIF-1α deficiency augmented CREB-mediated IL-10 production. While there are numerous binding sites on CBP, the contribution of other transcription factors to IL-10 production in the absence of HIF-1α is unlikely given that IL-10 protein is nearly lost with CREB inhibition or silencing. Our data suggests a novel mechanism of IL-10 regulation dependent on transcription factor competition for binding to CBP. In contrast to other models, HIF-1α is required to temper anti-inflammatory IL-10 production during the innate immune response to *H. capsulatum* infection. This finding is in accord with recent evidence that inhibition of HIF-1α elevates IL-10 secretion by LPS-treated BMDMφs (75).

Although IL-10 transcriptional induction and protein secretion occurs at 24 h in our *in vitro* studies, *Lyz2cre Hif1αfl/fl* BMDMφs only exhibit an increase relative to controls at 48 h. While the mechanism of this delay is unclear, several modes of IL-10 regulation may be responsible. The two most intriguing possibilities include control of IL-10 expression by microRNAs and HIF-1α-dependent regulation of mRNA stability. Both of these could account for the increase seen at both the transcriptional and resultant protein level. A variety of microRNAs which are regulated by HIF-1α have been shown to inhibit IL-10 production; these include miR-27a and Let-7 family members Let-7b, Let-7c, and Let-7f (85–89). While few studies have focused on the role of HIF-1α in controlling mRNA stability, it binds to VEGF mRNA and regulates transcript decay (90, 91). One or both of these mechanisms may drive the differential expression of IL-10 in *Lyz2cre Hif1αfl/fl* versus control BMDMφs.

Given the role of HIF-1α-dependent transcriptional regulation *in vitro*, the absence of an alteration of IL-10 MFI between cells from *Lyz2cre Hif1αfl/fl* and control mice was unexpected. Our inability to capture a change in IL-10 MFI in the presence of a dramatic increase in IL-10
may be the result of *in vivo* dynamics. The delayed transcript changes that are seen *in vitro* are observed in the absence of immigrating inflammatory cells. Moreover, infection *in vitro* is synchronized such that all cells are exposed simultaneously to the pathogen. The coordinated response does not occur in vivo since inflammatory cell recruitment is continuous and infection of these cells is asynchronous. This may obscure any changes in IL-10 within cells in the lung.

In summary, we identified HIF-1α as a vital transcription factor in the Mφ response to *H. capsulatum*. HIF-1α was transcriptionally upregulated and protein stabilized in *H. capsulatum*-infected Mφ exposed to normoxia. HIF-1α fortified the innate response to this fungus by tempering the production of IL-10 in Mφ. One clinical ramification of our work is in the arena of the potential use of HIF inhibitors to treat malignancies (92). Based on our findings, this intervention may pose a risk for those exposed to *H. capsulatum* or perhaps other intracellular pathogens in which IL-10 is a prominent feature of their immune regulation. Thus, a cautious approach may be warranted in the deployment of HIF-1α inhibitors.
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Footnotes

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Abbreviations used in this article: HIF-1α, Hypoxia inducible factor-1α; Mϕ, macrophage; CBP, CREB-binding protein; BMDMϕ, bone marrow derived macrophage; DC, dendritic cell; PMN, neutrophil

Address correspondence to Dr. George Deepe, Phone: 513-558-4706; e-mail address: george.deepe@uc.edu; and fax 513-558-2089.
**Figure Legends**

**FIGURE 1.** Mφ HIF-1α expression is required for murine survival during *H. capsulatum* infection. *A*, *Lyz2cre* and *Lyz2cre Hif1α*\(^{fl/fl}\) mice were infected and survival recorded when mice were moribund. (n=10-18 mice/group; representative of five experiments) *B*, Lungs were harvested at days 1, 3, and 7 post-infection to determine the number of CFUs. Data represent the mean ± SD (n=8-12 mice/group; representative of three experiments). *C*, Representative images of lung sections stained with H&E taken at 100x (C) magnification at 3 and 7 days post-infection. (n=5 mice/group; 3 experiments) *D*, Quantification of inflammatory area/total area and regions of inflammation/section. (n=5 mice/group; 3 experiments) *E* and *F*, Lung leukocytes were isolated and counted at days 3 and 7 post-infection. (n=6 mice/group; 4 experiments) *p*<0.05; **p**<0.01; ***p**<0.001

**FIGURE 2.** Elevated IL-10 in *Lyz2cre Hif1α*\(^{fl/fl}\) mice drives the increased fungal burden and decreased survival relative to control. Protein concentrations were determined in lung homogenates at days 3 (*A*) and 7 (*B*) post-infection. Data are represented as the mean ± SD. (n = 8-12 from 3 experiments) *C*, *Lyz2cre Hif1α*\(^{fl/fl}\) were infected with 2x10\(^6\) yeasts intranasally; *Lyz2cre* were infected with 2x10\(^6\) or 2x10\(^7\) (high dose) yeasts intranasally. Lungs were harvested at day 7 post-infection to determine the number of CFUs. The CFUs of the high dose and *Lyz2cre Hif1α*\(^{fl/fl}\) groups were compared to the control and to each other. (n=6-8 mice/group; representative of 3 experiments) *D*, IFN-γ, IL-1β, TNF-α and IL-10 protein concentrations in lung homogenates were determined at day 7 post-infection. (n=6-8 mice/group; representative of 3 experiments) *E*, Following administration of IL-10 neutralizing antibody or control IgG, *Lyz2cre* and *Lyz2cre Hif1α*\(^{fl/fl}\) mice were infected with 2x10\(^6\) yeasts intranasally. Lungs were
harvested at days 3 and 7 post-infection to determine the number of CFUs. Data represent the mean ± SD (n=8-12 mice/group; representative of three experiments). F, Survival was recorded when mice were moribund. (n=9-12 mice/group; representative of 3 experiments) *p<0.05; **p<0.01; ***p<0.001

FIGURE 3. Mφs from Lyz2cre Hif1α<sup>fl/fl</sup> mice are a major source of IL-10 in response to H. capsulatum. Lung leukocytes were isolated from infected Lyz2cre and Lyz2cre Hif1α<sup>fl/fl</sup> mice at days 3 and 7 post-infection, stained for IL-10, and counted. A, Representative dot plots of IL-10 staining in Hif1α<sup>fl/fl</sup> and Lyz2cre Hif1α<sup>fl/fl</sup> lung Mφs, neutrophils, and dendritic cells at day 7 post-infection. B and D, Graphs represent the absolute number and % of IL-10<sup>+</sup> cells in leukocyte populations at days 3 and 7 post-infection. C and E, Graphs represent the IL-10 MFI within those populations at those time points. Data represent the mean ± SD (n = 6-8) from three experiments. *p<0.05; **p<0.01; ***p<0.001

FIGURE 4. HIF-1α expression in Mφs, but not DCs, alveolar macrophages, or PMNs is required for murine survival during H. capsulatum infection. A, Lyz2cre, Lyz2cre Hif1α<sup>fl/fl</sup>, and Itgaxcre Hif1α<sup>fl/fl</sup> mice were infected and survival recorded when mice were moribund. (n=10-18 mice/group; representative of five experiments) B, Absolute number of PMNs was determined at day 7 post-infection after depletion. PMNs were characterized as Ly6G<sup>+</sup> CD11b<sup>+</sup> CD11c<sup>-</sup>. (n=9 mice/group; representative of 3 experiments) C, Lungs were harvested at day 7 post-infection to determine the number of CFUs. Data represent the mean ± SD. (n=9 mice/group; representative of 3 experiments) *p<0.05; **p<0.01; ***p<0.001
FIGURE 5. *H. capsulatum* induces nuclear HIF-1α in Mφs. *A* and *B*, mRNA expression of HIF-1α, VEGF, and PGK-1 was quantified by qRT-PCR relative to uninfected controls. *A*, GFP+ monocytes were collected at days 1, 3, and 7 following intranasal infection with GFP expressing yeast. (n=12 mice/group; representative of 3 experiments) *B*, BMDMφs were collected 24 h post-infection. (n=8 experiments/group) *C*, Immunoblot of HIF-1α and β-actin from whole-cell lysates of Mφs infected with *H. capsulatum* for 24 h. (representative experiment of 3) *D*, Confocal images of BMDMφs uninfected or infected for 24 h followed by nuclear staining (DAPI) and staining for HIF-1α. (representative experiment of 3) *p<0.05; **p<0.01; ***p<0.001

FIGURE 6. In the absence of HIF-1α, the transcription factor CREB drives elevated IL-10 production in response to *H. capsulatum* infection. *A*, BMDMφs were infected for 48 h then lysed to determine fungal burden (n=4-6 mice/group; representative of 3 experiments) *B*, *D*, *F* IL-10 concentration was determined in the cell culture supernatants by Magpix. *C*, *E* and *G*, mRNA expression of IL-10 was quantified by qRT-PCR relative to uninfected controls. *B*, BMDMφs were infected for 48 h. (n=6 mice/group; representative of 3 experiments) *C*, BMDMφs were infected for 8, 24, or 48 h. (n=6-9 mice/group; representative of 5 experiments) *D*, *Lyz2cre* and *Lyz2cre Hif1αββ*, and *Lyz2cre Hif1αββ Hif2αββ* double knockout BMDMφs were infected for 24 h. (n=6 mice/group; representative of 3 experiments) *E*, *F*, BMDMφs were infected for 48 h after treatment with DMOG (control), a HIF-1α-CBP interaction inhibitor (chetomin), or a CREB-CBP interaction inhibitor (KG-501). (n=9 mice/group; representative of 3 experiments). Treatment groups were compared to the DMOG control. *F*, BMDMφs were
infected for 48 h after treatment with scrambled siRNA (control) or CREB siRNA. (n=6 mice/group; representative of 3 experiments) *p<0.05; **p<0.01; ***p<0.001

**FIGURE 7.** IL-10 inhibits IFN-γ induced fungal control by Lyz2cre Hif1αβ/β BMDMφs. GFP-expressing *H. capsulatum* was utilized to determine the infected populations *in vivo* (*A, B*) or *in vitro* (*B, C*). *A*, Representative flow cytometry plots of infected Mφs at day 7 post-infection. *B*, Absolute number of infected Mφs was determined at day 7 post-infection in Lyz2cre and Lyz2cre Hif1αβ/β mice. (n=6 mice/group; representative of 3 experiments) *C* and *D*, Lyz2cre and Lyz2cre Hif1αβ/β BMDMφs were infected for 72 h with or without IFN-γ activation and IL-10 neutralization. Treatment groups were compared to the untreated control and to each other. (*C, n=6 mice/group; representative of 3 experiments; D, n=6 mice/group; representative of 3 experiments) *p<0.05; **p<0.01; ***p<0.001

**FIGURE 8.** NO production is not diminished in Lyz2cre Hif1αβ/β BMDMφs infected with *H. capsulatum*. *A*, iNOS transcript was determined in Lyz2cre and Lyz2cre Hif1αβ/β BMDMφs at 48 hours post-infection. (n=3 mice/group; representative of 3 experiments) *B* and *C*, Lyz2cre and Lyz2cre Hif1αβ/β BMDMφs were infected for 48 h with or without IFN-γ activation. Supernatants were tested for nitrite. (*B, n=3-8 mice/group; representative of 3 experiments; C, n=3 mice/group; representative of 3 experiments) *D*, iNOS transcript was assessed in whole lung homogenates at 1, 3, and 7 days post-infection. (n=3-6 mice/group; representative of 3 experiments) nd = not detected; *p<0.05; **p<0.01; ***p<0.001.
SUPPLEMENTAL FIGURE 1. Gating Strategy. Cells were identified using side (SSC-A) and forward scatter (FSC-A), followed by doublet exclusion using forward scatter height (FSC-H) against FSC-A. Cells were subsequently phenotypically characterized by the following surface markers: neutrophils (PMNs) were Ly-6G$^{hi}$, CD11b$^+$, F4/80$^-$; dendritic cells (DCs) were F4/80$^-$, CD11b$^{-/+}$, CD11c$^+$; Mφ were F4/80$^+$, CD11c$^-$, CD11b$^+$; CD4 T cells were CD3$^+$, CD4$^+$, CD8$^-$; CD8 T cells were CD3$^+$, CD4$, CD8^+$.

SUPPLEMENTAL FIGURE 2. IL-4 neutralization does not enhance survival of Lyz2cre Hif1α$^{β/β}$ mice. Lyz2cre Hif1α$^{β/β}$ mice were infected in the presence or absence of anti-IL-4 antibody and survival recorded when mice were moribund. (n=4-6 mice/group; representative of three experiments)

SUPPLEMENTAL FIGURE 3. CD11b expression on Lyz2cre Hif1α$^{β/β}$ lung Mφs or BMDMφs is not altered compared to Lyz2cre control cells. A, Lyz2cre and Lyz2cre Hif1α$^{β/β}$ mice were infected for 1, 3, or 7 days. Mφ were isolated from the lungs of these animals and CD11b expression was assessed. (n=4-6 mice/group; representative of three experiments) B, CD11b expression on BMDMφs was determined following 1, 2, 4, or 24 h of infection. (n=4-6 mice/group; representative of three experiments)

SUPPLEMENTAL FIGURE 4. IL-10 is responsible for decreased survival in Lyz2cre Hif1α$^{β/β}$ mice. Following administration of IL-10 neutralizing antibody or control IgG, Lyz2cre and Lyz2cre Hif1α$^{β/β}$ mice were infected with 2x10$^6$ yeasts intranasally. A, Survival was recorded when mice were moribund. Data in this plot was generated only with mice treated with IgG
control or anti-IL-10 rather than grouping IgG controls with untreated mice as in Fig. 2F. (n=9-12 mice/group; representative of 3 experiments)
FIGURE 5

A

Day 1  Day 3  Day 7

HIF-1α  VEGF  PGK-1

Fold change

***

B

Lyz2cre  Lyz2cre HIF1αΔ

HIF-1α  VEGF  PGK-1

Fold change

***

C

HIF-1α

β-actin

Uninfected Lyz2cre  M1a × CcL  Lyz2cre + 5x Hc  Lyz2cre M1a + 5x Hc

D

Uninfected KO  KO + 5x Hc

Uninfected WT  WT + 5x Hc

DAPI  HIF-1α
FIGURE 8

A

B

C

D

[Diagrams and data representing different biological conditions and responses, illustrating fold changes and nitrite levels across various groups and time points.]
SUPPLEMENTAL FIGURE 2

A

- Lyz2cre HIF1α^cre^floxed
- Lyz2cre HIF1α^cre^floxed
+ anti-IL-4

Percent survival vs Days post infection
SUPPLEMENTAL FIGURE 4

A

Lyz2cre + IgG
Lyz2cre + anti-IL-10
Lyz2cre HIF1α+/− + IgG
Lyz2cre HIF1α+/− + anti-IL-10

Percent survival

Days post infection
Dectin-1 mediated TNF-α production in response to *H. capsulatum* is essential for HIF-1α induction.
Introduction

Infection with a variety of pathogens has been associated with increased HIF-1α protein and/or expression of downstream targets. LPS can induce HIF-1α through increased transcription rather than protein stabilization in the setting of normoxia (1–4). While the mechanism of HIF-1α protein stabilization and/or transcriptional induction is unknown, it accumulates in the setting of infection with *Chlamydia pneumoniae*, Vesicular Stomatitis virus, Hepatitis B and C, Human Papilloma Virus, *Toxoplasma gondii*, *Leishmania amazonensis*, and the fungal pathogens *Aspergillus fumigatus* and *Candida albicans* (5–13). These studies suggest that, while HIF-1α plays an undeniable role in the cellular response to hypoxia, it may have been co-opted as a transcription factor in the response to pathogens as well.

*Histoplasma capsulatum* is a dimorphic fungus with worldwide distribution. Mφs are the primary phagocytic cell that engulfs *H. capsulatum*. Our lab has recently shown that Mφ HIF-1α is essential for fungal growth restriction and mouse survival, but the mechanisms that lead to induction of HIF-1α have not been elucidated (14).

Macrophages recognize *H. capsulatum* via CR3 (CD11b/CD18) and dectin-1. While CR3 is required for phagocytosis, CR3 and dectin-1 are both required for production of TNF-α, which occurs via the Syk/JNK/AP-1 signaling pathway. TNF-α is critical for host defenses to *H. capsulatum* in primary infection. TNF-α exerts multiple effects including activation of phagocytic cells, induction of apoptosis, and control of the CD4+ phenotype. TNF-α neutralization impairs survival of mice (15). In addition, TNF-α can drive HIF-1α transcriptional induction (16).

The critical role of this transcription factor in response to infection coupled with the incomplete understanding of the factors that drive HIF-1α protein accumulation in the setting of
pathogen exposure in normoxia led us to study the mechanism of HIF-1α accrual in response to *H. capsulatum*. We found that delayed transcriptional induction of HIF-1α is associated with release of a soluble mediator from infected cells. TNF-α signaling was necessary for *H. capsulatum*-dependent HIF-1α transcriptional induction. Therefore, we suggest that dectin-1 dependent TNF-α production in response to *H. capsulatum* is required for transcriptional induction of HIF-1α.
Materials and Methods

Mice

Male C57BL/6 and Tnfr1\textsuperscript{tm1Mak} breeding pairs were purchased from The Jackson Laboratory (Bar Harbor, ME). We thank Dr. Gurjit Khurana Hershey, Cincinnati Children’s Hospital Medical Center, for the Clec7a knockout mice. Animals were housed in isolator cages and maintained by the Department of Laboratory Animal Medicine, University of Cincinnati, accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. All animal experiments were performed in accordance with the Animal Welfare Act guidelines of the National Institutes of Health, and all protocols were approved by the Institutional Animal Care and Use Committee of the University of Cincinnati.

Preparation of \textit{H. capsulatum} and infection of mice

\textit{H. capsulatum} strain G217B and yeast cells of the same strain that express GFP were grown for 72 h at 37\textdegree C as described (24, 25). To infect mice, 6–8 week-old animals were inoculated intranasally with $2 \times 10^6$ yeasts or $2 \times 10^7$ yeasts (indicated as high dose) in ~30 µl of Hank’s Balanced Salt Solution (HyClone, Logan, UT). For \textit{in vitro} infection, cells were allowed to adhere to plates for 3 h. Cells were infected with 1-5 yeast per M\textit{\phi} for the indicated times.

Generation of bone marrow-derived M\textit{\phi}s (BMDM\textit{\phi}s) and \textit{in vitro} inhibition

Bone marrow was isolated from tibiae and femurs of 6–10-week-old mice by flushing with HBSS. Cells were dispensed into tissue culture flasks at a density of $1 \times 10^6$ cells/ml of RPMI-1640 supplemented with 10% fetal bovine serum, 0.1% gentamicin sulfate, 5 µM 2-mercaptoethanol, and 10 ng/ml of mouse GM-CSF (Peprotech, Rocky Hill, NJ). Flasks were
incubated at 37°C in 5% CO₂. Mϕs were harvested at day 7. Non-adherent cells were removed, ice-cold PBS was added, and cells were scraped from the flask. Cells were collected, washed with PBS, and dispensed into culture dishes. For inhibition studies, piceatannol (Tocris, Bristol, UK) was added to Mϕs 90 minutes before infection. For CBP interaction inhibition studies, chetomin (Santa Cruz, Dallas, TX) was added to Mϕs 30 minutes prior to infection and KG501 (Calbiochem, San Diego, CA) was added 90 minutes before infection.

**RNA Isolation, cDNA synthesis, and quantitative real-time reverse transcription PCR**

Total RNA from whole lungs of mice was isolated using TRIzol (Invitrogen, Carlsbad, CA) and from in vitro Mϕ cultures using the RNeasy Kit (Qiagen, Chatsworth, CA). Oligo(dT)-primed cDNA was prepared by using the reverse transcriptase system (Promega, Madison, WI). Quantitative real-time reverse transcription PCR analysis was performed using TaqMan master mixture and primers (Applied Biosystems, Foster City, CA). Samples were analyzed with ABI Prism 7500. The hypoxanthine phosphoribosyl transferase housekeeping gene was used as an internal control. The conditions for amplification were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min.

**Isolation of lung leukocytes**

Lungs were homogenized with the gentleMACS dissociator (Miltenyi Biotec, Auburn, CA) in 5ml of HBSS with 2 mg/ml of collagenase D (Roche, Mannheim, Germany) and 40U of DNase I (Roche) for 30 min at 37°C. The homogenate was percolated through a 40 mm nylon mesh (Spectrum Laboratories, Rancho Dominguez, CA) and washed three times with HBSS. Leukocytes were isolated by separation on Lympholyte M (Cedarlane, Burlington, ON).
Flow cytometry, cell sorting, and gating strategy

Cells from mouse lungs were incubated with CD16/32 to limit nonspecific binding. Leukocytes were then stained with the indicated antibodies at 4°C for 15-30 min in phosphate-buffered saline containing 1% bovine serum albumin and 0.01% sodium azide. Cells were stained with combinations of the following antibodies: FITC-conjugated Ly6G; FITC-conjugated Ly6C; FITC-conjugated CD11c; PerCP-conjugated CD11b; APC-conjugated CD3; PE-conjugated CD4; FITC-conjugated CD8; and APC-conjugated F4/80 (BD Biosciences, San Jose, CA). For intracellular IL-10 staining, cells were incubated with Cytofix/Cytoperm (BD Biosciences), washed in Permeabilization Buffer (BD Biosciences), and stained for 45 min with PE-conjugated IL-10 (R&D systems, Minneapolis, MN). Cells were washed and resuspended in 1% paraformaldehyde. Isotype controls were used. Data were acquired using BD Accuri C6 cytometer and analyzed using the FCS Express 4.0 Software (DeNovo Software, Los Angeles, CA). For cell sorting experiments, infected Mϕ were isolated from the lungs of control mice at day 7 post-infection using 5 laser FACS Aria II (BD Biosciences) following cell surface staining. Cells were identified using side (SSC-A) and forward scatter (FSC-A), followed by doublet exclusion using forward scatter height (FSC-H) against FSC-A. Mϕ were subsequently phenotypically characterized by F4/80⁺, CD11c⁻, CD11b⁺. As gated here, the Mϕ population does not include interstitial Mϕ or alveolar Mϕ.

Western blot

Following 24 h of infection, non-adherent cells were washed off with ice cold PBS. Cells were scraped off in ice cold PBS and spun down. Cells were lysed in RIPA buffer. Proteins were
separated by electrophoresis in SDS/PAGE and transferred to PVDF membranes. The membranes were used for immunodetection of HIF-1α (Novus Biologicals, Littleton, CO) and β actin (Santa Cruz).

Statistics

Statistical p values were calculated with one-way ANOVA for multiple comparisons and adjusted with Bonferroni’s or Holm Sidak correction and nonpaired Student’s t test where two groups were compared; *p < 0.05; **p < 0.01; ***p < 0.001; NS, not significant
Results and discussion

Transcriptional induction of Mφ HIF-1α in response to *H. capsulatum* is delayed both *in vivo* and *in vitro*.

Since HIF-1α protein can be elevated by stabilization or transcriptional induction after a stimulus, we sought to determine if there was any alteration in transcript following infection with *H. capsulatum*. To assess transcriptional induction of HIF-1α as well as several downstream targets including vascular endothelial growth factor (*Vegf-a*) and phosphoglycerate kinase 1 (*Pgk-1*) *in vivo*, we infected animals with *H. capsulatum* expressing GFP and sorted infected pulmonary Mφs (F4/80⁺ CD11b⁺ CD11c⁻ GFP⁺ or GFP⁻) at 1, 3, and 7 days post-infection. Infected cells exhibited transcriptional upregulation of *Hif-1α*, *Vegf-a*, and *Pgk-1* (Fig. 1A).

We have previously shown that HIF-1α is upregulated in BMDMφs after 24 h of infection with *H. capsulatum*. To determine if transcriptional induction is delayed *in vitro* we infected BMDMφs and assessed HIF-1α and its downstream target genes at 4, 8, 16, and 24 h post-infection (Fig. 1B). We found that *Hif-1α* transcript does not increase until 16 h and continues to increase by 24 h post-infection. Upregulation of both *Vegf-a* and *Pgk-1* was found at 24 h. The delay in transcriptional induction suggested that a soluble mediator might drive this response in Mφ.

A soluble mediator released from infected Mφ is capable of inducing HIF-1α in uninfected Mφs.

Soluble factors such as TNF-α can drive HIF-1α accumulation and activation (16, 26, 27). TNF-α is a critical cytokine in the immune response to *H. capsulatum* that is released by
MΦs (28, 29). To assess the potential contribution of a soluble mediator, we placed BMDMΦs into the lower wells of a transwell plate. We then placed BMDMΦs alone, *H. capsulatum* alone, or infected cells in the upper well (Fig. 2A). *Hif*-1α and *Vegf*-α transcript were elevated in uninfected cells exposed to infected but not uninfected cells (Fig. 2B).

To determine if uninfected cells exhibit transcriptional induction of HIF-1α or its downstream targets *in vivo*, we infected animals with *H. capsulatum* expressing GFP and sorted infected and uninfected pulmonary MΦs (F4/80+ CD11b+ CD11c− GFP+ or F4/80+ CD11b+ CD11c− GFP-) at 1, 3, and 7 days post-infection. *Hif*-1α and *Vegf*-α were both upregulated at day 7 post-infection in uninfected pulmonary MΦs (Fig. 2C).

**TNF-α signaling is required for MΦ induction of HIF-1α and its downstream targets.**

Since TNF-α has been implicated in HIF-1α induction and activation in other cell types, we sought to determine if this cytokine could enhance HIF-1α in BMDMΦs. We found that TNF-α caused an elevation in *Hif*-1α and *Vegf*-α transcript at 2 and 4 hours post-infection, but this response was not maintained at later time points (Fig. 3A). However, lack of TNF-α signaling through TNFR1 at 24 h post-infection with *H. capsulatum* led to reduced transcription of *Hif*-1α, *Vegf*-α, and *Pgk-1* (Fig. 3B).

In this study, we have demonstrated that a soluble mediator can drive transcriptional induction of HIF-1α in MΦs in response to *H. capsulatum* infection. We identified TNF-α as a potential candidate and established the need for this cytokine in transcriptional induction of HIF-1α. However, other soluble mediators such as IL-1β may also promote elevated HIF-1α protein (30). The influence of other mediators known to be elevated during *H. capsulatum* infection should be examined.
Dectin-1 signaling is required for HIF-1α transcription and protein accumulation.

Maximal Mφ TNF-α production in response to *H. capsulatum* requires dectin-1 signaling (29, 31). To determine the necessity of this receptor in HIF-1α induction, we infected control and dectin-1 deficient BMDMφs with *H. capsulatum*. HIF-1α and its downstream target genes were not induced in the absence of dectin-1 (Fig. 4A). The best characterized dectin-1 signaling pathway is through activation of Syk upstream of TNF-α (32). We, therefore, utilized a Syk inhibitor (piceatannol) to inhibit this signaling pathway. *Hif-1α* transcript was nearly absent while *Vegf-a* was reduced (Fig. 4B).

While we have demonstrated that dectin-1 signaling is required for HIF-1α induction, we have not examined the role of this signaling pathway in TNF-α production. To test this, we will analyze TNF-α in the supernatant from control and dectin-1 deficient BMDMφs with *H. capsulatum*. We anticipate that the reduction in HIF-1α and its downstream targets is due to a decrement in TNF-α production by infected Mφs that lack dectin-1 signaling.

We revealed a requirement for dectin-1 signaling in HIF-1α transcriptional induction during *H. capsulatum* infection. However, the Syk inhibitor that we utilized is not specific. In order to clarify the contribution of the canonical and non-canonical dectin-1 signaling pathways to HIF-1α induction, we will perform these experiments with a Raf-1 inhibitor as well as additional Syk inhibitor. To specifically address the impact of these pathways, we will perform siRNA silencing prior to infection; we anticipate that HIF-1α transcript will be reduced in the absence of canonical signaling, but not in the absence of non-canonical signaling.

Taken together these results suggest a model by which *H. capsulatum* drives TNF-α production in Mφs. TNF-α then signals through Mφ in an autocrine or paracrine manner to drive
transcriptional induction of HIF-1α. These data will be developed further in the discussion section of the thesis.
References


Figure Legends

FIGURE 1. Transcriptional induction of Mφ HIF-1α in response to *H. capsulatum* is delayed both *in vivo* and *in vitro*. mRNA of HIF-1α, VEGF, and PGK-1 was quantified by qRT-PCR relative to uninfected controls. *A*, GFP⁺ monocytes were collected at days 1, 3, and 7 following intranasal infection with GFP expressing yeast. (n=12 mice/group; representative of 3 experiments) *B*, BMDMφs were collected at 4, 8, 16, and 24 h post-infection. (n=3 experiments/group). *p<0.05; **p<0.01; ***p<0.001

FIGURE 2. A soluble mediator released from infected Mφs is capable of inducing HIF-1α in uninfected Mφs. *A*, Transwell experimental setup. In 1, uninfected BMDMφs were placed in both the upper and lower wells. In 2, *H. capsulatum* was placed above uninfected BMDMφs. In 3, infected BMDMφs were placed above uninfected BMDMφs. In 4, the upper well was left empty and infected BMDMφs were placed in the lower well. *B*, BMDMφs from the lower wells of the transwell were harvested and mRNA of HIF-1α, VEGF, and PGK-1 was quantified by qRT-PCR. (n=4 experiments/group) *C*, GFP⁺ monocytes were collected at days 1, 3, and 7 following intranasal infection with GFP expressing yeast. mRNA of HIF-1α, VEGF, and PGK-1 was quantified by qRT-PCR relative to uninfected controls. (n=12 mice/group; representative of 3 experiments) *p<0.05; **p<0.01; ***p<0.001

FIGURE 3. TNF-α signaling is required for Mφ induction of HIF-1α and its downstream targets. mRNA of HIF-1α, VEGF, and PGK-1 was quantified by qRT-PCR relative to uninfected controls. *A*, BMDMφs were collected at 2, 4, and 24 h following TNF-α treatment. (n=3
experiments/group) B, BMDMφs from control and TNFR1 knockout mice were infected with *H. capsulatum* and collected at 24 h post-infection. (n=3 experiments/group) *p<0.05; **p<0.01; ***p<0.001

**FIGURE 4.** Dectin-1 signaling is required for HIF-1α transcription and protein accumulation. mRNA of HIF-1α, VEGF, and PGK-1 was quantified by qRT-PCR relative to uninfected controls. A, BMDMφs from control and dectin-1 knockout mice were infected with *H. capsulatum* and collected at 24 h post-infection. (n=3 experiments/group) B, BMDMφs were treated with DMSO or piceatannol for 90 minutes and then infected with *H. capsulatum* and collected at 24 h post-infection. (n=3 experiments/group) *p<0.05; **p<0.01; ***p<0.001
FIGURE 3

A

B

WT + 5x Hc
TNFR1 KO + 5x Hc

Fold change

2 h
4 h
24 h

HIF-1α
VEGF
PGK-1

Fold change

HIF-1α
VEGF
PGK-1
CHAPTER 4

RNA-seq on BMDMφs from both uninfected and infected $Hif1α^{fl/fl}$ and Lyz2cre $Hif1α^{fl/fl}$ mice.
Introduction

Infection with a variety of pathogens has been associated with increased hypoxia-inducible factor (HIF)-1α protein and/or expression of downstream targets. HIF-1α accumulates in the setting of infection with *Chlamydia pneumoniae*, Vesicular Stomatitis virus, Hepatitis B and C, Human Papilloma Virus, *Toxoplasma gondii*, *Leishmania amazonensis*, and the fungal pathogens *Aspergillus fumigatus* and *Candida albicans* (1–9). These studies suggest that HIF-1α may have been co-opted as a transcription factor in the response to pathogens in addition to its role in hypoxia. While HIF-1α is beneficial for the host response to many of these pathogens, it has been co-opted by *Chlamydia pneumoniae*, *Toxoplasma gondii*, and *Leishmania amazonensis* (1, 6, 10).

*Histoplasma capsulatum* is a dimorphic fungus with worldwide distribution. Macrophages (Mφs) are the primary phagocytic cell that engulfs *H. capsulatum*. Prior to cellular activation, Mφs are permissive for intracellular fungal growth. After cellular activation with IFN-γ or GM-CSF, Mφs inhibit intracellular growth of *H. capsulatum* (11). Our lab has recently shown that Mφ HIF-1α is essential for fungal growth restriction and mouse survival (12). Following infection, Mφ HIF-1α was essential for restricting IL-10 production. Elevated IL-10 in the absence of HIF-1α was able to prevent IFN-γ-mediated Mφ activation.

While HIF-1α is necessary for a successful response to a wide variety of pathogens including *H. capsulatum*, the downstream factors that mediate this response have only been partially elucidated. Here we sought to determine the HIF-1α-dependent factors that influence the immune response to this fungal pathogen by performing RNA-seq on BMDMφs from both uninfected and infected *Hif1α*^{fl/fl} and *Lyz2^{cre/cre} Hif1α*^{fl/fl} mice. In addition to the target genes that regulate glycolysis and mediate a response to hypoxia, we have identified factors that regulate
the immune response and proteasome degradation. We identified miR-27 as a potential mediator of the diminished IL-10 found in the absence of HIF-1α.

MicroRNAs (miRNAs) are short non-coding RNAs that regulate mRNA stability and translation to control protein expression (13). miRNAs can be upregulated in response to pathogen associated molecular patterns (PAMPs) such as LPS or cytokines including TNF-α and IL-1β (14, 15). MicroRNAs have been shown to target IL-10 mRNA, which results in instability and degradation (16). MicroRNAs that directly regulate IL-10 post-transcriptionally include miR-106a, miR-4661, miR-98, miR-27, let7, and miR-1423p/5p (16). We identified a decrease in several let7 family members as well as miR-27 in infected Lyz2cre/cre Hif1αfl/fl BMDMφs relative to controls via RNA-seq. We confirmed the decrease in miR-27 with qRT-PCR. Future work will explore the impact of these factors on the host response to *H. capsulatum*. 
Materials and Methods

Mice

We thank Dr. Timothy Eubank, Ohio State University, for the Hif1α$^{fl/fl}$ and Lyz2$^{cre/cre}$ Hif1α$^{fl/fl}$ mice. Animals were housed in isolator cages and maintained by the Department of Laboratory Animal Medicine, University of Cincinnati, accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. All animal experiments were performed in accordance with the Animal Welfare Act guidelines of the National Institutes of Health, and all protocols were approved by the Institutional Animal Care and Use Committee of the University of Cincinnati. 6-10 week old Hif1α$^{fl/fl}$ and Lyz2$^{cre/cre}$ Hif1α$^{fl/fl}$ mice were sacrificed and bone marrow was isolated from tibiae and femurs by flushing with HBSS. Cells were dispensed into tissue culture flasks at a density of 1x10$^6$ cells/ml of RPMI-1640 supplemented with 10% fetal bovine serum, 0.1% gentamicin sulfate, 5 µM 2-mercaptoethanol, and 10 ng/ml of mouse GM-CSF (Peprotech, Rocky Hill, NJ). Flasks were incubated at 37°C in 5% CO$_2$. Mφs were harvested at day 6. Non-adherent cells were removed, ice-cold PBS was added, and cells were scraped from the flask. Cells were collected, washed with PBS, and dispensed into culture dishes.

Preparation of *H. capsulatum* and infection of Mφs

*H. capsulatum* strain G217B and yeast cells of the same strain that express GFP were grown for 72 h at 37°C as described (11, 17). Cells were allowed to adhere to plates for 3 h. Cells were infected with 5 yeast per Mφ. After 24 hour infection, RNA was collected using Qiagen RnEasy and submitted to UC Genomics, Epigenomics, and Sequencing Core.

RNA-seq
RNA-seq was performed by Genomics, Epigenomics and Sequencing Core (GESC) at the University of Cincinnati. RNA-seq methods were provided by the GESC.

**Target RNA enrichment - Isolation of polyA RNA**

NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs, Ipswich, MA) was used for polyA RNA purification with a total of 50 ng to 1 µg good quality total RNA as input. The Core used Apollo 324 system (WaferGen, Fremont, CA) and ran a PrepX PolyA script for automated polyA RNA isolation.

**Automated RNA-seq library preparation**

PrepX mRNA Library kit (WaferGen) combined Apollo 324 NGS automated library prep system was used for library preparation, which is an RNA ligation-based method to maintain strand specificity. In short, the isolated polyA RNA or rRNA/globin depleted RNA was RNase III fragmented, adaptor-ligated to maintain strand specificity and Superscript III reverse transcriptase (Lifetech, Grand Island, NY) converted into cDNA, followed by automated purification using Agencourt AMPure XP beads (Beckman Coulter, Indianapolis IN). The targeted cDNA fragment is around 200 bp.

Next, using the universal and index-specific primer with limited PCR cycle number (~13), sample-specific index was added to each adaptor-ligated cDNA sample and the amplified library was cleaned up by AMPure XP beads in Apollo 324 system with the final elution volume of 16 µl. To check the quality and yield of the purified library, one µl library was analyzed by Bioanalyzer (Agilent, Santa Clara, CA) using DNA high sensitivity chip. To accurately quantify
the library concentration for the clustering, the library was $1:10^4$ diluted in dilution buffer (10 mM Tris-HCl, pH 8.0 with 0.05% Tween 20), and qPCR measured by Kapa Library Quantification kit (Kapabiosystem, Woburn, MA) using ABI's 9700HT real-time PCR system (Thermo Fisher).

**Cluster Generation and HiSeq Sequencing**

To study differential gene expression, individually indexed and compatible libraries were proportionally pooled (20-50 million reads per sample in general) for clustering in cBot system (Illumina, San Diego, CA). Libraries at the final concentration of 15 pM were clustered onto a single read (SR) flow cell using Illumina TruSeq SR Cluster kit v3, and sequenced to 50 bp using TruSeq SBS kit on Illumina HiSeq system.

**Differential gene expression**

To analyze differential gene expression, sequence reads were aligned to the genome by using standard Illumina sequence analysis pipeline, which was analyzed by The Laboratory for Statistical Genomics and Systems Biology in the University of Cincinnati. The report consists of: 1) RNA-seq data quality control (QC) and sample clustering analyzing results; 2) All gene expression level in RNA samples; 3) Significantly differentially expressed genes between two treatment groups (if applicable); and 4) Gene Ontology or KEGG pathway analysis.

**Gene Set Analysis and Clustering Visualization**

Gene set enrichment analysis (GSEA) was performed using the javaGSEA Desktop application ([http://software.broadinstitute.org/gsea/downloads.jsp](http://software.broadinstitute.org/gsea/downloads.jsp)) (18, 19). The following gene sets were
downloaded from the Molecular Signatures Database version 5.1
(http://software.broadinstitute.org/gsea/msigdb/): hallmark gene sets (h.al.v5.1) and canonical
pathways (c2.cp.v5.1), resulting in 1370 gene sets total. GSEA was performed on complete
RNA-Seq expression data from all \textit{Hif1}\textalpha\textsuperscript{\textbeta/\textgamma} and \textit{Lyz2cre Hif1}\textalpha\textsuperscript{\textbeta/\textgamma} infected \textgamma\textphi samples. GSEA
phenotype analysis was performed using weighted enrichment statistic and the signal-to-noise
metric for ranking genes. Number of permutations was equal to 1000, gene set minimum size
was equal to 5, and all other settings were left at default. Gene set enrichment was then
visualized using Cytoscape version 3.2.1 (http://www.cytoscape.org/) and the Enrichment Map
plugin version 2.1.0 (http://www.baderlab.org/Software/EnrichmentMap) (20, 21). In order to
capture subtle differences between groups, cutoff values in Enrichment Map were relaxed to P-
Value = 0.1, Q-value = 0.25 and Overlap Coefficient = 0.25. Gene set clusters were examined
for major pathways and labeled manually.

**Pathway analysis**

Further analysis of significantly altered gene pathways was performed using the Database for
Annotation, Visualization and Integrated Discovery (DAVID) (22, 23). The raw data of the
experiment will be submitted to the NCBI Sequence Read Archive (www.ncbi.nlm.nih.gov/sra/)
with accession number xxxxx (to be submitted).
Results and discussion

Due to the striking nature of the immune defect found in Lyz2<sup>cre/cre</sup> Hif1α<sup>fl/fl</sup> mice, we decided to perform RNA-sequencing (RNA-seq) analysis on infected and uninfected Mφs from Hif1α<sup>fl/fl</sup> and Lyz2<sup>cre/cre</sup> Hif1α<sup>fl/fl</sup> mice in order to identify target genes for further investigation.

Here we found that a host of genes were differentially regulated in infected Lyz2<sup>cre/cre</sup> Hif1α<sup>fl/fl</sup> Mφs relative to controls (Fig. 1). However, immune related pathways were largely unaltered at the RNA level between these groups (Fig. 2-5). Indeed, only IRF7 was found to be differentially expressed in infected Lyz2<sup>cre/cre</sup> Hif1α<sup>fl/fl</sup> Mφs compared to uninfected Lyz2<sup>cre/cre</sup> Hif1α<sup>fl/fl</sup> Mφs, but not in Hif1α<sup>fl/fl</sup> Mφs compared to uninfected Hif1α<sup>fl/fl</sup> Mφs (Fig. 4, Fig. 5).

While this technique did not provide the insight into dysregulation of IL-10 production in Lyz2<sup>cre/cre</sup> Hif1α<sup>fl/fl</sup> Mφs that we anticipated, the alterations seen here will help elucidate future targets to study. In addition to our own RT-PCR confirmation that HIF-1α was knocked out in these Mφ, we found that glycolysis and pathways associated with the response to hypoxia were diminished in Lyz2<sup>cre/cre</sup> Hif1α<sup>fl/fl</sup> compared to Hif1α<sup>fl/fl</sup> Mφs (Fig. 6). Since this analysis largely identified glycolytic and hypoxia pathway targets we elevated the false discovery rate (FDR) in order to detect additional targets that may explain the altered immune response to <i>H. capsulatum</i> in Lyz2<sup>cre/cre</sup> Hif1α<sup>fl/fl</sup> mice compared to Hif1α<sup>fl/fl</sup> mice. This enabled us to detect a wide variety of genes that were elevated in the Lyz2<sup>cre/cre</sup> Hif1α<sup>fl/fl</sup> compared to Hif1α<sup>fl/fl</sup> Mφs. Most of the additional genes detected by elevating the FDR are involved in proteasomal degradation.

While we did not intend to target miRNA with our isolation procedure, we were able to detect miRNA in our reads. We found that Let7i, Let7bgh, Let7ad, and miR-27 were decreased in the Lyz2<sup>cre/cre</sup> Hif1α<sup>fl/fl</sup> compared to Hif1α<sup>fl/fl</sup> Mφs. These miRNAs have been shown to regulate IL-10 expression (24, 25). As an initial pass, we examined the expression of miR-27 in Lyz2<sup>cre/cre</sup>
*Hif1α*\(^{−/−}\) and *Hif1α*\(^{−/−}\) MΦs before, prior to, and after *H. capsulatum* infection. In these preliminary experiments, we found that miR-27 was unaltered in *Lyz2*\(^{cre/cre}\) *Hif1α*\(^{−/−}\) MΦs compared to *Hif1α*\(^{−/−}\) MΦs prior to infection, but was reduced in infected *Lyz2*\(^{cre/cre}\) *Hif1α*\(^{−/−}\) compared to *Hif1α*\(^{−/−}\) MΦs following infection. These results suggest that decreased expression of miR27a following infection of *Lyz2*\(^{cre/cre}\) *Hif1α*\(^{−/−}\) MΦs are correlated with increased IL-10. Future experiments will tests the hypothesis that miR-27 regulates IL-10 expression in a HIF-1α-dependent manner following infection with *H. capsulatum*. These results will be discussed further in the discussion of this thesis.
References


Figure legends

All data in this chapter were generated in collaboration with Mike Horwath.

**FIGURE 1.** Heat map of the 50 most differentially regulated genes in Mφs from *Hif1αββ* mice compared to Mφs from *Lyz2cre/cre Hif1αββ* mice infected with *H. capsulatum* for 24 hours. (n=4-5 mice/group)

**FIGURE 2.** Biocarta analysis of the inflammatory response in infected *Hif1αββ* and *Lyz2cre/cre Hif1αββ* Mφs compared to uninfected cells was performed. Significantly altered genes are denoted. (n=4-5 mice/group)

**FIGURE 3.** DAVID analysis of the cytokine-cytokine receptor signaling in infected *Hif1αββ* and *Lyz2cre/cre Hif1αββ* Mφs compared to uninfected Mφs was performed. Significantly upregulated cytokines, chemokines, and/or receptors are noted with an upward arrow. (n=4-5 mice/group)

**FIGURE 4.** DAVID pathway analysis of TLR signaling pathways altered in infected *Hif1αββ* Mφs compared to uninfected *Hif1αββ* Mφs. Significantly altered genes are denoted with an upward arrow. (n=4-5 mice/group)

**FIGURE 5.** DAVID pathway analysis of TLR signaling pathways altered in infected *Lyz2cre/cre Hif1αββ* Mφs compared to uninfected *Lyz2cre/cre Hif1αββ* Mφs. Significantly altered genes are denoted with an upward arrow. (n=4-5 mice/group)
FIGURE 6. Differential regulation of curated gene sets in RNA-Seq data from infected $Hif1\alpha^{fl/fl}$ and $Lyz2^{cre/cre} Hif1\alpha^{fl/fl}$ Møs were analyzed using GSEA, and visualized using Cytoscape with Enrichment Map plugin. Sets enriched in $Hif1\alpha^{fl/fl}$ Møs are visualized in blue, while sets enriched in $Hif1\alpha^{fl/fl}$ Møs and $Lyz2^{cre/cre} Hif1\alpha^{fl/fl}$ Møs are visualized in red. Enrichment significance is represented by node hue intensity. Gene set size is represented by node diameter, and overlap between gene sets is represented by edge width.

FIGURE 7. Expression of miR-27 was quantified by qRT-PCR relative to expression of the endogenous control sno234 in uninfected controls. BMDMøs were collected and analyzed after 24 h rest or 24 h post-infection. (n=2 experiments/group)
## Figures

### FIGURE 1

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<td>TNFSF6</td>
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FIGURE 2

T cell

Macrophage

IL-6
IL-10
TNF-α
IL-12
GM-CSF

TNF-α
IL-10
TGF-β
FIGURE 3
FIGURE 4 TLR signaling pathways altered in infected Hif1α/− Mφ compared to uninfected Hif1α/− Mφ
FIGURE 5 TLR signaling pathways altered in infected Lyz2cre Hif1αβ/β Mϕ compared to uninfected Lyz2cre Hif1αβ/β Mϕ.
FIGURE 6

MAPK, AP-1, and S1P Signaling

Glutathione/Xenobiotic Response

Mitochondrial Respiration

Translation/Protein Trafficking

Amino Acid Catabolism

Proteosomal Degredation

Glycolysis/Carbohydrate Metabolism

Response to Hypoxia
FIGURE 7.
CHAPTER 5

Discussion
Discussion

Successful resolution of *H. capsulatum* infection requires coordination between innate and adaptive immunity. While the factors governing the adaptive response have been extensively studied, molecular mechanisms that occur in innate immune cells prior to and after phagocytosis and/or cellular activation require further elucidation.

Inflammatory monocytes and macrophages (Mφs) constitute one of the largest cell populations that phagocytose *H. capsulatum* (1). These cells represent both a repository for the organism as well as a site for growth inhibition following activation (2). Once these cells are infected they can be found at the center of the developing granuloma (1). The center of the *H. capsulatum*-induced granuloma is hypoxic (3). Hypoxia inducible factor-1α (HIF-1α) is one of the fundamental transcription factors in the response to low oxygen tension.

HIF-1α has been implicated in the innate immune response to a wide variety of pathogens as outlined in Chapter 1. The work presented in this thesis builds upon the expanding knowledge of the function of this transcription factor. In the following sections the novel findings in this thesis will be summarized, followed by a discussion of potential future directions for this work.

**HIF-1α is induced in response to *H. capsulatum***

HIF-1α protein is elevated in Mφs following exposure to a wide variety of pathogens as reviewed in Chapter 1. This increase is associated with elevated transcription and/or enhanced protein stabilization. We found that HIF-1α protein was elevated in both the cytoplasm and the nucleus following Mφ infection with *H. capsulatum*. We were able to detect transcriptional induction in Mφs both *in vitro* as well as *ex vivo* isolated from infected mouse lungs. Although this may account for some of the HIF-1α elevation, the delay in transcriptional induction in *ex*
vivo cells suggests that stabilization may occur prior to this. Early stabilization may be necessary for production of cytokines and chemokines in the early immune response to *H. capsulatum*.

**Mφ HIF-1α is protective**

We anticipated a role for myeloid HIF-1α in shaping the adaptive immune response to mouse histoplasmosis since hypoxia is detected within liver granulomas induced by i.p. injection of yeast cells (3). However, protective immunity was subverted in the *Lyz2cre Hif1α<sup>fl/fl</sup>* mice prior to the onset of adaptive immunity. These data indicated that a maladaptive innate response led to the phenotype of *Lyz2cre Hif1α<sup>fl/fl</sup>* mice.

To hone in on the innate immune cell population responsible for the defective immune response in *Lyz2cre Hif1α<sup>fl/fl</sup>* mice, we evaluated the contribution of various myeloid lineage cells. Mφs, alveolar Mφs, neutrophils (PMNs), and resident DCs are important for phagocytosis of yeast cells following infection (4–6). These cells release cytokines and chemokines that facilitate mobilization of the inflammatory response to combat invasion by *H. capsulatum*. Since *Itgaxcre Hif1α<sup>fl/fl</sup>* mice did not manifest impaired survival we conclude that HIF-1α within DCs and alveolar Mφs is dispensable following *H. capsulatum* infection. The fact that elimination of PMNs from *Lyz2cre Hif1α<sup>fl/fl</sup>* mice did not alter fungal burden signifies that HIF-1α deficiency in this population was not a contributor to the failed protective immune response. These data strongly suggest that HIF-1α within Mφs is critical for *H. capsulatum* clearance. In the absence of this transcription factor mice not only exhibit dramatically elevated fungal burden, but they succumb to infection within 2 weeks.

Although the cause of death of *Lyz2cre Hif1α<sup>fl/fl</sup>* mice is currently unknown, our data suggest that several factors may be responsible. We suspect that the elevated fungal burden
impairs the appetite and the ability to get water. Although we have not presented the data, this is consistent with our finding that the Lyz2cre Hif1α\(\beta/\beta\) mice become smaller and less mobile over the course of *H. capsulatum* infection. We have not checked their temperature or blood pressure so we cannot rule out the contribution of these factors. There is no evidence that the mice die from hyperinflammation or massive tissue destruction since the lung histology of Lyz2cre Hif1α\(\beta/\beta\) mice is not significantly different from controls as presented in Chapter 2.

**HIF-1α is required for tempering IL-10 production**

The pivotal contribution of HIF-1α in response to *H. capsulatum* was not to drive inflammatory cytokine production, but rather to temper IL-10. This finding is quite unexpected since HIF-1α binds to hypoxia response elements (HREs) that exist in the promoters for several cytokines and chemokines important in the myeloid response to *H. capsulatum* including TNF-α, CCL2, and IL-10 (7–9). The loss of HIF-1α did not reduce pro-inflammatory cytokine production or cell recruitment to the lungs following infection. In fact, there was an increase in several of these prototypically protective cytokines in Lyz2cre Hif1α\(\beta/\beta\) lungs relative to control. These data contrast with infectious models in which decreased myeloid pro-inflammatory cytokine production is noted in the absence of HIF-1α; however, our data are congruent with a study of *Pseudomonas aeruginosa* infection in mice where the lack of HIF-1α in myeloid cells does not diminish the generation of inflammatory cytokines (10–12). In our model of histoplasmosis, the fungal burden, and not the absence of the transcription factor, was responsible for the enhanced pro-inflammatory cytokine production observed in the Lyz2cre Hif1α\(\beta/\beta\) mice. The exception was IL-10; this cytokine was elevated in the lungs of the conditional knockouts independent of the fungal load. This result establishes that the loss of HIF-
1α results in an early increase in IL-10, and this heightened response undermined the integrity of the innate immune response. A previous study by our group documented that the loss of IL-10 enhanced clearance of the fungus (13). However, the influence of this loss was not observed until adaptive immunity was operative. The current information clearly documents that early, exaggerated IL-10 can alter the function of Mφ.

While several studies demonstrate that HIF-1α induces IL-10 transcription, here we established that deficiency of this transcription factor alone or in conjunction with HIF-2α deficiency actually elevates IL-10 transcript and protein production during *H. capsulatum* infection (14, 15). Although counterintuitive, some data are emerging to support the notion that HIFs may moderate IL-10. First, inhibition of *Hif-1α* transcriptional induction in BMDMφs enhances IL-10 transcript and secreted protein following infection with *Mycobacterium tuberculosis* (16). Second, LPS-challenged mice deficient in myeloid HIF-2α exhibit an elevation in circulating IL-10 (17). Taken together, these results indicate that the influence of HIFs on IL-10 may be context dependent.

**cAMP response element binding protein (CREB)-dependent IL-10 induction**

Immune cell expression of IL-10 is regulated at the level of transcription by a wide variety of transcription factors as well as post-transcriptionally by a variety of microRNAs (miRNAs) (18). We identified transcriptional upregulation of IL-10 in BMDMφs from *Lyz2cre Hif1αφφ* mice relative to controls. We identified CREB as the primary transcription factor responsible for elevated IL-10. While this may explain the IL-10 elevation we see, miRNAs may also play a role in this upregulation. In chapter 4 we identified let7 and miR-27 as potential
candidates for IL-10 regulation following infection with *H. capsulatum*. We will outline experiments to test the contribution of these miRNAs in future directions.

**IL-10 inhibits Mφ immunity**

One of the principal issues raised by our findings is how IL-10 dampened innate immunity. Mφs must be activated by exogenous signals to exert anti-*Histoplasma* activity, and IFN-γ is central to the activation of Mφ (19, 20). One known effect of IL-10 is that it blunts IFN-γ-induced activation of Mφs thus thwarting the arming of these phagocytes to limit intracellular infection (21, 22). We asked if the heightened IL-10 in Mφs from *Lyz2cre Hif1α<sup>fl/fl</sup>* mice altered responsiveness to IFN-γ. Indeed, IFN-γ did not restrict fungal growth in these cells *in vitro* unless IL-10 was neutralized. *In vivo*, anti-IL-10 restored immunity as evidenced by a reduction in CFUs and an increase in survival. Although the bulk of IFN-γ produced after day 5 is largely released by CD4<sup>+</sup> T cells in an IL-12-dependent manner, there is an early production of this cytokine by unidentified sources (23–25). This initial generation appears to be important for early activation of Mφs to limit intracellular growth.

**PMNs are not required for effective clearance of *H. capsulatum***

Elimination of PMNs from control mice did not alter fungal burden. This contradicts prior studies whose results strongly suggest that PMNs are essential for *H. capsulatum* clearance (26, 27). However, those studies employed the antibody RB6 which is now known to recognize Ly-6G and Ly-6C. The latter is borne by inflammatory monocytes (28). Therefore it is quite likely that in those experiments both PMNs and inflammatory monocytes were depleted. The
mAb that we employed, 1A8, is considered to be more selective for Ly-6G, which should selectively deplete PMNs (28).

**Clinical relevance**

HIF-1α fortified the innate response to *H. capsulatum* by tempering the production of IL-10 in Mφs. One clinical ramification of our work is in the arena of the potential use of HIF inhibitors to treat cancer (29). Based on our findings, this intervention may pose a risk for those exposed to *H. capsulatum* or perhaps other intracellular pathogens in which IL-10 is a prominent feature of their immune regulation. Thus, a cautious approach may be warranted in the deployment of HIF-1α inhibitors.

While we found a dramatic increase in mouse mortality in the absence of myeloid HIF-1α, our work did not examine the impact of elevated HIF-1α. Given the increased incidence of histoplasmosis in individuals with an adaptive immune system defect, HIF-1α may represent a potential target for innate immune activation. Further studies to address the impact of HIF-1α elevation on the immune response to *H. capsulatum* would be necessary to assess this possibility.

Our work also demonstrates the deleterious role of IL-10 during *H. capsulatum* infection. There is currently no data on IL-10 in patients with disseminated *H. capsulatum*, but it represents a truly intriguing area of potential impact. IL-10 production is known to be highly variable between individuals (30). Human studies have demonstrated that between 50-70% of observed variability in IL-10 production is the result of genetic factors (30, 31). Individuals that are genetically pre-disposed to produce more IL-10 may be more at risk of infection or dissemination. IL-10 neutralization represents one potential avenue to reduce this risk. Anti-IL-
10 antibodies have been successfully administered in a small cohort of systemic lupus erythematosus (SLE) patients and the treatment was safe and well tolerated (32). Together these data suggest that IL-10 neutralization in patients with *H. capsulatum* may be beneficial.

**Future Directions**

Two major questions that are unanswered by our work are: 1. what is the mechanism of increased HIF-1α in the Mφ response to *H. capsulatum*? 2. Are there other mechanisms of IL-10 regulation in HIF-1α deficient Mφs?

As illustrated in Fig. 5.1, stabilization and transcription may both be implicated in the process of elevated HIF-1α. The contribution of these two processes may be critical in the timing of a HIF-1α-dependent response. Due to the constant synthesis and degradation of HIF-1α, increased stability may elevate protein levels in short order while transcription dependent change may be comparatively delayed. While transcription dependent changes may seem unnecessary when stabilization can act so rapidly, multiple groups have demonstrated that transcription dependent elevation of HIF-1α rather than stabilization is critical in immune cells in some settings (33, 34).
HIF-1α is regulated via transcription and protein stabilization. Receptor mediated signals are responsible for transcriptional activation and subsequent protein synthesis. PHD-dependent hydroxylation is responsible for ubiquitination and proteasomal degradation. In the absence of essential cofactors or in the presence of inhibitors, PHD activity decreases and this leads to an elevation of HIF-1α protein. HIF-1α can then bind to ARNT, translocate to the nucleus and drive transcription of downstream targets.

We hypothesize that *H. capsulatum*-mediated HIF-1α accumulation requires early stabilization that is not dependent on transcription. To directly assess the impact of protein synthesis and protein stabilization, we will perform Western blot analysis in *H. capsulatum* infected BMDMϕs treated with the protein synthesis inhibitor cycloheximide. If stabilization is
the primary means of elevating HIF-1α, cycloheximide would be expected to have little or no impact on the amount of HIF-1α protein detected. One technical difficulty with this and future experiments is the ability to detect mouse HIF-1α via Western blot. To avoid this problem we may utilize human derived monocytes or Mφs in this and future Western blot experiments. Our collaborators have had success in detecting HIF-1α in these cells following infection with *H. capsulatum*.

As mentioned previously, PHD-dependent hydroxylation of the oxygen dependent degradation domain (ODD) of HIF-1α leads to recognition by VHL and subsequent degradation of HIF-1α (35). Since diminished PHD2 expression may be responsible for decreased HIF-1α hydroxylation, we will examine transcriptional regulation of this protein via qRT-PCR as well as protein level via Western blot. To assess the contribution of decreased hydroxylation by PHD2, we will then perform Western blot analysis for HIF-1α hydroxylated at proline 564 in the presence of the proteasome inhibitor MG-132. Use of this inhibitor will allow us to analyze the hydroxylated form of HIF-1α that would normally be degraded in this and all future experiments that assess the hydroxylation of HIF-1α. If protein stabilization is responsible for elevated HIF-1α, we would expect to find a decrease in hydroxylated HIF-1α following infection with *H. capsulatum*.

Factors that determine the stability of HIF-1α include PHD cofactors oxygen and Fe$^{2+}$ iron as well as ROS and NO (35–39). Deficiency of PHD cofactors leads to decreased hydroxylation and, ultimately, increased HIF-1α (35). ROS promotes oxidation of PHD2 bound iron from Fe$^{2+}$ to Fe$^{3+}$, which leads to decreased hydroxylase activity (39). NO promotes HIF-1α accumulation via inhibition of PHD2 possibly via s-nitrosylation of this protein (38). However, the contribution of NO to HIF-1α is complicated since other studies have demonstrated
decreased HIF-1α when exogenous NO donors are utilized (40). To assess the involvement of these factors in the accumulation of HIF-1α protein, we will determine which factors promote elevation of this transcription factor.

We hypothesize that NO is responsible for HIF-1α stabilization following *H. capsulatum* infection. As demonstrated in Chapter 2, inducible nitric oxide synthase (iNOS) is transcriptionally induced in *H. capsulatum*-infected mouse Mφ. NO was detected in supernatants from these cells following activation with IFN-γ. In order to test the contribution of NO, we will perform a Western blot time course to assess HIF-1α protein and hydroxylation at proline 564 following treatment with the NOS inhibitor L-NAME. We will expect to find diminished HIF-1α protein and elevated HIF-1α hydroxylation if NO is required for protein stabilization. If we are forced to use human Mφ for Western blot experiments, one hurdle is the potential lack of NO produced by *in vitro* derived human monocytes and Mφ (41). If we find that NO is not produced by *H. capsulatum* infected human Mφ activated with IFN-γ, we may need to add NO to the culture to assess its ability to stabilize HIF-1α. Since NO can be produced by both endothelial nitric oxide synthase (eNOS) and iNOS, we will perform additional Western blot analysis for HIF-1α in the presence of an eNOS or iNOS selective inhibitor (L-NNA and 1400 W, respectively).

Another candidate that may drive HIF-1α stabilization is ROS, which is produced by Mφ within 20 minutes of *H. capsulatum* infection (42). To determine if ROS is required for elevated HIF-1α protein, we will analyze BMDMφ protein lysates for HIF-1α across time following infection and treatment with the ROS scavengers 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) or LPX. ROS scavenger activity will be confirmed with the OxyBlot assay for protein carbonyl groups, a marker of protein oxidation (43). If ROS is required for
elevated HIF-1α, we expect a decrease in HIF-1α protein from BMDMφs treated with Trolox or LPX.

In order to then identify the source of ROS, we will examine the two most common sources of ROS, NADPH-oxidase and mitochondria. NADPH-oxidase activity will be measured with a light detection assay as described in DeNicola et al; specificity will be determined with the enzyme specific inhibitor diphenylene iodonium (44). In order to specifically address the contribution of NADPH-oxidase, we will perform Western blot analysis of HIF-1α in infected BMDMφs derived from gp91phox knockout mice. MitoSOX will be utilized to examine the contribution of mitochondrial-derived ROS. While these are the most likely sources, the contribution of peroxisome and endoplastic reticulum derived ROS will be examined once other sources are ruled out. If we determine that ROS is important in HIF-1α stabilization in mouse Mφs, it would be interesting to explore in human Mφs where we have seen HIF-1α stabilization, but past work has demonstrated an absence of Mφ ROS production (45).

Iron sequestration represents an additional alternative that may drive HIF-1α stabilization. Pathogens may decrease intracellular iron availability through the secretion of siderophores that bind and sequester this metal. As mentioned in Chapter 1, bacterial siderophores have been shown to induce HIF-1α expression through iron depletion (46). In this study, the presence of siderophores was associated with a decrease in HIF-1α hydroxylation, which is likely responsible for diminished von Hippel Lindau recognition and subsequently reduced degradation (46). To examine the role of siderophores, we will perform Western blot analysis of BMDMφs infected with siderophore-deficient H. capsulatum in the presence of the protein synthesis inhibitor cyclohexamide. This inhibitor will allow us to determine the impact of siderophore-dependent iron sequestration on HIF-1α stabilization. One potential problem is that
cyclohexamide may inhibit growth of the pathogen. Since we are most interested in HIF-1α stabilization in the first few hours following infection when there is minimal fungal growth, this is unlikely to impact our results. One other potential confounding factor is the presence of other iron acquisition systems in *H. capsulatum*. While siderophore-silenced *H. capsulatum* does not grow well on iron-deficient medium, the presence of ferric reductases may allow sufficient depletion to inhibit PHD2 (47). In a separate series of experiments, we will add ferric ammonium citrate (FAC), which is commonly used to load these cells with iron, to infected BMDMφs and then perform Western blot analysis. If iron sequestration during infection is responsible for elevated HIF-1α, we would expect to find decreased HIF-1α protein in both of these experiments.

Our alternative hypothesis is: de novo protein synthesis is required for Mφ HIF-1α protein accumulation following *H. capsulatum* infection. We will directly assess this by blocking transcription with actinomycin D and performing Western blot analysis for HIF-1α following infection. If transcription is required for HIF-1α protein elevation, we would expect a decrease in protein with the use of this inhibitor. We will perform qRT-PCR to compare transcription of HIF-1α in infected cells that were treated with actinomycin D to those that were not treated. We would expect an increase in HIF-1α transcript if infection with *H. capsulatum* induces transcription. We will then explore the role of signaling pathways that may drive HIF-1α transcription.

Dectin-1 signaling has been implicated in HIF-1α elevation; β-glucan from *H. capsulatum* drives phosphorylation of Akt (p-Akt) downstream of dectin-1 (48). Phosphorylation of Akt was dependent on PI3K pathway signaling. Another group found that β-glucan from *H. capsulatum* drives Mφ transcription of TNF-α downstream of Syk signaling and dectin-1 (49).
We hypothesize that dectin-1 signaling in response to *H. capsulatum* β-glucan initiates Syk-dependent TNF-α production, which then drives transcription downstream of PI3K. This model is outlined in Fig. 5.2.

Macrophages recognize *H. capsulatum* via CR3 (CD11b/CD18) and dectin-1. CR3 and dectin-1 are both required for cytokine production, which occurs via the Syk/JNK/AP-1 signaling pathway. Soluble mediators such as TNF-α can subsequently signal through a PI3K pathway to drive HIF-1α transcriptional induction.

To address the requirement for dectin-1 in *H. capsulatum*-mediated HIF-1α induction, we will analyze *Clec7a*−/− and control BMDMφ protein lysates for HIF-1α across time following infection. In Chapter 3 we observed that dectin-1 was required for transcriptional induction of
HIF-1α. While we demonstrated that dectin-1 signaling through Syk was essential, the Syk inhibitor that we utilized has a variety of targets. We will, therefore, utilize the more specific Syk inhibitor Bay 61-3606 to determine the requirement for canonical dectin-1 pathway signaling in HIF-1α transcriptional induction in response to *H. capsulatum*.

We demonstrated with a transwell assay that a soluble mediator is required for transcriptional induction of HIF-1α. Since HIF-1α protein is elevated and downstream targets are transcriptionally induced at 24 h post-infection, any soluble cytokine that is responsible for this response must be produced by infected Mφ within 24 h following infection. We will perform Magpix analysis of supernatants from infected BMDMφs to determine the cytokines produced during the first day of infection. Two cytokines that we anticipate finding that are known to induce HIF-1α are TNF-α and IL-1β.

In Chapter 3, we demonstrated that TNF-α signaling was required for an elevation of HIF-1α transcript. Although not published, Betty Wu-Hsieh’s group found that BMDMφs produce TNF-α as early as 3 h post infection, which is further elevated by 6 h. To determine if TNF-α is required for protein accumulation, we will perform a Western blot for HIF-1α utilizing infected Mφs from TNF-α knockout mice. We would anticipate a reduction or loss of HIF-1α if TNF-α is required. These mice would also allow us to question the role of TNF-α in HIF-1α in *vivo*. We will infect control and TNF-α knockout mice and assess transcription of HIF-1α and its downstream targets in Mφs sorted from the lungs of infected animals. We would anticipate a decrement in HIF-1α and its targets in the absence of TNF-α if this cytokine is required for HIF-1α induction.

While our data points to a role for TNF-α, other soluble mediators such as IL-1β could be required for HIF-1α induction. If we see no reduction or only a partial reduction in HIF-1α in the
absence of TNF-α, we will perform similar experiments to those outlined in the above paragraph to examine the role of IL-1β.

Dectin-1 signaling in response to β-glucan from *H. capsulatum* was shown to drive PI3K-mediated HIF-1α accumulation (48). In order to determine the necessity of this pathway in HIF-1α induction following infection, we will perform p-Akt and p-mTOR Western blots in BMDMs utilizing inhibitors or knockouts from the relevant cytokines previously identified as essential. The experiments outlined here will allow us to assess the contribution of both dectin-1 and PI3K signaling pathways in the transcriptional upregulation of HIF-1α following MΦ infection with *H. capsulatum*.

**IL-10 regulation in HIF-1α deficient MΦs**

Although IL-10 transcriptional induction and protein secretion occurs at 24 h in our *in vitro* studies, *Lyz2cre Hif1αβ/β* BMDMs only exhibit an increase relative to controls at 48 h. While the mechanism of this delay is unclear, several modes of IL-10 regulation may be responsible. The two most intriguing possibilities include control of IL-10 expression by miRNAs and HIF-1α-dependent regulation of mRNA stability. Both of these could account for the increase seen at both the transcriptional and resultant protein level. A variety of miRNAs which are regulated by HIF-1α have been shown to inhibit IL-10 production; these include miR-27 and Let-7 family members Let-7b, Let-7c, and Let-7f (50–54). While few studies have focused on the role of HIF-1α in controlling mRNA stability, it binds to VEGF mRNA and regulates transcript decay (55, 56). One or both of these mechanisms may drive the differential expression of IL-10 in *Lyz2cre Hif1αβ/β* versus control BMDMs.
We hypothesize that decreased miRNA expression in Lyz2cre Hif1αβ/β BMDMφs allows for continued expression of IL-10. Several miRNAs have been shown to regulate IL-10 expression including miR-27 and the Let7 family members. In Chapter 4, we observed that miR-27 was decreased in Lyz2cre Hif1αβ/β versus control BMDMφs. We plan to perform additional qRT-PCR experiments to assess the amount of miR-27 as well as the Let7 family members Let7i, Let7bgh, and Let7ad, which were all decreased in Lyz2cre Hif1αβ/β versus control BMDMφs in our RNA-seq data. If there is an increase in these miRNAs, we could test their contribution to IL-10 production, by assessing IL-10 production from Lyz2cre Hif1αβ/β versus control BMDMφs following overexpression of the relevant pre-miRNA or inhibition of the miRNA with a locked nucleic acid (LNA). If these miRNAs are responsible for elevated IL-10 in Lyz2cre Hif1αβ/β BMDMφs, we would expect a decrease in IL-10 in the overexpression experiment and an increase in the inhibition experiment. In addition, we could determine if these miRNAs recognize IL-10 mRNA by performing a pull down experiment with subsequent qPCR analysis for these miRNAs. We would expect a decrease in these miRNAs in the Lyz2cre Hif1αβ/β BMDMφs compared to controls.

Alternatively, HIF-1α may directly decrease mRNA stability of IL-10. HIF-1α can bind to VEGF to regulate transcript decay (55, 56). To address this possibility, we will perform a pull down of HIF-1α and probe for IL-10 mRNA. We anticipate that IL-10 mRNA would be detected in this assay if HIF-1α regulates transcript decay. However, the short half-life and poor antibody detection may require additional stimulus such as LPS or hypoxia for enhanced HIF-1α. In addition, we will measure IL-10 mRNA stability. We would anticipate that mRNA stability is enhanced in Lyz2cre Hif1αβ/β versus control BMDMφs if this mechanism is involved in IL-10 elevation found in the absence of HIF-1α.
The experiments outlined in these future directions would help to clarify the mechanisms by which HIF-1α is stabilized in the immune response to *H. capsulatum* as well as the downstream effects that lead to elevated IL-10 in the absence of this essential transcription factor. Ultimately these would expand our understanding of the importance of HIF-1α in the Mφ response to *H. capsulatum*. A potential model that incorporates the data in this thesis with these future directions for Mφ regulation of HIF-1α in response to infection can be found in Figure 5.3.

**Proposed model**

Macrophage recognition of *H. capsulatum* via CR3 (CD11b/CD18) and dectin-1 may enhance HIF-1α either directly via NO/ROS mediated stabilization or via Syk signaling driven transcriptional induction. Transcriptional induction occurs via a soluble mediator, such as TNF-α or IL-1β. These cytokines can then act in an autocrine or paracrine manner to enhance HIF-1α transcription. In the absence of HIF-1α, CREB is able to bind to CBP to enhance IL-10
production, but the contribution of miRNA is not yet known. Ultimately elevated IL-10 reduces Mφ activation and ultimately leads to elevated fungal burden and mouse death.
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