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The Role Of Chemokines and Dendritic Cells In Regulation of IL-4 and Fungal Immunity

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Abstract

*Histoplasma capsulatum* is a dimorphic fungus that can cause lethal infection in immunocompromised hosts. In order to resolve infection, the host must mount a TH1 immune response that is accompanied by an influx of inflammatory cells to the primary site of infection, the lung. Chemokines regulate cell recruitment and TH1/TH2 polarization; however, the role of chemokines in control of *H. capsulatum* infection has not been explored. We have demonstrated that the chemokine receptor CCR2 is requisite for protective immunity to *H. capsulatum*. *H. capsulatum* infected CCR2−/− mice manifested defects in inflammatory cell recruitment, increased interleukin (IL)-4, and progressive infection. Increased IL-4 in CCR2−/− mice diminished host resistance as demonstrated by the ability of IL-4-neutralized CCR2−/− mice to resolve infection without altering cell recruitment. In contrast, mice lacking CCL2, a major CCR2 ligand, did not exhibit elevated IL-4 and resolved infection. Lack of both CCL2 and the CCR2 ligand CCL7 produced a phenotype similar to that observed in CCR2−/− mice demonstrating for the first time that CCL2 and CCL7 mutually regulate IL-4. We examined the cellular sources that contributed to IL-4 production in lungs of CCR2−/− mice. Surprisingly, numerous alveolar macrophages and dendritic cells (DC) secreted IL-4 in addition to CD4+ T cells at the peak of infection. We explored the mechanism by which IL-4 exacerbated infection. Elevations of IL-4 in CCR2−/− mice were associated with alternatively activated Mφ which exhibited increased arginase-1. Administration of L-arginine, an arginase-1 substrate, or BEC ((S)-(2-Boronoethyl)-L-cysteine, HCl), an arginase-1 inhibitor, partially decreased fungal burden in CCR2−/− mice indicating that increased arginase-1 expression is deleterious to host defense. We investigated the mechanism by which IL-4 becomes elevated in lungs of CCR2−/− mice. Infected CCR2−/− or CCL2−/− mice exhibited a marked decrement in the number of CD8+ conventional DC in lungs, therefore we hypothesized that these cells restrict IL-4 generation. Adoptive transfer of antigen (Ag)-loaded WT bone marrow-derived DC (Ag-BMDC) to WT or CCR2−/− mice resulted in suppression of IL-4 whereas adoptive transfer of BMDC that were not exposed to Ag had no effect. Transfer of Ag-BMDC lacking CCR2 also limited IL-4 generation. We asked if DC-mediated MHCII-Ag presentation to CD4+ T cells was necessary for IL-4 restriction. MHCII−/− Ag-BMDC did not limit IL-4 when transferred to CCR2−/− mice and depletion of CD4+ T cells prior to, but not at the initiation of infection, resulted in decreased IL-4 indicating that Ag-BMDC suppression of IL-4 is dependent on limiting production by CD4+ T cells early during infection. Fungal burden was not reduced in CD4-depleted CCR2−/− mice but CCR2−/− mice that received Ag-BMDC in addition to depletion of CD4+ T cells were able to control infection. Together, the data suggest that elevated IL-4 in CCR2−/− mice arises from decreased recruitment of DC capable of presenting antigen. Furthermore, CD4+ T cells dampen immunity in *H. capsulatum* infected-CCR2−/− mice.
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Abbreviations and Symbols

Commonly Used Abbreviations
Ag, antigen; Ag-BMDC, Ag-loaded BMDC, Arg-1, arginase-1; BEC ((S)-(2-Boronoethyl)-L-cysteine, HCl); BMDC, bone marrow-derived dendritic cell(s); BMDM; bone marrow-derived macrophage(s); cDC, conventional dendritic cell(s); CWM, cell wall membrane; DC, dendritic cell(s); GAG(s), glycosaminoglycans; GM-CSF, granulocyte macrophage-colony stimulating factor; HBSS, hanks balanced salt solution; \textit{Hc}, \textit{Histoplasma capsulatum}; hr, hour(s); HSP, heat shock protein; IFN, interferon; i.n., intranasal(ly); iNOS, inducible nitric oxide synthase; IL, interleukin; i.p. intraperitoneal(ly); M\phi, macrophage(s); mAb, monoclonal antibody; Mb, megabase(s); MFI, mean fluorescence intensity; MHC, major histocompatibility complex; ml, milliliter; NO, nitric oxide; ova, ovalbumin; PCR, quantitative real time polymerase chain reaction; RQ, relative quantification; SEM, standard error of the mean; TipDC, TNF and iNOS producing DC; TLR, toll-like receptor; TNF, tumor necrosis factor; TR, transferrin receptor; Tregs, t regulatory cells; UFA, unsaturated fatty acid

Symbols
\(\alpha\), alpha; \(\beta\), beta; \(\gamma\), gamma, \(\mu\), micro; \(\circ\), degree(s) Celsius
Chapter 1: Introduction

*Histoplasma capsulatum*

*Histoplasma capsulatum* is a dimorphic fungus that exists as a mold in nature and converts to the pathogenic yeast phase within the lungs of a mammalian host. The fungus is predominantly found in the Midwestern and Southeastern United States but exists on all continents except Antarctica (1-3). The mold phase of the fungus is found predominantly in soil that has been contaminated with bird and bat guano (4). Initially, to determine if *H. capsulatum* was present in soil, mice were inoculated with soil suspensions to amplify the number of organisms since the fungus grows poorly when isolated directly from the environment. Today it is possible to identify small amounts of *H. capsulatum* from environmental samples in a direct and less time consuming manner by two-stage PCR analysis (5).

The mold phase of the fungus is composed of mycelia fragments (1.2-1.5µm in diameter), microconidia (< 5µm), and larger macroconidia (6). The mold can be grown in the laboratory by culture at 25°C on appropriate media. Two mating types of the haploid mold, designated the (+) and (-) mating type have been identified, but the organism loses the ability to mate upon culture (7). The mold is the infectious form of the organism. Humans and other mammals become infected when small mycelia fragments and microconidia become aerosolized and inhaled (1).

The yeast form of the fungus was first described by Samuel Darling who viewed the organism microscopically within macrophages (Mφ), then termed histiocytes (8). The yeast are

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1 Excerpts from this chapter have been previously published; Szymczak, W.A., Deepe, G.S., Winters, M.S. The interface between virulence and host response to the pathogenic fungus *Histoplasma capsulatum*. *Current Fungal Infection Reports*. Vol. 2, No.3, September 2008. 159-164.
~1-3 µm in size and replicate by budding (6). Raising the temperature from 25°C to 37°C induces conversion of mold to yeast *in vitro* and presumably *in vivo*. The transformation of mold to yeast is necessary for the development of disease in mice (9) and most likely for causing symptoms in exposed individuals. Genetic study of the yeast form has revealed that the genome is approximately 25-30 Mb in size and is closely related to the dimorphic, soil-based fungi *Blastomycyes dermatidis*, *Coccidiodes immitis*, and *C. posadasii* that also cause lung disease (10).

**Mold to Yeast Transition**

Transition of fungal elements to the yeast phase occurs in the terminal bronchioles and alveoli of the lungs extracellularly and within infected resident phagocytic cells. Transcription of several genes is upregulated upon a shift from the mycelial to yeast phase which may be necessary for transition and subsequent virulence of the organism. Among them, heat shock protein (HSP)70 is one of the earliest. It is upregulated the first one to three hours after a temperature shift from 25°C to 37°C but then declines. Expression of a cysteine-dioxygenase is markedly enhanced and this elevation correlates with the requirement of cysteine for several strains of *H. capsulatum* during the switch (11). More contemporary approaches using microarrays have revealed numerous alterations in transcript expression that include genes involved in metabolism, nutrient acquisition, and cell cycle in other organisms (12).

Recent papers have enhanced knowledge regarding the dimorphic nature of *H. capsulatum*. First, a histidine kinase, *DRK1*, is necessary for the conversion of mycelial elements to yeast phase in *H. capsulatum* and *B. dermatitidis*. Changes in temperature, osmotic or oxidative stress, nutrient deprivation, redox potential, and host-derived factors have all been cited as possible signals recognized by DRK1 that elicit phase change. RNAi-silencing of *DRK1* in *H. capsulatum* causes defects in cell wall synthesis, reduces sporulation, decreases expression
of *H. capsulatum* virulence factors including calcium binding protein (CBP) and 1,3-alpha glucan, and prevents melanin pigmentation (13). Thus, DRK1 appears to have a global regulatory function on virulence.

Second, a transcriptional regulator, termed *Ryp1*, has been characterized as a global regulator of yeast phase gene expression including genes that are linked to virulence. It is expressed at high levels in yeast cells and associates with its own upstream regulatory units. Preliminary evidence suggests that it is required for optimal virulence (14).

The degree of saturation of fatty acids in the membrane of *H. capsulatum* may play a role in the mycelial to yeast phase switch. Addition of unsaturated fatty-acids (UFA) to the *H. capsulatum* membrane increases the time to convert from mycelial to yeast phase and attenuates *H. capsulatum* virulence (15). Furthermore, the hypovirulent Down's strain of *H. capsulatum* exhibits an increased amount of membrane UFA in comparison to the more virulent G217B strain. Membrane fatty acid composition may also influence organism survival in nutrient-limited environments. A decrease in the amount of UFA and increased saturated fatty acids in the membrane occur as yeasts are grown in culture over time and the medium becomes nutrient depleted (16). Alteration of the ratio of membrane fatty acids may ensure the ability of the yeast to maintain membrane structure sufficient for survival within nutrient-limited phagolysosomes of host-infected phagocytes.

**Histoplasmosis**

Up to 90 percent of individuals in endemic areas test positive to a histoplasmin antigen (Ag) skin test indicating a prior infection with *H. capsulatum* (1, 3). Most infected individuals will resolve the primary infection without experiencing any signs or symptoms. When symptoms of histoplasmosis do occur they are often mild and flu like (1, 2, 17). During primary
infection, the yeast is phagocytized by resident alveolar Mφ and dendritic cells (DC) in the lung (18). The organism then disseminates to other organs rich in phagocytic cells, even in immunocompetent hosts. Dissemination presumably arises through infected Mφ in which the yeast is capable of surviving prior to initiation of the cell mediated immune response. Within 7-18 days from the time of infection an Ag specific immune response is activated resulting in infection resolution (1).

Some individuals, primarily those that are immunocompromised, will not successfully control a primary infection (3, 17). A chronic respiratory infection will develop and/or the fungus will disseminate to mucosal surfaces, liver, spleen, adrenal gland or meninges causing an active infection at those sites. Individuals who are immunocompromised due to AIDS, chemotherapy, or immunosuppressive therapy are at a higher risk for developing an uncontrolled disseminated infection or reactivating latent organisms that remain from a prior exposure (CDC).

**Virulence Factors**

Although it has been a challenge to dissect virulence factors used by *H. capsulatum* to subvert the host immune response, major strides have been made in creating gene disruptants. Transformation with bacterial plasmid vectors is challenging due to poor homologous recombination and random rather than targeted integration (19). Therefore, gene inactivation and complementation experiments, which are necessary to fulfill Koch's molecular postulate to prove virulence, are cumbersome for some strains such as G217B. The first *H. capsulatum* gene disrupted, *URA5*, encodes orotidine-5'-monophosphate pyrophosphorylase, a component of the pyrimidine biosynthetic pathway. Loss of *URA5* induced by UV radiation of the G217B strain or targeted disruption in the G184A strain, results in decreased growth in mouse and human Mφ lines and in a mouse model of infection. Virulence was successfully restored in these strains by
either addition of uracil or by restoring *URA5* expression by transformation (20). URA5 selection was used in the transformation scheme to knock-out the first identified virulence factor, *CBP1*. Disruption of *CPB1*, the yeast-phase specific gene encoding calcium binding protein, was accomplished using a telomeric linear plasmid and a two-step genetic selection to enrich for stable recombinants with disrupted alleles at the correct locus. This gene is suspected to be involved in calcium acquisition in conditions of limited calcium such as that within the phagolysosome or for modification of phagolysosome conditions. Transformants lacking the *CBP1* gene failed to grow in conditions of low calcium, did not cause lysis of a mouse Mφ-like cell line, and were not recoverable from mouse lungs after infection with a low dose of yeast. Complementation restored the ability of the yeast to destroy Mφ *in vitro* and caused a virulent infection *in vivo* formally demonstrating a role in virulence (21).

The ability to silence gene expression by RNAi interference has become a valuable tool in the determination of virulence factors. Genes expressed only in the yeast phase and/or expressed differentially in low virulent strains can now be more easily knocked-down. The gene encoding α-(1-3)-glucan synthase, *AGS1*, was the first gene to be successfully knocked down by RNAi in *H. capsulatum*. Silencing in *H. capsulatum* strain G186 decreases the expression of α-(1-3)-glucan, a cell wall polysaccharide only found in the yeast phase. This component of the cell wall was thought to play a role in virulence since some spontaneous mutants lacking α-(1-3)-glucan were avirulent. Loss of *AGS1* expression decreases the ability of yeast cells to lyse Mφ *in vitro* and reduces the severity of infection in lungs of mice (22). Interestingly, the G217B strain of *H. capsulatum*, a commonly used laboratory strain, lacks α-(1-3)-glucan suggesting that α-(1-3)-glucan either disrupts localization of another cell wall component necessary for virulence or that the strain has adapted another virulence mechanism to compensate. α-(1-3)-
glucan impairs host cell-mediated immunity by concealing β-glucans. These carbohydrates are present in many fungi including numerous strains of *H. capsulatum*. β-glucans act as pattern recognition receptors and bind to the surface molecule dectin-1 on Mφ. One consequence of this interaction is the induction of TNF-α which is critical for protective immunity (23). The presence of α-(1-3)-glucan in *H. capsulatum* inhibits TNF-α production through the lack of binding to Mφ via the dectin-1 receptor (24).

*YPS3*, which encodes a protein that is both localized to the cell wall and secreted, is also a virulence determinant (25). Originally identified as a yeast-phase specific gene by subtractive DNA technology, it was considered to be a hallmark of yeast cells. Although silencing this gene does not inhibit the destruction of Mφ *in vitro*, the burden of the silenced strain in spleens and livers of infected mice at early time points post infection is far less than controls. Therefore, it is postulated that *YPS3* may be important for hematogenous dissemination.

Other virulence determinants surely exist and await discovery. For example, melanin production by pathogenic fungi reduces susceptibility to potent antifungal agents and it may alter the host response (26-28). For *Cryptococcus neoformans* and other fungi, ample evidence exists to indicate that this compound is a virulence determinant (29, 30). Melanized *H. capsulatum* yeast cells have been found in infected mice and humans (31), but a direct association with virulence has not been established.

Mating type may mediate *H. capsulatum*-induced pathogenesis. Like *Cryptococcus*, a dominant mating type of *H. capsulatum*, the - mating type, is more prevalent in clinical isolates from patients with pulmonary disease than the + strain (32). No difference in virulence exists between the + and - strains of *H. capsulatum* in mice, but unrelated strains were used in these studies warranting re-examination (33). The mating type locus was more recently characterized
in *H. capsulatum* by its regulation of MAT transcription factors in response to extracts predicted to contain pheromones (34). Studies elucidating pathways utilized during mating may provide important information regarding the contribution of this process to the pathogenic potential of *H. capsulatum*.

Secretion of proteases may influence the virulence of some *H. capsulatum* strains. Extracellular proteases with a preference for cleavage of azocollagen during *in vitro* growth are released by Class 1 isolates of *H. capsulatum* (35). These isolates are unique in that they have only been recovered from immunocompromised patients and have never been found in the soil. It is postulated that Class 1 isolates remain in a latent state in immunocompetent hosts (19). The ability to cleave collagen *in vivo* may provide an advantage for the organism to reactivate and disseminate from latent infection as binding of human Mφ to collagen activates anti-*H. capsulatum* activity (36).

**Ingestion and Intracellular Survival**

*H. capsulatum* encounters arms of innate and acquired immunity during the course of infection. Despite the attack of the host immune system, the fungus is able to establish a persistent state. The early interaction between yeast cells and the host immune system is between cells of the phagocytic lineage: neutrophils, immature DC, and Mφ. The fate of the fungus differs in each population. Neutrophils inhibit the growth of the organism (37), human DC are capable of degrading organisms through phagolysosomal formation *in vitro* (38), but Mφ prior to activation, are the only professional phagocytic cell population in which *H. capsulatum* can replicate freely (39). Mφ are assumed to be a reservoir used by the fungus to avoid the immune system. Perhaps the first encounter between fungus and host is with alveolar Mφ that
comprise ~95% of resident cells within the alveolar space making these cells the primary line of defense against the pathogen.

Although *H. capsulatum* replicates freely in both mouse and human Mφ prior to activation, the interaction between human and mouse Mφ differs considerably. Entry of unopsonized *H. capsulatum* yeast cells into murine Mφ via interaction between fungal HSP60 (40) and Mφ β2-integrin receptor CD11/CD18 does not elicit superoxide production whereas opsonized yeasts do (41). However, phagocytosis of *H. capsulatum* by human monocytes and cultured Mφ stimulates the respiratory burst (42). Despite the toxic products resulting from the human Mφ respiratory burst, *H. capsulatum* replication occurs. Thus, the organism can resist the deleterious effects of the respiratory burst.

*H. capsulatum* inhibits phagolysosomal fusion in human Mφ (43). Although fusion is not affected in mouse Mφ, *H. capsulatum* yeasts survive despite being in contact with lysosomal hydrolases. *H. capsulatum* modulates the acidification of phagolysosomes in mouse Mφ, sustaining a pH ~ 6.5 (44), to possibly avoid the effects of the lysosomal hydrolases which are most active in an acidic environment. A pH of 6.5 is observed in the phagosomes of human Mφ containing *H. capsulatum*, but surprisingly, phagosomal acidification was not necessary to mediate fungistatic and/or fungicidal activity after activation (45).

The cytokines necessary to activate the antifungal activity of human and mouse Mφ infected with *H. capsulatum* differ *in vitro*. Mouse, but not human, Mφ are activated by IFN-γ to inhibit *H. capsulatum* growth. The response by mouse Mφ varies according to the site from which the Mφ are obtained. Peritoneal Mφ are activated by IFN-γ alone, while splenic Mφ require a priming agent (46). One possible explanation for the effect of IFN-γ on peritoneal Mφ
is that it induces nitric oxide synthase (NOS) production whereas IFN-γ treatment of human Mϕ does not (47, 48). Mouse Mϕ also are activated by Mϕ colony stimulating factor (M-CSF) to inhibit *H. capsulatum* intracellular growth (49) while human Mϕ are activated by IL-3, GM-CSF, or M-CSF (50). Fungal growth is also markedly inhibited by this phagocytic population when it is attached to collagen. This activity is unaltered by exogenous cytokines. One mechanism to explain this finding is a sharp increase in the amount of phagolysosomal fusion (36).

Iron sequestration initiated by IFN-γ activation of Mϕ is one means used by the host to defend itself against *H. capsulatum* infection (51). Chelation of Fe(II) or Fe(III) inhibits fungal growth within Mϕ (52). IFN-γ activated Mϕ downregulate transferrin receptors which may decrease iron transport into the cell. Although the amount of iron within infected cells has not been directly measured, *H. capsulatum* may overcome the iron deficient environment. This fungus can manipulate the pH of the phagolysosome enhancing the potential for the organism to acquire iron from transferrin. *H. capsulatum* secretes siderophores and produces ferric reducing compounds, both of which facilitate the capture of iron from host sources of transferrin and hemin (53).

**Adaptive Host Defense**

Cell mediated immunity is critical for resolution of *H. capsulatum* infection. T cells, specifically T cells bearing α/β T cell receptors, are pivotal for controlling *H. capsulatum* infection. When CD4⁺ T cells are depleted, mice succumb within two weeks of primary infection (54). CD8⁺ T cells, although not necessary for survival of primary infection, assist in control of fungal replication (55) by production of IFN-γ and granzyme B (56). Support for the contribution of CD8⁺ T cells arises from experiments demonstrating that major histocompatibility (MHCII)⁻/⁻ mice that have scarce CD4⁺ T cells that cannot be activated control
fungal replication (56). Both CD4\(^+\) and CD8\(^+\) T cells influence resolution of a secondary exposure to *H. capsulatum*. Unlike primary infection, mortality requires depletion of both CD4\(^+\) and CD8\(^+\) T cells (57).

The influence of humoral immunity in control of *H. capsulatum* is limited since B cell-deficient mice are not more or less susceptible to infection. However, administration of monoclonal antibody (mAb) as immunotherapy improves host resolution. Treatment with an mAb against a histone H2B-like protein or Hps60 of *H. capsulatum* reduces fungal burden and prolongs survival (58, 59). Hsp60 mAb modulates the immune response by increasing production of IL-2, IL-12, and TNF-\(\alpha\) while depressing generation of IL-4 and IL-10 in mice and enhances phagolysosomal fusion of M\(\phi\) *in vitro*. These data reveal that antibodies can enhance immunity to *H. capsulatum* infection and represent potential vaccine candidates (59).

Interaction between T cells and Ag presenting cells is required for an appropriate host type-1 immune (T\(H\)1) response to *H. capsulatum* infection. To dampen host immunity, *H. capsulatum* can limit this protective response. *H. capsulatum* cell entry through CD11b (CR3) inhibits IL-12 production, a T\(H\)1 cytokine, *in vitro* (60). It is not known if binding to CR3 results in an inhibitory signal or blocks a positive signal for IL-12 production, but by limiting IL-12 production, the organism would be faced with a less robust adaptive immune response.

Host production of TNF-\(\alpha\) is necessary for host defense. Neutralization of TNF-\(\alpha\) *in vivo* results in increased fungal burden and 100% mortality in naïve and immune mice infected with *H. capsulatum*. Treatment of humans and mice with TNF-\(\alpha\) inhibitors reactivates *H. capsulatum* (61, 62). Possible mechanisms that explain the involvement of TNF-\(\alpha\) in host defenses during *H. capsulatum* infection are beginning to be elucidated. Neutralization of this cytokine during primary infection reduces alveolar M\(\phi\) nitric oxide (NO) production (23) and induces a
population of CD4$^+$CD25$^+$ T cells that alter protective immunity in an Ag specific manner (63). TNF-α also contributes to host defense by promoting apoptosis, which is a key factor in successful resolution of infection (64).

Other cytokines that are necessary for protective immunity in vivo include GM-CSF and IL-1β. Impaired immunity in the absence of GM-CSF results from elevations of the Th2 cytokine IL-4 and the immune dampening cytokine IL-10 and these cytokines are associated with increased fungal burden in IL-1β$^{-/-}$ mice as well (65, 66). There are numerous scenarios in which these cytokines are increased and contribute to the demise of animals in experimental infection. In secondary, but not primary infection, TNF-α neutralization elevates IL-4 and IL-10, and neutralization of the latter two concomitantly restores immunity (23). Neutralization of either IL-4 or IL-10 also improves immunity in mice in which apoptosis is inhibited (64). Both IL-4 and IL-10 may impair immunity by inducing production of suppressor of cytokine signaling (SOCS)-1 and SOCS3 that dampen signaling generated by protective Th1 cytokines (67-70). Additionally, IL-4 alternatively activates Mφ resulting in inhibited killing of intracellular or some extracellular organisms (71-73).

The tissue hallmark of an effective adaptive immune response is the granuloma, a circumscribed collection of cells from innate and adaptive arms of immunity that contain organisms within the innermost area of the lesion. Granulomas are postulated to sequester microbes from uninfected tissues, but one consequence of granuloma formation is that yeasts may be protected from the immune response. Yeasts remain viable with granulomas and if the host becomes immunocompromised at a later time point, reactivation of the fungus can result, most likely from disintegration of these structures.
In mice, the evolution of the granulomatous response has been characterized. By the second week post infection, tissues manifest mature granulomas that are comprised of multiple cell populations including Mφ, CD4⁺, CD8⁺, and regulatory (CD4⁺CD25⁺) T cells. IFN-γ, IL-17, TNF-α, and several chemokines are produced by cells within the granuloma (74). Conversely there is little T_H2 cytokine production. At this time, little is known about how each of these components contributes to granuloma formation or maintenance.

Chemokines

Chemokines are small (8-10 kilodalton) cytokines produced by hematopoietic and structural cells to induce cell motility. Chemokines are grouped into four categories based on the position of the first two conserved cysteine residues. The first and second cysteine residues of CC chemokines are consecutive whereas the cysteines of the CXC subfamily of chemokines have an amino acid separating the residues. Over 90 percent of chemokines belong to the CC and CXC families (75).

Chemokines bind to pertussis toxin-sensitive G-protein coupled receptors. Signaling through these receptors influence cell migration by inducing actin rearrangement (76). Chemokine-chemokine receptor interactions are much more complex than a simple ligand-receptor interaction. Many chemokines bind to multiple receptors and conversely, chemokine receptors manifest promiscuity for these molecules. Some chemokines form dimers and/or oligomers resulting in synergistic signaling (76, 77). For example, oligomers of CCL2, CCL4, CCL5, and CXCL10 enhance leukocyte recruitment in vivo (78, 79). New evidence reveals that chemokines exert a more potent effect in the presence of chemokines whose agonistic receptors are absent. The non-signaling chemokines may amplify the response by inhibiting degradation of the signaling chemokines (80).
Chemokine receptors form higher-order structures and undergo conformational changes upon ligand binding that affect function. CXCR4 and CCR2 can form homo and heterodimers. Conformational changes in both CXCR4 and CCR2 arise upon binding of an agonist for either receptor which initiates signaling cascades downstream of both receptors (81). It is postulated that chemokine receptor dimer formation can dampen chemokine signaling. For example, in cells in which CCR2 and CCR5 are co-expressed and form dimers, binding of CCR2 and CCR5 ligands becomes competitive (82). Chemokine receptors are hypothesized to have multiple conformations that allow the initiation of different signaling cascades upon interaction with differing chemokines. Binding of CCL2, CCL7, CCL8, and CCL12 are agonists for CCR2 whereas CCL11 is an antagonist (83).

Chemokines also interact with glycosoaminoglycans (GAG) associated with extracellular matrix. Heparan sulfate is the most common cell surface GAG to which chemokines bind (84). The association of chemokines with GAG is postulated to participate in the establishment of chemokine gradients at the site of production (85, 86). The GAG-chemokine complex may be required for expression of chemotactic activity and for priming T cells to interact with Ag presenting cells (79, 87).

Chemokine signaling is constitutive or inducible. The former contributes to the formation and homeostasis of secondary and tertiary lymphoid organs (88) while inducible chemokines are produced upon infection (74, 89-93), allergic inflammation (94, 95) tissue injury (96, 97) and cancer (98, 99). Inducible chemokines mediate leukocyte recruitment to sites of infection and draining lymph nodes and are elicited by Toll-like receptor (TLR) signaling (100-102) and/or the pro-inflammatory cytokines TNF-α, IFN-γ and IL-1β (76, 103, 104). Apoptosis, which accompanies infection, magnifies chemokine production through Fas-FasL interactions.
(105). In contrast, constitutive chemokines are downregulated upon infection. The production of homeostatic chemokines in lymphoid tissues is decreased by IFN-γ which is associated with reorganization of T cells and DC to most likely promote interaction and lessen competition with other cells for space within the structure (106).

Chemokines exert multiple functions in the immune response in addition to chemotaxis such as DC maturation, Mφ activation, neutrophil degranulation, B cell antibody class switching, T_H1 vs. T_H2 cell polarization, and T cell activation (75, 107) (Fig. 1). Chemokines also contribute to the asthmatic response by causing hematopoiesis, basophil histamine release, and airway remodeling (108). Because of their numerous purposes, it is not surprising that altered chemokine/chemokine receptor function depresses immunity to viral (109, 110), bacterial (92, 111), fungal (112, 113), protozoan (114, 115), and helminth (116) infections. In contrast, the absence of chemokine signaling improves resolution in some models. Lack of CCR2 accelerates viral clearance in one influenza model by impairing the recruitment of monocytes and subsequent release of tissue damaging nitric oxide (117), although in another model utilizing the same strain of virus, immunity is impaired (117). Improved resolution of Leishmania infection ensues in the absence of CCR5 due to decreased recruitment of immune response dampening regulatory T cells (118).

Cell Recruitment During H. capsulatum Infection

The immune response must be balanced during infection. It is necessary to recruit leukocytes to sites of infection to activate an adaptive immune response but the recruitment must be regulated such that tissue damage is limited. The function of chemokines in cell recruitment or control of H. capsulatum infection has not yet been explored, but leukotrienes, lipid chemoattractant molecules, have been shown to direct cell recruitment. The leukotriene inhibitor
MK886 completely inhibits mononuclear cell recruitment to the peritoneum upon injection of *H. capsulatum* yeast intraperitoneally (i.p.), but only partially inhibits eosinophil and neutrophil recruitment suggesting that other mediators participate (119). Decreased mononuclear cell recruitment in MK866 treated mice could be an indirect result of decreased chemokine production since leukotrienes induce the production of CCL2 (120), a monocyte chemoattractant.

The influence of leukotrienes on *H. capsulatum* infection varies dependent on the route of infection; therefore these molecules may have organ specific functions. Blockade of leukotrienes during pulmonary infection does not result in a similar phenotype to that described in MK866 treated mice infected with yeast i.p. Mice infected intratracheally exhibit increased inflammatory cell recruitment to the lungs and exacerbated infection. The absence of leukotrienes is associated with increased production of TNF-α, IL-1, and IL-6 and decreased IL-2, IL-5, IL-12, and IFN-γ. It remains to be explored why yeast clearance is impaired in these mice, and it is not clear if the aberrant cytokine levels are a result of increased burden, altered cell recruitment, or a direct effect of the absence of leukotrienes (121).

The mediators of leukocyte recruitment to lungs during pulmonary infection, the primary exposure route for humans are not yet identified. We have begun to explore the role of chemokines in cell recruitment and control of pulmonary *H. capsulatum* infection. Only two reports regarding chemokines and *H. capsulatum* infection have been published; the first demonstrates that CCL3 and CCL11 are produced in the peritoneum of *H. capsulatum*-i.p. infected mice (90), the second study provides evidence that both CC and CXC chemokines are produced by cells contained within liver granulomas (74). Our laboratory has found that chemokines comprise the greatest family of genes upregulated in lungs over the first week of infection (George S. Deepe, Jr., unpublished data). Of these chemokines, ligands for the
chemokine receptor CCR2, as well as the receptor are all highly upregulated, thus we hypothesized that CCR2 is necessary for infection resolution.

The Biological Properties of CCR2 and its Ligands

The chemokine receptor CCR2 is expressed at highest levels on circulating monocytes (122), but is expressed by many cell types. The chemokines CCL2, CCL7, CCL8, and CCL12 are agonists for CCR2 in the mouse (123-125). LPS downregulates CCR2 expression (126). In humans, two alternatively spliced variants of CCR2 exist; CCR2a and CCR2b. Murine CCR2 exhibits 80% sequence identity to human CCR2b, the predominant surface expressed isoform (123). Polymorphisms in both CCR2a and CCR2b are present in humans and are associated with myocardial infarction, decreased development of the lung disease sarcoidosis, protection against multiple sclerosis, and protection against progression to AIDS (127).

CCR2 mediates monocyte egress from bone marrow in mice through CCL2 and CCL7 which have additive roles in chemotaxis (92, 128-130). It is postulated that CCL2 and CCL7 either act sequentially to promote movement or signal in independent bone marrow niches (92). Monocytes that egress from bone marrow can differentiate into DC in the periphery (131). Consequently, the absence of CCR2 results in decreased numbers of monocytes and monocyte-derived DC at sites of infection and draining lymph nodes. Diminished recruitment of Mφ, CD4⁺ and CD8⁺ T cells has also been reported in some infection models (91, 113, 117, 132).

Dampened recruitment of monocytes and monocyte-derived DC is associated with impaired clearance of intracellular pathogens. It is speculated that impaired immunity in CCR2⁻/⁻ mice infected with *Toxoplasma gondii* is a direct result of decreased degradation of organisms by monocytes since recruitment is impaired and monocytes have anti-*Toxoplasma* activity *in vitro* (115). In contrast, it is the diminished recruitment of a population of monocyte-derived DC that
produce large quantities of TNF-α and iNOS, termed TNF and iNOS producing DC (TipDC), that is associated with reduced clearance of *Listeria monocytogenes* infected CCR2<sup>−/−</sup>, CCL2<sup>−/−</sup>, and CCL7<sup>−/−</sup> mice (129, 130). It is not known if TipDC are infected with *L. monocytogenes in vivo*, but impaired clearance of *Listeria* may be a consequence of diminished TNF-α and iNOS paracrine activity on infected cells in the spleen. Aside from their protective function, TipDC mediate CD8<sup>+</sup> T cell proliferation in influenza-infected mice. Decreased recruitment of the former impairs infection resolution as a consequence of diminished CD8<sup>+</sup> T cell cytolytic activity (133).

In addition to decreased leukocyte recruitment, CCR2 is essential for T<sub>H</sub>1 polarization in some, but not all, infections and in eliciting experimental autoimmune encephalitis, a T<sub>H</sub>1 mediated disease (105). *C. neoformans*, *Leishmania major*, or *Mycobacterium tuberculosis*-infected CCR2<sup>−/−</sup> mice manifest decreased production of the T<sub>H</sub>1 cytokine IFN-γ and exacerbated infection (111, 113, 132, 134). Increased T<sub>H</sub>2 cytokine production accompanies the diminished T<sub>H</sub>1 response in *C. neoformans* infected CCR2<sup>−/−</sup> and CCL2<sup>−/−</sup> mice and *L. major* infected CCR2<sup>−/−</sup> mice (113, 132, 134). It has not been determined if exacerbated infection is primarily a result of decreased IFN-γ activity or if increased T<sub>H</sub>2 cytokine production or decreased cell recruitment amplify these infections.

CCR2 signaling can influence T cell polarization by various mechanisms. In a model of influenza, CCR2<sup>+</sup> monocyte-derived DC migrate into lymph nodes where they induce T<sub>H</sub>1 cells by secreting IL-12, a T<sub>H</sub>1 promoting cytokine (135). However, since polarization is not effected in some infection models, T<sub>H</sub>1 cytokine production by T cells may be induced by other accessory cells or by innate cells in a compensatory manner. It is hypothesized that CCR2 limits the number of T<sub>H</sub>2 cells at infection sites through internalization of CCL7. Since CCL7 recruits T<sub>H</sub>2
cells to the sites of infection most likely through binding to CCR3, decreased levels of CCL7 in the extracellular milieu would diminish Th2 cell recruitment (104). Some evidence suggests that CCR2 signaling directly inhibits production of the Th2 cytokine IL-4. In vitro studies demonstrate that CCR2 expression on bone marrow-derived DC (BMDC) is needed to limit autocrine production of IL-4 and subsequent production by T cells, but the mechanism has not been explored (136). Further studies need to be performed to determine if CCR2+ DC limit IL-4 generation in infection models where IL-4 is elevated.

**Dendritic Cell Mediated Regulation of T cell Polarization**

DC polarization of naïve T cells in infected mice initially transpires in lymph nodes (137). DC are directed to sites of infection and then to lymph nodes by chemokines. Recruitment of immature DC to lungs is directed by the chemokine receptor CCR6 (112). CXCR3 induces DC emigration through binding of CXCL10 (110). Although the absence of CCR2 impairs lung dendritic cell numbers (93, 129), CCR2 may not be necessary after bone marrow egress since chemotaxis from blood to tissues is independent of CCR2 (129). Within lungs, DC phagocytosis of Ag induces maturation of DC which is associated with upregulation of the chemokine receptor CCR7 (138). The chemokines CCL19 and CCL21 bind to CCR7 to direct chemotaxis towards lymph nodes (139). Prostaglandin E2 also induces recruitment (140).

Within lymph nodes DC polarize T cells to produce Th1 or Th2 cytokines. Cytokine production and contact-dependent signaling participate in DC mediated polarization. Production of IL-12 by DC promotes IFN-γ production from CD4+ T cells and the ensuing Th1 immune response (141, 142). However, IL-12−/− mice can mount a Th1 immune response (143), thus other signals influence polarization. Initiation of Th2 responses by DC is enhanced by IL-4. In the presence of IL-4, DC signal to CD4+ T cells to produce more IL-4 (144, 145). DC
production of the chemokines CCL17 and CCL22 drive T\(_\text{H}2\) immunity since these chemokines recruit IL-4 producing T cells (94). Notch signaling between Notch receptors on T cells (146) and Notch ligands on DC also influence polarization. Expression of the Notch ligands Jagged 1 and Delta 4 on DC are associated with T\(_\text{H}1\) priming (147, 148) whereas the Notch ligand Jagged 2 is associated with increased T\(_\text{H}2\) cytokine expression in the mouse (148). Decreased expression of the maturation marker CD40 on DC is associated with increased T\(_\text{H}2\) cytokine production as well (149, 150).

Upon infection, the initial signals that instruct DC-mediated polarization of immune responses are initiated by pathogens, but the mechanisms that govern whether a T\(_\text{H}1\) or T\(_\text{H}2\) response will predominate are not fully understood. It is believed that pathogen associated molecular patterns are recognized by the DC resulting in downstream events that skew polarization. For example, stimulation by the bacterial product LPS induces TLR4 signaling resulting in IL-12 production by DC (148) and limits T\(_\text{H}2\) development through inhibition of GATA-3-induced IL-4 production (151). Conversely, fungal proteases stimulate DC biased T\(_\text{H}2\) polarization by inhibiting DC maturation and IL-12 production (152). Another recently identified mechanism by which DC recognition of pathogens induces cell-mediated immune responses is by inducing changes in DC structure. Secreted ribonucleases from helminth eggs promote T\(_\text{H}2\) polarization by inducing cytoskeletal changes in DC that inhibit Ag-dependent interaction with CD4\(^+\) T cells and subsequent production of T\(_\text{H}1\) cytokines (153).

Adoptive transfer of DC prior to infection can skew polarization of the immune response demonstrating their pivotal role in immunity. Addition of mature, Ag presenting BMDC to T\(_\text{H}2\) biased Balb/c mice prior to \textit{L. major} infection results in a switch to a T\(_\text{H}1\) polarized response and control of infection (148). In contrast, transfer of immature, Ag-presenting BMDC to mice prior
to *C. neoformans* infection results in a T\(_{H2}\) response and decreased yeast clearance (154). These studies suggest that the number and maturation state of the DC and/or the type of Ag presented influence polarization.

In our studies, we have explored how CCR2 mediated recruitment of DC effects immune polarization in response to *H. capsulatum* infection. T cell production of IFN-\(\gamma\) is crucial for immunity to primary *H. capsulatum* infection and for vaccine-mediated protective responses (155-157), however it is not known if DC inhibit IL-4 generation by CD4\(^+\) T or other cells. Herein, we demonstrate that DC limit IL-4 generation in response to *H. capsulatum* infection in an Ag-dependent manner. Our data suggest that decreased recruitment of DC capable of presenting Ag to lungs of CCR2\(^-/-\) mice results in elevated IL-4 and impaired immunity.
**Figure 1.** Diagram of the multiple functions of chemokines.
Chapter 2: The CCL7-CCL2-CCR2 Axis Regulates IL-4 Production in Lungs and Fungal Immunity

Abstract

Expression of the chemokine receptor CCR2 can be detrimental or beneficial to infection resolution. Herein, we examined if CCR2 was required for control of infection by the dimorphic fungus *Histoplasma capsulatum*. *H. capsulatum*-infected CCR2<sup>−/−</sup> mice manifested defects in inflammatory cell recruitment, increased IL-4, and progressive infection. Increased IL-4 in CCR2<sup>−/−</sup> mice primarily contributed to decreased host resistance as demonstrated by the ability of IL-4-neutralized CCR2<sup>−/−</sup> mice to resolve infection without altering inflammatory cell recruitment. Immunity in mice lacking the CCR2 ligand CCL2 was not impaired despite decreased inflammatory cell recruitment. Neutralization of the CCR2 ligand CCL7 in CCL2<sup>−/−</sup> mice, but not WT, resulted in increased IL-4 and fungal burden. Thus CCL7 in combination with CCL2 limits IL-4 generation and exerts control of host resistance.

Introduction

Infection by the dimorphic fungus *Histoplasma capsulatum* occurs when conidia and mycelial fragments from disturbed soil are coincidentally inhaled by a host and deposited within the lung (1). The lung environment supports conversion of conidia to the virulent yeast phase (9). Alveolar Mφ are a first line of host defense against the invading pathogen, but *H. capsulatum* yeast cells replicate and disseminate to other organs, most likely transported by resting Mφ (42, 44, 158). The host must mount a Th1 immune response to activate Mφ and resolve infection (23, 57, 65, 156, 159). CD4<sup>+</sup> T cells and IFN-γ must be present in order for

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<sup>2</sup> Parts of this chapter have been previously published; W.A. Szymczak and George S. Deepe, Jr., The CCL7-CCL2-CCR2 axis regulates IL-4 production in lungs and fungal immunity. *The Journal of Immunology*. 183: 1964-74.
mice to survive infection (57). Other T_{H1} cytokines including TNF-\(\alpha\) and GM-CSF are also required for resolution of infection (23, 65).

*H. capsulatum* infection induces a complex inflammatory response that consists of numerous cell populations (160). Recruitment of immune cells into infected tissues is governed by multiple mediators including chemokines. These soluble factors are small molecules that induce chemotaxis, activate T cells, and direct or maintain T_{H1} or T_{H2} immunity during infection upon engagement with their cognate receptors (161, 162). In mice, the chemokine receptor CCR2 directs the migration of myeloid lineage cells by engaging the chemokine ligands CCL2, CCL7, CCL8, and CCL12 (75, 123-125). CCL2 is a major ligand of CCR2, and CCR2 is the only known receptor for CCL2 that promotes signaling. The latter appears to be more potent in monocyte recruitment than the other ligands that engage CCR2 (125).

Mice lacking CCR2 exhibit disturbances in monocyte egress from bone marrow and monocyte recruitment to sites of infections (115, 117, 129). Alterations in M\(\phi\), DC, and T cell recruitment in CCR2^/- and CCL2^/- mice during infection have been reported (93, 113, 130, 132, 163, 164). Recently, CCL7 has been shown to mediate monocyte egress from bone marrow upon *L. monocytogenes* infection (92). Aside from its role in inflammation, CCR2 is essential for promoting a T_{H1} response and limiting infection by several pathogens (111, 113, 132, 134, 164). Conversely lack of CCR2 promotes protective immunity by altering the influx of monocyte-derived cells that generate noxious inflammatory mediators. The net result is a decrement in the severity of immunopathology and as a consequence improved survival (117).

We have sought to determine if signaling through the chemokine receptor CCR2 is necessary for resolution of *H. capsulatum* infection. CCR2^/- mice manifested higher fungal burdens, diminished inflammatory cell recruitment, and succumbed to infection. The major
defect in immunity in CCR2−/− mice was increased production of IL-4 in the lungs. Neutralization of IL-4 facilitated resolution of infection in CCR2−/− mice, but it did not alter the defects in inflammatory cell recruitment to the lung. Although loss of CCL2 or CCL7 alone was not essential for control of infection, the combined absence of CCL2 and CCL7 resulted in increased IL-4 and fungal burden. These data demonstrate that CCL7 and CCL2 must engage CCR2 for optimal immunity to *H. capsulatum*. CCL2 and CCL7-mediated signaling through CCR2 is requisite for limiting IL-4 production.

**Results**

**Fungal burden and course of infection in CCR2−/− and CCL2−/− mice.** To determine if CCR2 was necessary for control of *H. capsulatum* infection, we infected CCR2−/− and WT mice with a sub-lethal number of yeast cells intranasally (i.n.) and assessed fungal burden and survival. At day 7 post-infection, CCR2−/− mice exhibited a 0.85log10 increase in CFU in lungs compared to WT (Fig. 2A). The burden in spleens was similar between the two strains (Fig. 2A). Between days 7 and 14 post infection, fungal burden in lungs and spleens of CCR2−/− mice increased whereas the burden diminished in lungs and spleens of WT mice (Fig. 2A). CCR2−/− mice infected with a sub-lethal number of yeast were unable to resolve infection; all CCR2−/− mice succumbed by day 20 (Fig. 2B). Lungs from moribund CCR2−/− mice contained > 10⁸ CFU.

CCL2 is a major ligand of CCR2 (125), and impaired immunity is often ascribed to loss of CCL2 signaling (113, 115, 129). To ascertain if CCL2 was requisite for protective immunity to *H. capsulatum* infection, CCL2−/− mice were infected with a sub-lethal number of yeast. CCL2−/− mice manifested a transient increase in fungal burden in the lungs and spleen only at day 14 (Fig. 1A). By day 21, the number of CFU in lungs of CCL2−/− mice was similar to WT. All CCL2−/− mice survived (Fig. 2B).
**Inflammatory response in CCR2<sup>-/-</sup> and CCL2<sup>-/-</sup> mice.** Because CCR2<sup>-/-</sup> mice exhibit disturbed inflammatory cell recruitment to infected tissues (114, 115, 129, 132, 134, 163, 164) we assessed the absolute number and phenotype of inflammatory cells in the lungs during *H. capsulatum* infection. At days 3 and 7, fewer leukocytes were recovered from lungs of infected CCR2<sup>-/-</sup> or CCL2<sup>-/-</sup> mice compared to WT (Fig. 3A). Between days 3 and 7, the number of leukocytes increased ~4-fold in CCR2<sup>-/-</sup> and CCL2<sup>-/-</sup> mice, but this value was still decreased by 4.5-fold compared to WT.

The diminished number of inflammatory cells in lungs of CCR2<sup>-/-</sup> and CCL2<sup>-/-</sup> mice prompted us to examine specific leukocyte populations in lungs. The percentage of CD4<sup>+</sup> and CD8<sup>+</sup> T cells was similar among WT, CCR2<sup>-/-</sup> and CCL2<sup>-/-</sup> mice (Fig. 3B), but the numbers (mean ± SEM x 10<sup>4</sup>) were decreased (p< 0.05) in CCR2<sup>-/-</sup> (CD4<sup>+</sup>; 5.8 ± 0.2, CD8<sup>+</sup>; 6.4 ± 0.1, n=12) and CCL2<sup>-/-</sup> mice (CD4<sup>+</sup>; 4.6 ± 1.0, CD8<sup>+</sup>; 5.2 ± 0.1, n=7) in comparison to WT (CD4<sup>+</sup>; 23.4 ± 0.3, CD8<sup>+</sup>; 18.3 ± 0.3, n=11). We analyzed the number of activated CD4<sup>+</sup> T cells in CCR2<sup>-/-</sup> and CCL2<sup>-/-</sup> mice since defects in T cell activation have been attributed to loss of CCR2 (163, 165). CD3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup>CD69<sup>+</sup> T cells (mean x 10<sup>4</sup> ± SEM) were decreased (p<0.001) in both CCR2<sup>-/-</sup> (0.64 ± 0.13, n=10) and CCL2<sup>-/-</sup> (1.41 ± 0.39, n=7) mice in comparison to WT (5.56 ± 0.68, n=11).

We next determined the percentage and absolute number of phagocytic cells in the lungs of CCR2<sup>-/-</sup> and CCL2<sup>-/-</sup> mice. At day 7 the percentage and number of inflammatory monocytes, TNF and iNOS producing DC (TipDC), and CD8<sup>-</sup> eDC were decreased in CCR2<sup>-/-</sup> mice (Fig. 3B, 3C, Table I). The mean fluorescence intensity (MFI) of I-A<sup>b</sup> expression (mean ± SEM) on CD11c<sup>+</sup> cells from CCR2<sup>-/-</sup> mice (568 ± 25, n=8) was decreased (p<0.001) in comparison to WT (864 ± 36, n=8). The relative percentage of neutrophils and tissue Mφ was increased in lungs of
CCR2\(^{-/-}\) mice in comparison to WT (Fig. 3C); however, the number was similar to WT (Table I). The relative percentage of alveolar Mφ was similar in WT and CCR2\(^{-/-}\) mice (Fig. 3C) although the number was decreased in CCR2\(^{-/-}\) mice (Table I).

Assessment of leukocyte populations in the lungs of CCL2\(^{-/-}\) mice revealed disturbances similar to that of infected CCR2\(^{-/-}\) mice. The percentage and number of inflammatory monocytes and TipDC were decreased in CCL2\(^{-/-}\) mice in comparison to WT although not as severely as in lungs of CCR2\(^{-/-}\) mice (Fig. 2B, 2C, Table I). The number of CD8\(^{-}\) cDC in lungs of CCL2\(^{-/-}\) mice was decreased in comparison to WT (Table I). However, the percentage of CD8\(^{\alpha^{-}}\) cDC (Fig. 3C) and MFI (mean ± SEM) I-A\(^{b}\) expression of CD8\(^{\alpha^{-}}\) cDC from WT (1331 ± 151, \(n=4\)) and CCL2\(^{-/-}\) mice (1205 ± 195, \(n=4\)) were similar (p>0.05). Neutrophil number, which was not affected by loss of CCR2, was reduced in CCL2\(^{-/-}\) mice (Table I).

To determine if the decrement in inflammatory cell number in CCR2\(^{-/-}\) and CCL2\(^{-/-}\) mice resulted from global disturbances in chemokine expression, we examined transcription of chemokines in lungs at day 7 post infection. The generalized impairment in inflammatory cell recruitment was not associated with a loss of CCL2, CCL3, CCL4, CCL7 or CXCL10. Mean \(\log_{10}\) (± SEM) transcription of CCL2 (1.67 ± 0.2, \(n=6\)), CCL7 (1.27 ± 0.14, \(n=6\)), and CXCL10 (2.6 ± 0.21, \(n=6\)) in lungs of WT mice relative to uninfected controls, was similar (p>0.05) in CCR2\(^{-/-}\) mice (CCL2; 1.97 ± 0.11, CCL7; 1.59 ± 0.07, CXCL10; 2.75 ± 0.13, \(n=8\)). Transcription of CCL3 (1.99 ± 0.07, \(n=7\)) and CCL4 (2.04 ± 0.06, \(n=7\)) in CCR2\(^{-/-}\) mice was increased (p<0.001) in comparison to transcription in WT (CCL3; 1.45 ± 0.1, CCL4; 1.53 ± 0.07, \(n=7\)). CCL5 was the only chemokine reduced (p<0.001) in CCR2\(^{-/-}\) mice (0.69 ± 0.03, \(n=7\)) in comparison to WT (1.18 ± 0.09, \(n=7\)).
We asked if the impaired number in leukocyte populations in the lungs of CCR2−/− mice was present earlier than day 7 post infection. At day 3, the percentage of monocytes, TipDC, and CD8− cDC in lungs of CCR2−/− mice were decreased as compared to WT (Fig. 3D).

**Histology of lungs from infected-CCR2−/− mice.** Histopathology of lungs from WT and CCR2−/− mice 7 days post infection indicated similar alterations in lung pathology between the two groups. WT and CCR2−/− mice manifested moderate to severe multifocal peribronchiolar pyogranulomatous pneumonia, moderate to severe lymphocyte cuffing, and mild to moderate edema.

**Cytokine profile in the lungs of CCR2−/− and CCL2−/− mice.** We analyzed cytokine transcripts in lungs by qRT-PCR to determine if the absence of CCR2 or CCL2 affected the polarization of the immune response upon *H. capsulatum* infection. CCR2−/− or CCL2−/− mice did not manifest a decrement in transcription of IL-10, IL-12, IFN-γ, TNF-α, or GM-CSF over the course of infection as compared to WT (Fig. 4A). Transcription of IL-1β, a pro-inflammatory cytokine that contributes to host immunity to *H. capsulatum* infection (66), was not different between CCR2−/− mice and WT. In contrast, an elevation in IL-4 was observed at days 3, 5, and 7 in CCR2−/− mice (Fig. 4A). GM-CSF transcription was also significantly increased in CCR2−/− mice at day 3 (Fig. 4A). Increased IL-4 in the lungs of CCR2−/− mice was not a result of a pre-existing bias in expression of this cytokine. Mean log_{10} (± SEM) transcription of IL-4 within the lungs of uninfected CCR2−/− mice was only 0.07 ± 0.1 (n=4) greater than WT mice and not statistically significant (p>0.05).

One explanation for the increase in IL-4 may be the elevated fungal burden in lungs. Accordingly, we infected WT mice with 1 x10⁷ yeast i.n., which results in a ~10-fold increase in fungal burden in the lungs of WT mice 7 days post infection. IL-4 transcription (mean log_{10} ±
SEM) at day 7 in WT mice infected with $1 \times 10^7$ yeast cells was increased ($0.21 \pm 0.81, n=4$) compared to uninfected controls but not significantly different ($p>0.05$) as compared to WT mice infected with $2 \times 10^6$ yeast cells ($0.46 \pm 0.07, n=3$). These results indicate that fungal burden alone cannot account for increases in IL-4.

The elevation in IL-4 was confined to lungs. Mean log$_{10}$ ($\pm$ SEM) IL-4 transcription in spleens of infected WT mice ($0.13 \pm 0.1, n=4$) did not differ ($p>0.05$) from that of CCR2$^{+/−}$ mice ($0.095 \pm 0.03, n=4$) 7 days post infection.

We assessed protein concentrations of several cytokines to confirm the findings with qRT-PCR. IL-4 was increased within the lungs of CCR2$^{+/−}$ mice at day 7 post infection (Fig. 4B). IL-10, IL-12, IFN-$γ$, TNF-$α$, GM-CSF, and IL-1$β$ did not differ between CCR2$^{+/−}$ and WT mice (Fig. 4).

To ensure that a defect in T$_H1$ polarization and subsequent IFN-$γ$ production did not occur in lungs of CCR2$^{+/−}$ mice, we performed intracellular staining to detect IFN-$γ$ producing CD4$^+$ T cells 7 days post infection. The number of IFN-$γ$ producing CD4$^+$ T cells (mean $\pm$ SEM x $10^4$) did not differ ($p>0.05$) between WT ($7.9 \pm 0.1, n=4$) and CCR2$^{+/−}$ mice ($5.1 \pm 0.3, n=4$). We examined IFN-$γ$ transcription from FACS sorted CD3$^+$ T cells from lungs at day 7. Transcription of IFN-$γ$ (log$_{10}$ $\pm$ SEM) in CD3$^+$ T cells from CCR2$^{+/−}$ mice was only $0.013 \pm 0.007$ lower relative to WT ($n=8$ pooled mice per group).

Neutralization of IL-4 restores protective immunity in CCR2$^{+/−}$ mice. Neutralization of IL-4 produced a decrement in fungal burden and restored protective immunity in CCR2$^{+/−}$ mice (Fig. 5A and 5B). All CCR2$^{+/−}$ mice given mAb to IL-4 and WT mice were sacrificed after 42 days and lungs cultured for the presence of $H.\ capsulatum$. CFU was $< 10^2$ in WT mice and
ranged from $< 10^2$ to $10^3$ in CCR2$^{-/-}$ mice. The data indicate that neutralization of IL-4 promoted clearance of the fungus.

We ascertained if the salutary effect of IL-4 neutralization was a consequence of an improvement in the inflammatory response to \textit{H. capsulatum}. Treatment with mAb to IL-4 did not increase the number of leukocytes in lungs of CCR2$^{-/-}$ mice at day 7 (Fig. 5C). The percentages of CD8$^\alpha$ cDC, TipDC, and monocytes in IL-4 neutralized-CCR2$^{-/-}$ mice were similar to those of infected controls (Fig. 5D).

We assessed fungal burden in lungs and spleens of IL-4$^{-/-}$ mice to determine if neutralization of IL-4 in CCR2$^{-/-}$ could result in effects on immunity unrelated to lack of the chemokine receptor. The absence of IL-4 caused a modest and transient alteration in fungal burden in lungs as compared to WT (Fig. 5E).

\textbf{Regulation of IL-4 by CCR2 ligands.} To ascertain which ligands mediated control of IL-4 production and immunity to \textit{H. capsulatum} infection, we neutralized CCR2 ligands in WT or CCL2$^{-/-}$ mice. Neutralization of CCL7 or CCL7 and CCL12 concomitantly in WT mice or neutralization of CCL12 in CCL2$^{-/-}$ mice did not impair immunity (Fig. 6A, 6B, 6C) despite a decrement in the absolute number of inflammatory lung leukocytes compared to WT mice (Fig. 6D). In contrast, neutralization of CCL7 in CCL2$^{-/-}$ mice resulted in increased IL-4 transcription and fungal burden as compared to goat-IgG treated WT or CCL2$^{-/-}$ mice (Fig. 6A and 6B). In addition, the relative percentage of CD8$^-$ cDC was decreased and the relative percentage of neutrophils increased in CCL7-neutralized CCL2$^{-/-}$ mice in comparison to controls (Fig. 6C).

\textbf{Discussion}

These data demonstrate that CCR2, but not CCL2 alone, was essential for resolution of \textit{H. capsulatum} infection. Although CCR2$^{-/-}$ and CCL2$^{-/-}$ mice displayed severe reductions in the
number of inflammatory cells in lungs, only the former developed a progressive infection associated with elevated IL-4. The deleterious effect of IL-4 was evidenced by the ability of CCR2\(-/-\) mice to survive infection upon IL-4 neutralization independent of an effect on inflammatory cell recruitment. Disruption of CCL7 and CCL2 signaling mimicked the absence of CCR2. These results demonstrate that CCL7 in conjunction with CCL2, are involved in the regulation of IL-4 and immunity to *H. capsulatum* infection.

Reduced recruitment of myeloid lineage cells, disturbed cytokine production, and exacerbated infection observed in CCR2\(-/-\) mice have been at least partially attributed to loss of CCL2 signaling (113, 115, 129). An intact CCR2-CCL2 axis is obligate for resolution of *T. gondii* infection. Lack of CCR2 or CCL2 decreases recruitment of Gr-1\(^+\) monocytes in association with an inability to control infection (115). Monocyte recruitment is decreased in CCR2\(-/-\) and CCL2\(-/-\) mice infected with *L. monocytogenes*, but it is the deficit in TipDC that results in decreased TNF-\(\alpha\) and iNOS production and impaired immunity (129, 130). In contrast, the absence of CCL2 was not a major influence on the course of histoplasmosis. CCL2\(-/-\) mice exhibited a transient increase in fungal burden in lungs and spleens but they cleared infection and survived. Elimination of yeast cells transpired even though phagocyte recruitment, including monocytes and TipDC, was profoundly disturbed.

In experimental *C. neoformans* infection, the absence of CCR2 or CCL2 is accompanied by a poor T\(_{H1}\) response and a heightened T\(_{H2}\) response in addition to decreased recruitment of M\(\phi\), DC and CD8\(^+\) T cells in CCR2\(-/-\) mice or CD4\(^+\) T cells in CCL2-neutralized mice (91, 132). Trapping of T\(_{H1}\), but not T\(_{H2}\) cells in lymph nodes causes a skewing to type 2 immunity in lungs of CCL2-neutralized mice whereas CCR2\(-/-\) mice lack T\(_{H1}\) polarization (113). Whether the decreased inflammatory cell recruitment or the T\(_{H1}\) to T\(_{H2}\) shift, or both contribute to increased
fungal burden remains unresolved. We found that the absence of CCL2 did not induce a polarized cytokine response, a finding quite different than that in experimental cryptococcosis.

Similar to *C. neoformans* and *L. major* infection of CCR2−/− mice, *H. capsulatum* infection resulted in increased generation of IL-4. However, elevated IL-4 in *H. capsulatum*-infected CCR2−/− mice was not accompanied by decreased generation of TH1 cytokines including IFN-γ as occurs during *C. neoformans* and *L. major* infection (113, 132, 134). Thus a reduction in these mediators is not the reason for exacerbation of infection. The data suggest that IL-4 is a highly potent influence on immunity even when TH1 cytokine levels are maintained. However, CCR2−/− mice do not succumb to infection as quickly as mice lacking IFN-γ or TNF-α (23, 156) suggesting that TH1 cytokines partially impair fungal replication in the absence of CCR2. Infection of CCR2−/− mice with *H. capsulatum* also differs from *M. bovis* infection of CCR2−/− mice in which a more aggressive infection is attributable to a decrease in IFN-γ without a concomitant increase in IL-4.

The absence of CCR2 or CCL2 during *H. capsulatum* infection did not impair the generation of the pro-inflammatory mediators IL-12, IFN-γ, TNF-α, GM-CSF, or IL-1β. Therefore, the impairment in leukocyte migration and/or retention in lungs cannot be attributed to the loss of these molecules. Moreover, production of several chemokines that may contribute to the inflammatory cell recruitment was not diminished.

Decreased DC number in lungs may contribute to defects in T cell activation and expansion observed in CCR2−/− and CCL2−/− mice. Immature DC kill yeast cells (166), and the severe reduction in the number of TipDC and CD8α⁺ cDC in CCR2−/− and CCL2−/− mice may limit their capacity to exert this action and thereby reduce the quantity of Ag that can be presented to T cells. Thus, the paucity of T cells in CCR2−/− and CCL2−/− mice may result from a decreased
number of DC-T cell interactions ensuing in decreased expansion and not only decreased T cell emigration.

Disturbances in cell recruitment were not directly responsible for the accelerated mortality in infected CCR2\(^{-/-}\) mice. The inflammatory response both from a qualitative as well as quantitative assessment was unchanged in mice that received mAb to IL-4. Additional support for the argument that the substandard inflammatory response did not contribute greatly to impaired immunity is that CCL2\(^{-/-}\) mice manifested many of the same alterations in inflammatory cell recruitment yet did not succumb to infection. Nevertheless, a decrease in the numbers of cells concurrent with increased IL-4 may contribute to the failure to resolve infection. More IL-4 molecules may be available to interact with fewer inflammatory cells, therefore increasing the amount of cytokine engaging receptors on individual cells and modulating their effector function. The above argument is supported by the finding that transgenic mice overexpressing IL-4 only in lungs resolve \textit{H. capsulatum} infection despite exhibiting higher fungal burden in comparison to controls. These animals do not manifest a deficit in inflammatory cells (167).

IL-4 is detrimental to resolution of \textit{Histoplasma} infection when it was aberrantly increased as observed in infected CCR2\(^{-/-}\) mice or when increased in combination with IL-10 as occurs in the absence of GM-CSF (65). Neutralization of IL-4 in WT mice had no observable effect on immunity thus neutralization in CCR2\(^{-/-}\) mice improved infection outcome by reversing defects that resulted directly from loss of CCR2 and subsequent IL-4 elevation. It is unlikely that altered production of other cytokines such as GM-CSF in conjunction with IL-4 impaired outcome in CCR2\(^{-/-}\) mice since GM-CSF is a protective cytokine in murine histoplasmosis (168).
Our data suggest that CCR2−/− mice succumbed to infection as a consequence of increased IL-4 in lungs resulting in an inability to control yeast replication but not dissemination since an increase in IL-4 and fungal burden was restricted to the lung. Lung histopathology was similar in CCR2−/− and WT mice as well as T_{H1} and T_{H17} (data not shown) cytokine levels therefore it is unlikely that CCR2−/− mice succumbed as a result of hyper-inflammation. The lack of CCR2 mediated cell recruitment most likely constrained lung inflammation.

CCL7 is considered a crucial molecular effector in host resistance depending on the model of infection. The functional attributes of this chemokine are diverse. In murine listeriosis, CCL7 promotes immunity by attracting monocytes and TipDC to infected tissues. This activity is similar to that observed with CCL2. The absence of either CCL2 or CCL7 is associated with an elevated bacterial burden compared to controls but the magnitude is not the same as detected in CCR2−/− mice (92). Alternatively, CCL7 production at the site of *Leishmania* infection promotes persistence of infection through recruitment of IL-4^+^ T_{H2} leukocytes (104). The findings herein differ considerably from those aforementioned studies. In contrast to *L. monocytogenes* infection, CCL7 was dispensable for protective immunity in murine histoplasmosis as assessed by fungal burden. The inability to control infection required the combined absence of CCL2 and CCL7. Furthermore, the impairment in immunity could not be directly attributed to altered monocyte or TipDC trafficking. Our results differed considerably from those in experimental leishmaniasis. In histoplasmosis, CCL7 in conjunction with CCL2 was responsible for restraint, not production, of IL-4. To our knowledge, these pivotal findings are the first demonstration of a cooperative role of CCL2 and CCL7 in regulating IL-4 production and consequently, control of an intracellular infection.
CCL2 and CCL7 may regulate IL-4 directly through engagement of CCR2 on DC. Bone marrow-derived DC lacking CCR2 produce IL-4 and stimulate T cell IL-4 production to a greater extent than WT bone marrow-derived DC (136) suggesting signaling through CCR2 is necessary for limiting IL-4 production. Alternatively, IL-4 production may be limited indirectly through CCR2\(^+\) cells recruited by CCL2 and CCL7. In support of the latter hypothesis, the relative percentage and number of CD8\(^-\) cDC in lungs of CCR2\(^{-/-}\) and CCL7- neutralized CCL2\(^{-/-}\) mice are similarly depressed to a greater extent than that observed in CCL2\(^{-/-}\) mice.

Our studies contribute to the understanding of the complexity of host control of intracellular organisms and highlight the necessity of regulating IL-4 during *H. capsulatum* infection. Downstream effects of loss of signaling through CCR2 vary depending on what organism is eliciting infection, the dose of organism, and the route of infection suggesting a multi-functional role of CCR2. Further studies examining the initial stimulus that results in increased IL-4 production in CCR2\(^{-/-}\) mice upon *H. capsulatum* infection may identify previously unknown roles of CCR2 in controlling infection.
Figure 2. CCR2 mediates resolution of *H. capsulatum* infection; CCL2 alone is dispensable. Mice were infected with 2 x 10^6 yeasts and fungal burden in lungs and spleens assessed at day 7 and 14 post infection in WT and CCR2^-/- mice or at day 7, 14, and 21 in WT and CCL2^-/- mice (A). (n=8-10). **p<0.001. Data represent the mean ± SEM of 2-3 experiments. Survival of infected mice was assessed (B). (n=8-9).
Figure 3. Inflammatory response are impaired in lungs of infected CCR2−/− and CCL2−/− mice. Lung leukocytes were isolated 3 and 7 days post infection and enumerated (A). \( n=12-20 \). Representative FACS plots of leukocytes from lungs of WT, CCL2−/−, and CCR2−/− mice 7 days post infection to demonstrate gating for monocyte, Mφ and DC populations. The majority of CD11c+ cells in lungs of infected mice were DC at day 7 as demonstrated by I-A\(^{b}\)hi expression (117, 169). Two distinct populations of CD11c+ cells were identified by the level of Ly6C expression. TipDC were Ly6C\(^{hi}\)CD11c\(^{int}\)I-A\(^{b}\)hi (130, 133). CD8\(^{+}\)cDC were Ly6C\(^{int}\)CD11c\(^{hi}\)I-A\(^{b}\)hi (93, 117). Inflammatory monocytes were defined as CD11c\(^{+}\)Ly6C\(^{hi}\) (129, 131). Monocyte and DC populations were CD11b\(^{hi}\), CD8\(^{+}\)cDC were CD8\(^{−}\), and many TipDC produced TNF-\(\alpha\) when stained intracellularly (data not shown). Alveolar Mφ were CD11c\(^{+}\)I-A\(^{b}\)\(^{lo/int}\)Mac3\(^{hi}\) (170, 171) and tissue Mφ were Mac3\(^{+}\)CD11c\(^{−}\) (170). Whereas alveolar Mφ were CD11b\(^{lo/int}\), tissue Mφ were CD11b\(^{int/hi}\) (data not shown) (B). Percentages of examined leukocyte populations 7 days post infection in lungs of WT, CCR2−/− and CCL2−/− mice determined by flow cytometry. CD4\(^{+}\) and CD8\(^{+}\) T cells also expressed CD3, neutrophils were CD11b\(^{hi}\)CD11c\(^{−}\)Ly6G\(^{hi}\) (C). \( n=8-12 \). Leukocyte populations 3 days post infection in WT and CCR2−/− mice (D). \( n=8-12 \). **p<0.001 vs. WT. ## p<0.001 vs. CCL2−/−. Data represent the mean ± SEM of 2-3 experiments.
Table I. Absolute Number of Myeloid Populationsa

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Mean Cell Number ± SEM x 10^5</th>
<th>WT</th>
<th>CCL2⁻/⁻</th>
<th>CCR2⁻/⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophil</td>
<td>1.67 ± 0.12</td>
<td>1.11 ± 0.13b</td>
<td>1.78 ± 0.23c</td>
<td></td>
</tr>
<tr>
<td>Monocyte</td>
<td>0.59 ± 0.67</td>
<td>0.23 ± 0.05b</td>
<td>&lt;0.01b,c</td>
<td></td>
</tr>
<tr>
<td>TipDC</td>
<td>1.63 ± 0.10</td>
<td>0.54 ± 0.09b</td>
<td>0.10 ± 0.01b,c</td>
<td></td>
</tr>
<tr>
<td>CD8⁻ cDC</td>
<td>1.56 ± 0.29</td>
<td>0.71 ± 0.10b</td>
<td>0.15 ± 0.03b,c</td>
<td></td>
</tr>
<tr>
<td>Alveolar Mϕ</td>
<td>0.40 ± 0.07</td>
<td>0.21 ± 0.03b</td>
<td>0.12 ± 0.02b,c</td>
<td></td>
</tr>
<tr>
<td>Tissue Mϕ</td>
<td>1.57 ± 0.20</td>
<td>1.07 ± 0.20</td>
<td>1.57 ± 0.21</td>
<td></td>
</tr>
</tbody>
</table>

aAbsolute numbers of leukocytes 7 days post infection. Data represent the mean ± SEM of 2-3 experiments. (n=8-12).

b\(p<0.001\) vs. WT

c\(p<0.001\) vs. CCL2⁻/⁻
Figure 4. The absence of CCR2, but not CCL2, results in increased IL-4 in lungs; the T\textsubscript{H}1 immune response is not impaired. Cytokine transcription in lungs of CCR2\textsuperscript{−/−} and CCL2\textsuperscript{−/−} mice. Whole lung RNA was prepared at the indicated time points post infection for use in subsequent qRT-PCR analysis. Transcription is expressed as log\textsubscript{10} RQ relative to uninfected WT lung (A). Cytokine protein levels in lung homogenates 7 days post infection. Protein concentrations were determined by ELISA (B). (n=6-12). **p<0.001. *p <0.05. Data represent the mean ± SEM of 2-3 experiments.
Figure 5. Neutralization of IL-4 in CCR2−/− mice prevents mortality from *H. capsulatum* infection. CCR2−/− mice treated with 1mg anti-IL-4 or 1mg rat IgG and WT mice were infected with 2 x 10^6 yeasts and fungal burden assessed 7 days post infection (A). (n=7-8). Survival of infected mice was monitored (B). (n =6). At day 7 inflammatory cell recruitment was evaluated. Absolute numbers of leukocytes within lungs were enumerated (C) and relative percentages of leukocyte populations were analyzed by flow cytometry (D). Fungal burden in lungs and spleens of WT and IL-4−/− mice over the course of infection (E) (n=8-12). **p <0.001, *p <0.05. Data represent the mean ± SEM of 2-3 experiments.
Figure 6. CCL7 in combination with CCL2 is necessary for immunity to *H. capsulatum* infection. Mice were infected with 2 x 10^6 yeasts and treated with the indicated neutralizing antibodies or goat-IgG. Fungal burden was assessed 7 days post infection (A). (n=3-6). Log_{10} RQ IL-4 transcription from whole lungs 7 days post infection (B). (n=3-6). Relative percentages of lung leukocyte populations 7 days post infection (C). (n=3-6). Absolute number of lung leukocytes 7 days post infection (D). (n=3-6). **p <0.001 vs. WT + goat IgG and CCL2^{-/-} + goat IgG, *p <0.05 vs. WT + goat IgG and CCL2^{-/-} + goat IgG, #p<0.05 vs. WT + goat IgG. Data represent the mean ± SEM of 2 experiments (n=5-6 per group) with exception of WT + anti-CCL7 and WT + anti-CCL7 & anti-CCL12 groups (n=3, 1 experiment).
Chapter 3: IL-4 is Produced by Phagocytic Cells and is Associated with Alternative Mφ Activation which is Deleterious to Host Defense

Abstract

Production of IL-4 by CD4⁺ T cells, NKT cells, basophils, and eosinophils is beneficial for the clearance of extracellular helminthes and some extracellular pathogens. We sought to determine which populations of leukocytes contributed to excess IL-4 production in lungs of *H. capsulatum* infected CCR2⁻/⁻ mice. Surprisingly, numerous alveolar Mφ and DC generated IL-4. The number of IL-4⁺ leukocytes was similar in WT and CCR2⁻/⁻ mice; however transcription of IL-4 was increased in DC, alveolar Mφ, tissue Mφ and CD4⁺ T cells in comparison to the respective WT populations. We examined the mechanism by which IL-4 impairs yeast clearance in CCR2⁻/⁻ mice. IL-4-mediated impairment of immunity was associated with increased expression of the alternative activation markers arginase-1, YM1, and transferrin receptor by phagocytic cells. Administration of L-arginine, an arginase-1 substrate, or BEC ((S)-(2-Boronoethyl)-L-cysteine, HCl), an arginase-1 inhibitor, decreased fungal burden in CCR2⁻/⁻ mice. Thus, CCR2⁻/⁻ phagocytic cells as well as CD4⁺ T cells produce greater quantities of IL-4 per cell than the respective WT populations upon *H. capsulatum* infection. Furthermore, increased IL-4 in CCR2⁻/⁻ mice is associated with alternative activation of phagocytic cells which is deleterious to host defense.

Introduction

The cytokine IL-4 participates in induction and maintenance of T_{H2} immune responses. IL-4 acts as a growth factor for type 2 polarized T cells and induces eosinophil recruitment and immunoglobulin IgE class switching. IL-4 signals through the type I (IL-4Rα/γc) and type II
(IL-4Rα/IL-13Rα) receptors. Signaling results in the phosphorylation of STAT6, which upon subsequent nuclear translocation, activates the Th2 specific transcription factors GATA3 and c-maf (172-174). IL-4 signaling results in the production of IL-4, IL-5 and IL-13 and leads to the repression of IFN-γ and a Th1 response (145, 175); however IL-4 is not required for the generation of a Th2 response. The initial appearance and number of Th2 cells is not affected by the absence of IL-4 in various Th2 biased models of infection (176).

Infection by Th2 eliciting pathogens results in IL-4 production by eosinophils, CD4+ T cells, and basophils (174). NKT cells, γδ T cells, and mast cells produce IL-4 as well. Although not commonly thought to be producers, phagocytic cells are capable of IL-4 production (73, 136, 177).

IL-4 mediates resolution of infection resulting from intestinal helminths by inducing smooth muscle contractility and mucous secretion in the intestine to promote worm expulsion (178). On the other hand, IL-4, when produced in excess, is detrimental to host resistance against intracellular pathogens (65, 72, 179). One mechanism by which IL-4 impairs immunity against intracellular pathogens is through alternative activation of Mφ (72, 73). IL-4 and IL-13 signaling induce an alternatively activated Mφ phenotype consisting of highly expressed arginase-1 (Arg-1), MHCII, transferrin receptor (TR), Mφ mannose receptor, YM1, and FIZZ1 (72, 73, 180). Alternatively activated Mφ promote wound repair (181) and dampen cytokine mediated tissue injury in helminth infected mice (182), but they accelerate growth of intracellular pathogens due to increased expression of Arg-1. The enzyme promotes growth of pathogens by decreasing the availability of L-arginine, an essential substrate for the generation of toxic NO (183, 184). The result is decreased generation of NO and subsequent pathogen degradation. Arg-1 also increases biosynthesis of polyamines that are scavenged by intracellular microbes.
resulting in enhanced intracellular growth (185). In contrast, classical Mφ activation is generated by IFN-γ and results in pathogen degradation due to increased iNOS, the enzyme necessary for NO production (186). Thus an increased proportion of alternative to classically activated Mφ favors pathogen persistence.

Our previous studies have demonstrated that *H. capsulatum* infected CCR2−/− mice do not resolve infection as a consequence of elevated IL-4 and fungal burden in lungs (Chapter 2). We sought to determine the cellular sources responsible for aberrant IL-4 production in lungs of *H. capsulatum* infected-CCR2−/− mice and the mechanism by which IL-4 impairs yeast clearance. Although phagocytic cells are not typically considered to be major sources of IL-4, we found that phagocytic cells from lungs of *H. capsulatum*-infected CCR2−/− mice generated greater quantities of IL-4 than WT populations. Production of IL-4 by CD4+ T cells was also increased in CCR2−/− mice, however depletion of CD4+ cells at the time of infection did not decrease fungal burden suggesting that production of IL-4 by non-T cell sources contributes to exacerbated infection. Furthermore, increased IL-4 in lungs of CCR2−/− was associated with alternatively activated phagocytic cells with elevated Arg-1. Specifically, increased Arg-1 contributed to increased yeast growth in lungs of CCR2−/− mice.

**Results**

*Characterization of IL-4+ cells in CCR2−/− mice.* To determine the sources of IL-4 in lungs of WT and CCR2−/− mice, IL-4 secreting cells were isolated from pools of infected lungs at day 7 of infection and analyzed for surface phenotype (Fig. 7A). Approximately 60% of the IL-4+ cells in CCR2−/− mice were CD11c+ and ~50% of those were Mac3hi (Fig. 7A, 7B). The IL-4 secreting CD11c+ cells also expressed intermediate to high levels of CD11b and I-A^b^ demonstrating that these cells were from the myeloid lineage (Fig. 7A). The percentage of IL-4
secreting cells expressing CD11c and Mac3$^{hi}$ or Mac3$^{lo/int}$ was similar in WT and CCR2$^{-/-}$ mice (Fig. 7B). CD11c$^{+}$Mac3$^{+}$ tissue Mφ (170), CD3$^{+}$CD4$^{+}$ T cells, NK1.1$^{+}$CD3$^{-}$ natural killer cells (NK) cells, NK1.1$^{+}$CD3$^{+}$ NKT cells, and CD11c$^{+}$FCEr1$^{+}$FSC/SSC$^{lo}$ basophils secreted IL-4 in lungs of both WT and CCR2$^{-/-}$ mice although these populations comprised a lesser proportion of the total IL-4$^{+}$ cells than those expressing CD11c (Fig. 7B).

We sorted IL-4 secreting populations that were CD11c$^{+}$Mac3$^{hi}$ or CD11c$^{+}$Mac3$^{lo/int}$ from CCR2$^{-/-}$ mice and performed cytospins to confirm the morphology of both populations (Fig. 7C). The IL-4-secreting, CD11c$^{+}$Mac3$^{hi}$ cells were highly vacuolated, and manifested a high cytoplasm to nuclei ratio characteristic of alveolar Mφ. Many yeast cells were associated with CD11c$^{+}$Mac3$^{hi}$ cells (Fig. 7C, arrows). Approximately 45% of the Mac3$^{+}$CD11c$^{hi}$ cells appeared to contain *H. capsulatum* yeast cells.

The IL-4-secreting CD11c$^{+}$Mac3$^{lo/int}$ population exhibited a typical DC morphology. CD11c$^{+}$Mac3$^{lo/int}$ cells were smaller, not as highly vacuolated, the cytoplasm to nuclei ratio was lower, and characteristic dendrites were observed. Approximately 23% of these cells were infected when we analyzed cytospins. Slides were examined for eosinophils, which express CD11c (174), but no cells with characteristic granules were detected.

The above experiments defined by phenotype the populations that generated IL-4. To quantify IL-4 production by the various populations, lung leukocytes were sorted and transcription assessed by qRT-PCR. Because sorting of phagocytic cells could result in DC-T cell clusters, cells expressing CD3 were excluded to ensure that T cell contamination did not account for IL-4 transcription in sorted phagocytic cell populations. Sorted CD11c$^{+}$ Mac3$^{hi}$ alveolar Mφ, CD11c$^{+}$Mac3$^{lo/int}$ DC, and Mac3$^{+}$CD11c$^{-}$ tissue Mφ, as well as CD4$^{+}$CD3$^{+}$ T cells
from CCR2−/− mice 7 days post infection exhibited increased IL-4 transcription in comparison to the respective WT population (Fig. 8).

To confirm that phagocytic cells produce IL-4 in response to \textit{H. capsulatum} infection, we infected BMDM and BMDC with increasing concentrations of yeast for 24 hr and then measured IL-4 transcription. IL-4 transcription by bone marrow-derived cells increased above uninfected levels with a low infection ratio of 1 yeast per 10 BMDM or BMDC (Fig. 9). CCR2−/− BMDM and BMDC exhibited elevated basal transcription of IL-4 as previously reported (136), but upon infection with \textit{H. capsulatum}, IL-4 transcription was similar to the respective WT populations (Fig. 9).

**Depletion of CD4+ T cells from CCR2−/− mice.** To determine if IL-4 production by CD4+ T cells contributed to the demise of CCR2−/− mice we depleted CD4+ T cells and assessed fungal burden in the lungs. CFU (mean log_{10} ± SEM) in the lungs of CD4+ T cell depleted- CCR2−/− mice 7 days post infection (8.6 ± 0.08, n=8) was similar (p>0.05) to infected CCR2−/− mice given rat IgG (8.2 ± 0.2, n=8).

**Expression of IL-4 regulated genes and proteins in lungs of CCR2−/− mice.** We determined the impact of IL-4 by evaluating other downstream targets of this cytokine. Arginase-1 (Arg-1) is induced by IL-4 (187) and promotes survival of intracellular organisms (184, 185). Because Arg-1 utilizes the same substrate as iNOS, L-arginine (L-Arg), increased Arg-1 can deplete cells of L-Arg resulting in decreased NO production (183, 187, 188). Increased Arg-1 can also be beneficial to intracellular organisms as it contributes to polyamine synthesis (185). Transcription of Arg-1 was increased in lung leukocytes from CCR2−/− mice in comparison to WT 7 days post infection, although not earlier (Fig. 10A). IL-4 can inhibit iNOS generation (188), but iNOS transcription was similar in leukocytes from lungs of WT and
CCR2\(^{-/-}\) mice at days 3, 5, and 7 post infection (Fig. 10A). Examination of NO production revealed no disturbances from lung leukocytes from CCR2\(^{-/-}\) mice in comparison to WT (Fig. 10B).

IL-4 induces alternative activation of M\(\phi\), and these cells are defective in their ability to degrade intracellular organisms (72, 73). Elevated Arg-1 expression often accompanies alternative activation of M\(\phi\) (73, 189). To determine if phagocytic cells were alternatively activated in CCR2\(^{-/-}\) mice we analyzed transcription of YM1 and FIZZ1, two markers of alternative activation in sorted phagocytic populations as well as Arg-1 (180). YM1 and Arg-1 were increased in CD11c\(^+\)Mac3\(^{hi}\) alveolar M\(\phi\) and Mac3\(^+\)CD11c\(^-\) tissue M\(\phi\) from CCR2\(^{-/-}\) mice in comparison to the respective WT populations (Fig. 11A). YM1 and Arg-1 were increased in CD11c\(^-\)Mac3\(^{lo/int}\) DC from CCR2\(^{-/-}\) mice in comparison to WT DC (Fig. 11A). There was no difference in FIZZ1 transcription between WT or CCR2\(^{-/-}\) tissue M\(\phi\) (Fig. 11A). TR expression (mean MFI ± SEM), another marker of alternative activation (72), was increased on alveolar M\(\phi\) as well as DC from CCR2\(^{-/-}\) mice in comparison to WT although expression was similar on tissue M\(\phi\) (Fig. 11B).

To determine if amplified Arg-1 contributed to the inability to control fungal burden, we treated WT and CCR2\(^{-/-}\) mice with L-arginine (L-Arg) or D-arginine (D-Arg) as a control. Administration of L-Arg to CCR2\(^{-/-}\) mice decreased lung fungal burden at day 8 in comparison to untreated or D-Arg-treated CCR2\(^{-/-}\) mice. The fungal burden of L-Arg-treated WT mice was similar to untreated WT mice (Fig. 12A). Treatment with BEC, an inhibitor of Arg-1, resulted in a modest but significant (p < 0.007) decrease in lung CFU in CCR2\(^{-/-}\) mice in comparison to untreated CCR2\(^{-/-}\) controls (Fig. 12A).
Discussion

Our studies demonstrate that *H. capsulatum* induces IL-4 production from phagocytic cells and that Mφ and DC contributed to elevated IL-4 in lungs of CCR2−/− mice. Although CD4+ T cells in CCR2−/− lungs also produced excess IL-4, these cells alone did not mediate exacerbated infection. We found that increased IL-4 in lungs of CCR2−/− was associated with alternatively activated phagocytic cells expressing increased Arg-1. Furthermore, elevated Arg-1 impaired yeast clearance in CCR2−/− mice.

CD11c+Mac3hi and CD11c−Mac3lo/int cells contributed to IL-4 production in *H. capsulatum* infected WT and CCR2−/− mice. Others have reported Mac3+CD11c+ leukocytes in the lung as alveolar Mφ (170), although Mac3 surface expression has been reported on DC (190). By morphology the Mac3−CD11chi cells were characteristic of Mφ while the Mac3−CD11clo/int cells appeared to be DC. The finding that alveolar Mφ and DC were generators of IL-4 was unanticipated. As further confirmation, we also demonstrated that bone marrow-derived phagocytes also produce IL-4 when infected with yeast. Our results extend and expand other reports indicating that Mφ and DC can generate IL-4 (73, 136, 177). Conventional sources of IL-4 such as basophils also contributed to IL-4 production. Because we did not determine if the population we termed basophils expressed c-kit, a mast cell marker, it is possible that mast cells produced IL-4 as well.

The increase in IL-4 in CCR2−/− mice most likely results from an enhanced production on a per cell basis. The number of IL-4 generating leukocytes is not significantly increased in lungs of CCR2−/− mice, yet all phagocytic populations and CD4+ T cells transcribed more IL-4 than respective WT populations. The number of phagocytic cells secreting IL-4 in CCR2−/− mice...
was greater than non-phagocytic populations, but the quantity produced does not appear to be
greater since all leukocyte populations transcribed equivalent amounts of IL-4.

A great deal of literature regarding the biological activity of IL-4 stems from studies in
which CD4+ T_{h}2 cell are the principal source of the cytokine. In *Histoplasma*-infected CCR2^{-/-}
mice, CD4+ cells were a source of IL-4 during *H. capsulatum* infection, however loss of these
cells in CCR2^{-/-} mice did not diminish the fungal burden. Thus, it appears that IL-4 produced by
other leukocyte populations contributes to impaired immunity.

IL-4 induces alternatively activated Mφ that are advantageous for the clearance of
helminths, but promote survival of intracellular pathogens (72, 73, 182). In addition, *in vitro*
evidence demonstrates that *H. capsulatum* replication is enhanced in bone-marrow derived Mφ
treated with IL-4 (M. Winters and G.S. Deepe, unpublished observation). Alternatively activated
cells manifest a profile of phenotypic and genotypic characteristics that discriminate them from
classically activated Mφ. Among these are enhanced expression of Arg-1 although this is not an
exclusive property of alternative activation (187). Elevated expression of Arg-1 may enhance
permissiveness of Mφ for intracellular pathogens including *Francisella tularensis* and *C.
neoformans* (73, 189). The importance of Arg-1 expression in host defenses to intracellular
pathogens has been reinforced by studies demonstrating that mice lacking Arg-1 in Mφ are more
efficient in clearing *T. gondii* or *M. tuberculosis* (191). The observed increase in YM1, Arg-1,
and expression of TR in Mφ from *H. capsulatum* infected CCR2^{-/-} mice suggest alternative
activation.

Our findings demonstrate that elevated Arg-1 in alternatively activated phagocytes
imparts immunity to *H. capsulatum*. Arg-1 inhibition by BEC slightly diminished the fungal
burden in lungs of CCR2^{-/-} mice consistently in three independent experiments indicating the
detrimental impact of increased activity. Furthermore, treatment of CCR2$^{-/-}$ mice with L-arg decreased lung CFU suggesting that L-arg depletion by Arg-1 is disadvantageous to control of infection. However, L-arg addition may have additional effects on immunity since CFU was more sharply reduced in L-arg treated CCR2$^{-/-}$ mice. Treatment with L-arg or BEC did not lessen burden in lungs of WT mice, or spleens of WT or CCR2$^{-/-}$ mice. These findings signify that a threshold level of Arg-1 activity must be reached before impaired immunity occurs. The data indicate that there is an Arg-1-independent effect of elevated IL-4 on host defenses since CFU in lungs of BEC or L-arg treated mice were not diminished to the values found in WT.

Depletion of L-arg by Arg-1 can limit generation of NO consequently promoting intracellular pathogen growth. For example, L-Arg administration reduces the number of trypanosomes in circulation, and this modulation is associated with increased NO production by peritoneal Mφ (184). Thus one hypothesis to explain the decrease in fungal burden in lungs of *H. capsulatum* infected-L-Arg and BEC treated CCR2$^{-/-}$ mice is enhanced NO. Although our data failed to detect differences in NO or iNOS between CCR2$^{-/-}$ and WT mice, it is possible that NO production may be compromised in a subset of phagocytic cells that depend on it for yeast degradation. Accordingly, infected TipDC from infected CCR2$^{-/-}$ mice exhibited decreased iNOS transcription in comparison to WT TipDC (W. Szymczak, unpublished results) and were infected with yeast. Production of NO by TipDC could also be necessary for yeast degradation in closely surrounding infected cells. Additionally, depletion of L-arg in the microenvironment by myeloid cells could result in T cell cycle arrest, hyporesponsiveness, and decreased activation (192, 193).

Other mechanisms not yet examined may contribute to increased survival of *H. capsulatum* within alternatively activated Mφ. Increased transferrin receptor expression may
promote growth by enhancing intracellular iron uptake. Increased iron availability has been associated with increased growth of *M. tuberculosis* and iron is critical for growth of *H. capsulatum* (194, 195). IL-4 most likely has effects independent of alternative activation as well. For example, IL-4 can inhibit autophagy which is necessary for the degradation of intracellular pathogens (196). IL-4 can also inhibit apoptosis of T regulatory cells (197) which function to dampen the immune response and thus promote pathogen outgrowth.

Our studies have revealed that downstream effectors of IL-4 have a negative impact on immunity in CCR2<sup>−/−</sup> mice. These data highlight the importance of regulation of cytokines during immune responses and future studies dissecting downstream effects of IL-4 in infected CCR2<sup>−/−</sup> mice may reveal further unknown roles of IL-4 in dampening immunity against *H. capsulatum*. 
Figure 7. Phagocytes secrete IL-4 in lungs of *H. capsulatum* infected mice. Representative plots of IL-4 secreting cells from lungs of WT and CCR2<sup>−/−</sup> mice 7 days post infection. IL-4 positive cells were gated on to determine phenotype (A). Percentage of IL-4<sup>+</sup> leukocytes expressing the indicated surface markers (B). (*n*=3-5, 2-4 lungs pooled per group). 100x magnification of FACS sorted populations from the lungs of 10 pooled CCR2<sup>−/−</sup> mice 7 days post infection. Arrows indicate *H. capsulatum* yeast (C). (*n*=2-4, 8-12 pooled lungs per group). **p<0.001, *p<0.05 Data represent the mean ± the SEM of 3-5 experiments.
Figure 8. Transcription of IL-4 is elevated in sorted CD4$^+$ T cells and phagocytic populations in CCR2$^{-/-}$ mice. Log$_{10}$ RQ IL-4 transcription from FACS sorted populations. Data are expressed relative to WT lung RNA from uninfected mice. ($n=2-4$, 8-12 pooled lungs per group). **p<0.001, *p<0.05 Data represent the mean ± the SEM of 3-5 experiments.
Figure 9. *H. capsulatum* infection of WT and CCR2^{−/−} bone marrow-derived Mϕ (BMDM) and bone marrow-derived DC (BMDC) results in IL-4 production in a yeast dependent manner. Log10 transcription of IL-4 24 hr post infection. (*n*=3-4).
Figure 10. Arginase-1, but not iNOS or NO, is elevated in lung leukocytes of CCR2\(^{-/-}\) mice. Log₁₀ RQ transcription of Arg-1 and iNOS from isolated lung leukocytes from WT and CCR2\(^{-/-}\) mice days 3, 5, and 7 post infection (A). (\(n=6-8\)). Measurement of NO products nitrate and nitrite produced by \textit{ex vivo} stimulated lung leukocytes isolated from WT and CCR2\(^{-/-}\) mice 7 days post infection (B). (\(n=5\)). **\(p<0.001\), *\(p<0.05\). Data represent the mean ± SEM of 2-4 experiments.
Figure 11. CCR2\(^{-/-}\) Phagocytic populations exhibit markers of alternative activation. Log\(_{10}\) RQ Arg-1, iNOS, YM1, and FIZZ1 transcription from FACS sorted populations 7 days post infection relative to whole lung transcription from uninfected WT mice (A). \((n=3-4, 8-12\) pooled lungs per group). MFI TR expression of phagocytic populations from WT and CCR2\(^{-/-}\) lungs 7 days post infection (B) \((n=8)\). **p<0.001, *p<0.05. Data represent the mean ± SEM of 2-4 experiments.
Figure 12. Arg-1 inhibition increases fungal clearance in CCR2^{-/-} mice. Fungal burden in lungs of L-Arg, D-Arg, and BEC treated WT and CCR2^{-/-} mice 8 days post infection (E). (n=8-11). **p<0.001, *p<0.05 Data represent the mean ± SEM of 2-4 experiments.
Chapter 4: Ag Presenting DC Regulate IL-4 in H. capsulatum-Infected Mice

Abstract

Excessive production of IL-4 impairs clearance of the fungal pathogen *Histoplasma capsulatum* in mice lacking the chemokine receptor CCR2. This elevation is associated with decreased recruitment of DC, therefore we investigated the role of these cells in IL-4 regulation. Adoptive transfer of WT or CCR2−/− BMDC loaded with heat-killed yeast (Ag-BMDC) to CCR2−/− mice increased the number of lung DC and suppressed generation of IL-4. Conversely, IL-4 remained elevated in CCR2−/− mice that received BMDC that were not exposed to a source of *H. capsulatum* Ag (Ag-free BMDC). Despite limiting IL-4 production, transferred Ag-BMDC did not enhance yeast clearance in CCR2−/− mice, and transfer of Ag-free BMDC increased fungal burden. CCR2−/− mice that received Ag-BMDC manifested an increased number of infected DC whereas transfer of Ag-free BMDC resulted in an increased number of infected DC and CD11c'CD11b+ phagocytes in comparison to CCR2−/− mice. To determine if Ag-BMDC regulation of IL-4 was dependent on MHCII-Ag presentation to CD4+ T cells, we transferred Ag-BMDC lacking MHCII expression to CCR2−/− mice. MHCII−/− Ag-BMDC did not limit IL-4. In addition, depletion of CD4+ T cells beginning prior to, but not at the time of infection, suppressed IL-4. However, fungal burden was only reduced in CD4-depleted CCR2−/− mice that also received Ag-BMDC. Taken together, the data suggest that the number of Ag presenting DC in lungs is critical for regulating IL-4 production by CD4+ T cells early during infection and that CD4+ T cells exert a deleterious impact on immunity.

Introduction

Infection by the intracellular fungal pathogen *H. capsulatum* can cause a fatal disseminated infection in immunocompromised individuals (61). In mice, impaired immunity
results from a diminished TH1 and/or an increased TH2 immune response (23, 65, 156, 167, 179). Production of IFN-γ, a TH1 cytokine, promotes infection resolution by activating Mφ harboring yeast (156, 159). These Mφ, termed “classically activated Mφ,” produce NO resulting in yeast degradation (46). TNF signaling also promotes production of NO and protective immunity (179). In contrast, excessive production of IL-4, a TH2 cytokine, is associated with alternative Mφ activation (198) and dampens the protective immune response (23, 64, 65, 198).

A principal source of IL-4 is the CD4+ T cell (174, 199, 200). Basophils produce IL-4 and induce CD4+ T cells to generate the cytokine in an Ag-dependent manner upon helminth infection or allergic airway inflammation (199, 201, 202). In addition, presentation of microbial Ag by DC influences T cell polarization, but the mechanisms that determine whether TH1 or TH2 cells will dominate are not fully understood. In the presence of IL-4, DC stimulate CD4+ T cells to transcribe IL-4 resulting in an amplified TH2 response (144). Increased surface expression of the Notch ligand Jagged 1 on human DC (203) or Jagged 2 on murine DC (148) and decreased expression of the maturation marker CD40 (149) promotes a TH2 phenotype as well. In contrast, pathogens that induce DC production of IL-12 promote TH1 polarization and simultaneously inhibit IL-4 (141).

The chemokine receptor CCR2 contributes to DC recruitment to inflamed tissues. This receptor is necessary for monocyte egress from bone marrow (129); hence lack of CCR2 results in reduced numbers of monocytes and monocyte-derived DC in infected tissues and lymph nodes (92, 115, 117, 133). In some infections and autoimmunity models, CCR2 is required for maintaining a TH1 response. Diminished recruitment of monocyte-derived DC to lymph nodes in influenza infected-CCR2−/− mice results in decreased IL-12 and TH1 cytokine production (135). Impaired recruitment of DC to lungs of C. neoformans infected-CCR2−/− mice is associated with a
detrimental shift from a T\textsubscript{H1} to a T\textsubscript{H2} dominating response (93, 113, 132) and \textit{H. capsulatum} infected-CCR2\textsuperscript{-/-} mice manifest a similar decrease in DC that correlates with elevated IL-4 and impaired immunity despite significant production of T\textsubscript{H1} cytokines (198).

We have investigated whether decreased recruitment of DC to lungs of \textit{H. capsulatum} infected CCR2\textsuperscript{-/-} mice influences regulation of IL-4. Adoptive transfer of BMDC to CCR2\textsuperscript{-/-} mice not exposed to Ag (Ag-free BMDC) did not suppress IL-4 and exacerbated infection. However, transfer of \textit{H. capsulatum} Ag exposed BMDC (Ag-BMDC) suppressed IL-4. Expression of CCR2 or \textit{in vitro} maturation of Ag-BMDC was not required for IL-4 regulation. We investigated the mechanism by which Ag-BMDC decrease IL-4 generation in lungs. Ag-BMDC expression of MHCII was necessary for limiting IL-4 suggesting that suppression was accomplished by interaction with MHCII-restricted CD4\textsuperscript{+} T cells. Furthermore, depletion of CD4\textsuperscript{+} T cells beginning prior to infection reduced IL-4. The immune dampening effects of CD4\textsuperscript{+} T cells were demonstrated in CD4-depleted CCR2\textsuperscript{-/-} mice that received Ag-BMDC. These mice exhibited an increased number of CD8\textsuperscript{+} T cells which was associated with improved fungal burden and survival.

\textbf{Results}

\textit{IL-4 production in lungs of \textit{H. capsulatum}-infected mice subsequent to adoptive transfer of BMDC.} Our previous data demonstrated that elevated IL-4 in lungs of \textit{H. capsulatum}-infected mice deficient in CCR2 or CCL7 and CCL2 impairs yeast clearance (198). Increased IL-4 in these mice was associated with a profound decrease in the proportion of CD8\textsuperscript{-} cDC in lungs. In contrast, IL-4 production, the percentage of CD8\textsuperscript{-} cDC in lungs, and survival was not affected by the absence of CCL2 suggesting that recruitment of DC is important for regulation of IL-4 and infection resolution. To determine if an increased number of DC in lungs
could influence IL-4 generation, we adoptively transferred Ag-BMDC i.n. to CCR2−/− mice prior
to infection (Fig. 13A). Ag-BMDC were fully matured in vitro before transfer with anti-CD40
and LPS to promote pro-inflammatory cytokine-producing BMDC capable of restricting IL-4 as
described previously (148). These cells manifested an upregulation in I-A b, CD40, CD80, and
CD86 (Fig. 14). Transfer of mature WT Ag-BMDC, but not immature Ag-free BMDC,
suppressed IL-4 transcription in lungs (Fig. 13B). CCR2−/− Ag-BMDC also decreased IL-4 in
lungs when transferred to CCR2−/− mice (Fig. 13B). We confirmed that transfer of WT Ag-
BMDC suppressed IL-4 proteins levels in addition to transcription (Fig. 13C).

We questioned if transfer of Ag-BMDC would reduce IL-4 in WT mice that exhibit
relatively low levels of IL-4 in comparison to CCR2−/− mice. IL-4 transcription (log10 RQ ±
SEM) 7 days post infection in lungs of WT mice that received Ag-BMDC (0.56 ± 0.05, n=6) was
decreased (p<0.05) in comparison to WT mice (1.07 ± 0.1, n=6) or WT mice receiving Ag-free
BMDC (1.13 ± 0.16). As a control, we assessed the ability of Ag alone to limit IL-4 and found
that exposure to heat-killed H. capsulatum Ag independent of BMDC had no effect (Fig. 13B).

Requirements for stimulating donor bone marrow-derived cells to suppress IL-4. We
investigated how Ag exposure and maturation contributed to IL-4 regulation. Transfer of WT
BMDC matured with LPS and anti-CD40 to CCR2−/− mice did not reduce (p>0.05) IL-4 (log10
RQ ± SEM) in lungs (2.18 ± 0.12, n=3) in comparison to CCR2−/− controls (1.90 ± 0.19, n=6). In
contrast, Ag-BMDC not fully matured still limited IL-4 transcription (0.80 ± 0.17, n=6). To
determine if BMDC needed to be exposed specifically to H. capsulatum Ag, we treated BMDC
with ovalbumin (ova-BMDC) prior to transfer. Inoculation of CCR2−/− mice with ova-BMDC did
not reduce IL-4 levels (Fig. 13B).
We asked if the *H. capsulatum* soluble Ag cell wall membrane (CWM) (204) would mimic the effect of heat-killed yeast. BMDC exposed to CWM prior to transfer suppressed \( p<0.05 \) IL-4 \( \log_{10} \text{RQ} \pm \text{SEM} \) in lungs of CCR2\(^{-/-}\) mice \( (0.95 \pm 0.10, n=6) \) in comparison to CCR2\(^{-/-}\) mice not receiving BMDC \( (1.72 \pm 0.22, n=6) \). We postulated that the immunodominant *H. capsulatum* Ag Hsp60 (157) may be responsible for IL-4 regulation; however, BMDC exposed to Hsp60 alone could not significantly limit IL-4 generation in lungs of CCR2\(^{-/-}\) mice \( (1.50 \pm 0.18, n=6) \).

Since Mφ also present Ag, we measured the ability of BMDMφ to regulate IL-4. Transfer of WT Ag-BMDMφ to CCR2\(^{-/-}\) mice decreased \( p<0.05 \) IL-4 transcription \( \log_{10} \text{RQ} \pm \text{SEM} \) in lungs \( (0.65 \pm 0.12, n=6) \) in comparison to CCR2\(^{-/-}\) \( (2.26 \pm 0.16, n=6) \) or CCR2\(^{-/-}\) receiving Ag-free BMDMφ \( (2.0 \pm 0.17, n=6) \).

IL-12 production by DC is associated with dampening IL-4 generation (141, 152, 205); therefore IL-4 regulation in CCR2\(^{-/-}\) mice receiving BMDC could be a result of IL-12 production rather than DC-mediated Ag presentation. To address this possibility we measured IL-12 transcription by BMDC prior to transfer. Mature ova-BMDC and mature untreated BMDC that did not reduce IL-4 \textit{in vivo} actually generated the greatest amounts of IL-12 (Fig. 13D).

**Percentage of endogenous and donor BMDC in lung.** An increase in the number of DC in lungs of adoptively transferred CCR2\(^{-/-}\) mice correlated with IL-4 reduction. CCR2\(^{-/-}\) mice that received WT Ag-BMDC manifested an increase in the relative percentage and absolute number of lung DC 7 days post infection whereas transfer of WT Ag-free BMDC did not elevate DC beyond that of CCR2\(^{-/-}\) controls (Fig. 15A and 15B). We asked if the increased number of lung DC were donor Ag-BMDC or host lung DC (Fig. 15C). The percentage of transferred CD45.1 Ag-BMDC or CD45.1 Ag-free BMDC detected in lungs was greatest prior to infection. Between
days 1 and 7 post infection the relative percentage of transferred BMDC to other leukocytes was reduced. At day 7, donor Ag-BMDC in lungs represented less than 0.05% of the total lung leukocytes.

**Phenotype of endogenous CCR2<sup>−/−</sup> lung DC after H. capsulatum infection.** We hypothesized that lung DC in CCR2<sup>−/−</sup> mice may differ functionally from those present in lungs of WT mice, thus exaggerating the dysregulation of IL-4. Our previous studies revealed that MHCII expression is decreased on lung CD8<sup>+</sup> cDC from infected-CCR2<sup>−/−</sup> mice thus at least one phenotypic difference exists (198). Utilizing GFP expressing-*H. capsulatum*, we examined the percentage of infected DC expressing CD40 in lungs of CCR2<sup>−/−</sup> mice. Decreased expression of CD40 can promote T<sub>H2</sub> polarization upon Ag-dependent engagement of CD4<sup>+</sup> T cells (149). The percentage of GFP<sup>+</sup> DC that were CD40<sup>+</sup> (± SEM) in lungs of CCR2<sup>−/−</sup> mice was decreased (p<0.05) at day 1 (21.5 ± 1.2, n=7) and day 3 (61.1 ± 3.9, n=7), but not at day 7 (94.4 ± 1.7, n=8) in comparison to WT (day 1: 53.5 ± 3.4, day 3: 87.29 ± 3.7, day 7: 98.1 ± 1.2, n=7-8).

**Fungal burden in CCR2<sup>−/−</sup> mice immunized with BMDC.** Human DC are capable of degrading *H. capsulatum* yeast *in vitro* (166). This led us to hypothesize that transfer of BMDC may improve clearance of *H. capsulatum* in mice. Like human DC, BMDC restrict the growth of *H. capsulatum in vitro* (Fig. 16). Transfer of Ag-free BMDC had no effect in WT and worsened fungal burden in lungs and spleens of CCR2<sup>−/−</sup> mice (Fig. 17). Unexpectedly, addition of Ag-BMDC did not improve fungal burden in WT or CCR2<sup>−/−</sup> mice (Fig. 17) despite lessened IL-4. We addressed the possibility that exposure to heat-killed *H. capsulatum* had a deleterious effect that negates any Ag-BMDC-mediated yeast clearance since heat-killed yeast suppress Mϕ (60) and DC IL-12 generation (Fig. 13C). Immunization with Ag prior to infection impaired yeast clearance in WT and CCR2<sup>−/−</sup> mice (Fig. 17).
Analysis of infected populations in CCR2−/− and CCR2−/− mice that received BMDC.

One hypothesis to explain increased fungal burden in CCR2−/− mice that received Ag-free BMDC is that the donor cells become infected and support yeast replication, possibly as a result of the elevated IL-4 in lungs. To address this question, we infected mice with GFP expressing-*H. capsulatum* and examined the proportion of infected CD45.1+ donor Ag-free BMDC in lungs of CCR2−/− (CD45.2) mice. At day 1 post infection, an increased percentage of donor Ag-free BMDC are infected in comparison to Ag-BMDC in lungs of CCR2−/− mice (Fig. 18). By day 3, the majority of donor Ag-free BMDC and Ag-BMDC were not infected (Fig. 18).

We investigated how the absence of CCR2 and adoptive transfer of BMDC affected the distribution of yeast among phagocytes since this could help to identify populations providing a reservoir for yeast growth. At day 7 post infection the majority of *H. capsulatum* was associated with DC in lungs of WT or CCR2−/− mice that received Ag-BMDC whereas CCR2−/− mice manifested a decreased proportion of yeast within DC (Fig. 19A). CCR2−/− mice inoculated with Ag-free BMDC exhibited a further reduction in the percentage of yeast distributed within DC (Fig. 19A). We next examined the percentage of DC and CD11c−CD11b+ cells that were infected in the lung. The CD11c−CD11b+ population is comprised of CD11c−CD11b+Ly6Ghi neutrophils and CD11c−CD11b+Mac3+ tissue Mφ (198). We found that the percentage of DC associated with yeast was increased in lungs of CCR2−/− mice immunized with Ag-free BMDC in comparison to WT and CCR2−/− mice (Fig. 19B). The percentage of infected CD11c−CD11b+ phagocytes was increased in CCR2−/− mice in comparison to WT and increased further in CCR2−/− mice that received Ag-free BMDC (Fig. 19B). Additionally, a remarkable increase in the number of infected CD11c−CD11b+ phagocytes was observed in CCR2−/− mice that received Ag-free BMDC in comparison to WT or CCR2−/− mice (Fig. 19C). Both CCR2−/− mice immunized
with Ag-BMDC or Ag-free BMDC exhibited an increase in the absolute number of infected DC in comparison to CCR2<sup>−/−</sup> but not WT mice (Fig. 19C).

Although FACS analysis allowed us to examine the number and types of cells infected, the amount of yeast per cell could vary between infected populations indicating cells in which yeast degradation may be impaired. To determine the number of yeasts per cell we sorted GFP<sup>+</sup> phagocytes from lungs of CCR2<sup>−/−</sup> and WT mice 7 days post infection. Analysis of sorted samples revealed that DC and CD11c<sup>+</sup>Mac3<sup>+</sup> tissue Mφ from CCR2<sup>−/−</sup> mice contained a greater (p<0.05) number of yeast per cell (± SEM) (DC: 3.19 ± 0.18, tissue Mφ: 4.11 ±0.57, n=100 cells), than respective WT populations (DC: 2.08 ± 0.14, tissue Mφ: 1.9 ± 0.18, n=100 cells) whereas the number of yeast within CD11c<sup>+</sup>I-A<sup>bint</sup> alveolar Mφ (CCR2<sup>−/−</sup>: 3.00 ± 0.19, WT: 3.06 ± 0.25, n=100 cells) and neutrophils (CCR2<sup>−/−</sup>: 1.63 ± 0.10, WT: 1.48 ± 0.08, n=100) was similar (p>0.05).

**Cytokine production in lungs of BMDC immunized CCR2<sup>−/−</sup> mice.** We assessed pro-inflammatory cytokine production in lungs of mice receiving BMDC since reduction could contribute to yeast persistence. Transfer of Ag-BMDC resulted in decreased levels of TNF-α and IFN-γ in lungs in comparison to CCR2<sup>−/−</sup> or WT mice 7 days post infection (Fig. 20A). Ag-free BMDC transfer to CCR2<sup>−/−</sup> mice did not alter generation of these cytokines (Fig. 20A). Transcription of IL-10 which is deleterious to host resistance was not modulated in immunized mice and despite the ability of Ag-BMDC to suppress IL-4, transcription of the Th2 cytokine IL-13 was not reduced in lungs of CCR2<sup>−/−</sup> mice that received Ag-BMDC (Fig. 20A).

We examined TNF-α and IFN-γ levels in WT mice given Ag-BMDC to determine if dampening of the Th1 cytokine response was attributable to the absence of CCR2. TNF-α and IFN-γ were not decreased in lungs of WT mice receiving Ag-BMDC or Ag-free BMDC (Fig.
To determine if decreased TNF-α or IFN-γ were an effect of exposure to heat-killed *H. capsulatum* Ag, we measured cytokine production in Ag immunized mice not receiving BMDC. Ag alone did not disturb cytokine production in CCR2<sup>−/−</sup> mice (Fig. 20C).

**DC interaction with CD4<sup>+</sup> T cell is critical for reducing IL-4.** DC interaction with CD4<sup>+</sup> T cells through MHCII-Ag binding to the TCR activates T cells and regulates cytokine production (135, 141, 175). To determine if DC-mediated regulation of IL-4 was dependent on MHCII-Ag presentation we transferred MHCII<sup>−/−</sup>-Ag-BMDC into CCR2<sup>−/−</sup> mice. Ag-BMDC lacking MHCII did not suppress IL-4 (Fig. 21A). We then asked if DC interaction with CD4<sup>+</sup> T cells was necessary for IL-4 restriction. We had previously demonstrated that CCR2<sup>−/−</sup> CD4<sup>+</sup> T cell transcription of IL-4 is greater than that of WT after *H. capsulatum* infection (198). CD4<sup>+</sup> T cell depletion in CCR2<sup>−/−</sup> mice starting at the time of infection did not suppress (p>0.05) IL-4 transcription (log<sub>10</sub> RQ ± SEM) in lungs (CCR2<sup>−/−</sup>: 2.21 ± 0.33, CD4-depleted CCR2<sup>−/−</sup>: 1.97 ± 0.06) or as demonstrated previously, the fungal burden (198). However, if depletion was started prior to infection, IL-4 levels were significantly reduced (Fig. 21A). Subsequently, we examined how quickly CD4<sup>+</sup> T cells are depleted from lungs after mAb treatment in CCR2<sup>−/−</sup> mice. After 24 hours, the number of CD4<sup>+</sup> T cells is reduced by ~50% whereas by 48 hrs > 90% are absent (n=4).

Although CD4-depletion reduced IL-4 in CCR2<sup>−/−</sup> mice, fungal burden was not significantly improved. Conversely, the addition of Ag-BMDC to CCR2<sup>−/−</sup> mice depleted of CD4<sup>+</sup> T cells prior to infection reduced IL-4 and fungal burden in lungs in comparison to untreated CCR2<sup>−/−</sup> mice (Fig. 21A, 21B) and improved survival (Fig 21C). As a control, we investigated if an interaction between DC and CD8<sup>+</sup> T cells regulated IL-4. Depletion of CD8<sup>+</sup> T cells in CCR2<sup>−/−</sup> mice did not reduce IL-4 whereas CD8-depleted CCR2<sup>−/−</sup> mice that received Ag-
BMDC manifested decreased IL-4 in comparison to untreated CCR2<sup>+/−</sup> mice (Fig. 21A). Fungal burden was elevated in CD8-depleted CCR2<sup>+/−</sup> mice immunized with Ag-BMDC in comparison to CCR2<sup>+/−</sup> mice (Fig. 21B).

CD4<sup>+</sup> T cells are critical for resolution of *H. capsulatum* infection (57), thus the finding that fungal clearance was improved in CD4-depleted CCR2<sup>+/−</sup> mice injected with Ag-BMDC was unexpected. We investigated the mechanisms responsible for improved immunity. Transcription of IFN-γ and TNF-α was reduced in CD4-depleted CCR2<sup>+/−</sup> mice that received Ag-BMDC in comparison to CCR2<sup>+/−</sup> mice (Fig. 21A), but decreased fungal burden was associated with an increased number of CD8<sup>+</sup> T cells in lungs (Fig. 21D).

**Discussion**

In this study, we demonstrated that IL-4 production in lungs of *H. capsulatum* infected-CCR2<sup>+/−</sup> mice was reduced sharply by adoptive transfer of *H. capsulatum*-exposed phagocytes. However, transfer of Ag-BMDC did not improve fungal clearance. CCR2<sup>+/−</sup> mice that received Ag-BMDC manifested diminished T<sub>H1</sub> cytokine production which most likely contributed to the inability to reduce fungal burden despite reduced levels of IL-4. In contrast, transfer of Ag-free BMDC did not suppress IL-4 and actually exacerbated infection in lungs and spleens of CCR2<sup>+/−</sup> mice. Increased fungal burden in CCR2<sup>+/−</sup> mice that received Ag-free BMDC was associated with early invasion of Ag-free BMDC and increased infection of CD11c<sup>−</sup>CD11b<sup>+</sup> phagocytes. We determined that Ag-BMDC mediated regulation of IL-4 was dependent on MHCII-Ag presentation to CD4<sup>+</sup> T cells. Furthermore, CCR2<sup>+/−</sup> mice that received Ag-BMDC and were depleted of CD4<sup>+</sup> T cells prior to infection exhibited decreased fungal burden in lungs indicating that CD4<sup>+</sup> T cells inhibit fungal clearance.
The absence of CCR2 results in decreased recruitment of DC to infected tissues in mice exposed to *H. capsulatum* or other organisms (92, 93, 132, 135, 198). In lungs of CCR2−/− mice infected with *C. neoformans* or *H. capsulatum*, elevated IL-4 accompanies decreased DC infiltration. These results imply that the number of DC at the site of infection is important for cytokine regulation (93, 132, 198). Increasing the number of DC in lungs by adoptive transfer of Ag-free BMDC was not sufficient for IL-4 suppression; however transfer of Ag-BMDC to CCR2−/− mice infected with *H. capsulatum* reduced IL-4 indicating that DC must be presenting Ag to regulate cytokine production. The failure of Ag-free BMDC, ova-BMDC or MHCII−/− Ag-BMDC to limit IL-4 further supports the contention that *H. capsulatum* Ag-specific presentation is required. The importance of MHCII expression in this scenario is highlighted by the finding that CD8+ cDC from the lungs of CCR2−/− mice manifest decreased expression of these surface molecules (198). This finding indicates that Ag presentation by the DC may be impaired. In contrast, modulation of IL-4 was independent of IL-12 generation since ova-BMDC and mature BMDC produced the latter yet could not restrict the former. Collectively, the data support the contention that decreased recruitment of DC capable of Ag presentation and altered MHCII expression on those cells that are recruited promotes the type 2 immune profile observed in CCR2−/− mice. Transfer of Ag-BMDMφ reduced levels of IL-4 demonstrating that regulation is not restricted to DC, but the number of alveolar and tissue Mφ in lungs of CCR2−/− mice is not decreased so it is unlikely that insufficient suppression by Mφ is the primary cause of elevated IL-4.

IL-4 regulation by Ag-BMDC was associated with an increased number of lung DC at the peak of infection. Donor Ag-BMDC constituted only a small proportion of lung DC at this time point, hence the majority of lung DC observed at day 7 post infection were host-derived. The
fact that host DC were more prominent may be a result of inefficient transfer or cell death of donor Ag-BMDC before day 7. Alternatively, Ag bearing DC may have trafficked to lymph nodes. Indeed donor CD45.1 DC were detected in mediastinal lymph nodes by day 3 post infection (data not shown). We previously demonstrated that IL-4 neutralization in CCR2\(^{+/−}\) mice does not alter cell recruitment (198), therefore the increased number of DC in lungs of CCR2\(^{+/−}\) mice inoculated with Ag-BMDC cannot be attributed strictly to reduced IL-4. However decreased TNF-\(\alpha\) in lungs of these mice may promote DC survival since this cytokine induces apoptosis during *H. capsulatum* infection (64). An additional possibility is that the increased number of CD8\(^{+}\) T cells promotes DC survival. Some evidence suggests that T cell interaction with mature, Ag-presenting DC is critical for preventing DC death (206).

Expression of CCR2 on Ag-BMDC was not required for limiting IL-4 generation. Previous *in vitro* studies had indicated a role for CCR2 in regulation of IL-4 production by BMDC and BMDC induction of T cell IL-4 (136); however our findings unequivocally demonstrate that any deficiency preventing IL-4 regulation was overcome in our model. The data support the postulation that CCR2 regulation of IL-4 is mediated indirectly through recruited DC.

DC induce IL-4 production by CD4\(^{+}\) T cells and NK cells (148, 152, 153, 203). The DC present in lungs of CCR2\(^{+/−}\) mice exhibit a phenotype consistent with Th2 cytokine instruction. A decreased proportion of CD8\(^{−}\) cDC in lungs of CCR2\(^{+/−}\) mice expressed CD40, a co-stimulatory molecule that restricts IL-4 production by CD4\(^{+}\) T cells (149). DC also exhibit decreased MHCII expression (198) suggestive of a partially decreased maturation state although CD80 and CD86 presentation were not impaired (data not shown). Decreased maturation of DC is associated with increased generation of IL-4 by CD4\(^{+}\) T cells (152). We also demonstrated previously that
CCR2⁺ CD8⁺ cDC transcribe more IL-4 than WT. IL-4 signaling via IL-4Rα receptors on DC promotes DC-mediated T\(_h\)2 polarization (145).

Inoculation with Ag-BMDC limited IL-4 without promoting the T\(_h\)1 immune response in WT and CCR2⁻/⁻ mice. This finding differs from \(L. \) major infection in which transfer of mature, Ag specific BMDC to BALB/c mice decreases IL-4, increases IFN-\(\gamma\), and improves infection (148). Surprisingly, in recipients of Ag-BMDC, IFN-\(\gamma\) and TNF-\(\alpha\), two cytokines that are critical for resolution of \(H. \) capsulatum infection (23, 159), were reduced. This alteration was not observed in the lungs of WT mice that received Ag-BMDC. The decrement in these two pivotal cytokines could explain, in part, why fungal burden was not improved in CCR2⁻/⁻ mice despite a reduction in IL-4. The decrement in TNF-\(\alpha\) and IFN-\(\gamma\) was not caused by T cell mediated suppression. Both CD4-depleted and CD8-depleted CCR2⁻/⁻ mice that received Ag-BMDC manifested a similar reduction in these cytokines.

The exacerbated fungal burden in CCR2⁻/⁻ mice that received Ag-free BMDC implies that elevated IL-4 creates an environment permissive for yeast growth. Uncontrolled yeast replication in untreated CCR2⁻/⁻ mice and CCR2⁻/⁻ mice that received Ag-free BMDC was associated with an increased number of infected CD11c⁺CD11b⁺ phagocytes in comparison to WT. This population contains CD11c⁺Mac3⁺ tissue M\(\phi\) that in CCR2⁻/⁻ mice harbor more yeast per cell than the respective WT population. Tissue M\(\phi\) may be a selective safe haven for yeast cells in the presence of IL-4. The detrimental effects of this cytokine on this population may be a result of alternative activation. In this regard, our previous studies demonstrated that a proportion of M\(\phi\) from infected CCR2⁻/⁻ mice exhibit an alternatively activated phenotype that is associated with impaired fungal clearance and survival of mice (198).
Similar to *H. capsulatum*, transfer of immature BMDC to *C. neoformans* infected mice exacerbates burden (154) demonstrating that donor BMDC can dampen immunity; however, *H. capsulatum* infection differs from *C. neoformans* in that only Ag-free BMDC transfer and not Ag-BMDC to CCR2<sup>−/−</sup> mice resulted in a worsened condition. Unlike transfer of Ag-exposed BMDC to *C. neoformans* infected mice, a further elevation in IL-4 was not observed in lungs and therefore cannot account for the higher fungal burden. Possibly, Ag-free BMDC provide a reservoir for yeast replication early in infection in an environment of excess IL-4. Indeed many of the donor Ag-free BMDC in lungs were infected with yeast 1 day after infection. In addition, CCR2<sup>−/−</sup> mice manifested an increased number of yeast per DC suggesting that DC can promote yeast survival when IL-4 is elevated.

CD4<sup>+</sup> T cells as well as phagocytes contribute to elevated IL-4 in lungs of CCR2<sup>−/−</sup> mice at the peak of infection (198). Our data herein provide evidence that CD4<sup>+</sup> T cells production of IL-4 early during infection promotes production at later time points. IL-4 levels were reduced in CCR2<sup>−/−</sup> mice by depletion of CD4<sup>+</sup> T cells prior to, but not at the time of infection. Complete elimination of CD4<sup>+</sup> T cells required 48 h following mAb administration. Thus, mice treated at the time of infection would still have IL-4-producing CD4<sup>+</sup> T cells that amplify the type 2 response early after exposure to the fungal pathogen. The timing of CD4<sup>+</sup> T cell elimination was critical. The narrow window of 48 hr provided a sufficient stimulus for the propagation and maintenance of the type 2 immune response.

CD4<sup>+</sup> T cells are required for resolution of *H. capsulatum* infection in WT mice (57); thus the finding that Ag-BMDC reduced fungal burden in CCR2<sup>−/−</sup> mice devoid of CD4<sup>+</sup> cells despite dampened IFN-γ and TNF-α production was unexpected. The increased number of CD8<sup>+</sup> T cells in lungs of these mice correlated with control of yeast replication. Although CD8<sup>+</sup>
T cells are dispensable for protective immunity in immunocompetent mice, these cells exhibit Ag-specific cytolytic activity against yeast-infected Mφ and produce IFN-γ and granzyme B which contributes to control of *H. capsulatum* infection in MHCII−/− mice that lack CD4+ T cells (56). Arming of CD8+ T cells to enhance fungal immunity requires cross-presentation by DC (56). Similarly, in a vaccination model, CD8+ cells function to eliminate *H. capsulatum* or *Blastomyces* in a CD4+ T cell-independent manner. (207). We hypothesize that the addition of Ag-BMDC in the absence of CD4+ T results in control of fungal burden due to increased proliferation and activation of CD8+ T cells by Ag-BMDC. Possibly, the removal of CD4+ T regulatory cells, which inhibit CD8+ T cell expansion (208) and *H. capsulatum* clearance (63), increased the number of CD8+ T cells in lungs and improved immunity.

In conclusion, addition of Ag presenting BMDC can suppress excessive IL-4 production by CD4+ T cells in lungs of CCR2−/− mice infected with *H. capsulatum*, thus the reduced recruitment of DC to lungs at least partially impairs IL-4 regulation. The addition of Ag-BMDC in conjunction with the loss of CD4+ T cells improved immunity demonstrating that CD4+ T cells can inhibit infection resolution, possibly by suppressing DC-dependent CD8+ T cell functions. These data highlight the opposing roles of CD4+ T cells in immunity to *H. capsulatum* infection and demonstrate the pivotal functions of DC in regulation of IL-4 and immunity.
Figure 13. Adoptive transfer of Ag-BMDC to CCR2−/− mice suppresses IL-4. Schematic of adoptive transfer experiments (A). Log_{10} relative quantification (RQ) IL-4 transcription from whole lungs 7 days post infection relative to uninfected lung expression. Prior to infection BMDC were untreated (Ag-free BMDC), or matured and exposed to heat-killed *H. capsulatum* (Ag) or ovalbumin (ova-BMDC) (B). (n=6-9). Protein levels of IL-4 in whole lung homogenates were determined by ELISA (C). (n=6). IL-12 transcription by WT BMDC prior to transfer relative to untreated WT BMDC (D). **p=0.002 vs. CCR2−/−, *p<0.05 vs. CCR2−/−, #p<0.05 vs Ag-BMDC not mature. Data represent the mean ± SEM of 2-3 experiments.
Figure 14. BMDC exposure to heat-killed \textit{H. capsulatum} (Ag) and the maturation (mat) stimuli LPS and anti-CD40 results in increased expression of maturation markers. WT and CCR2\textsuperscript{−/−} (KO) BMDC were untreated or exposed to LPS, anti-CD40, and Ag for 4 hr. Infected BMDC were inoculated with a 2:1 ratio of yeast to BMDC. Groups that were treated with maturation stimuli were infected after the 4 hr incubation. Data represent 1 of 3 similar experiments.
Figure 15. IL-4 regulation is associated with an increased number of lung DC. Relative percentage of lung CD11c⁺, CD11b⁺ I-Abhi DC 7 days post infection (A), \((n=8-9)\). Absolute number of lung DC at day 7 (B), \((n=8-9)\). **p<0.001 vs. CCR2⁻/⁻. Data represent the mean ± SEM of 2-3 experiments. Representative FACS plots of isolated lung leukocytes stained with CD11c and CD45.1 to identify transferred donor Ag-BMDC and Ag-free BMDC prior to and at days 1,3 and 7 post infection (C), (CD45.1 transfer experiments were performed 1-2 times with 3-4 mice per group).
Figure 16. *H. capsulatum* (Hc) growth is restricted by BMDC. $1 \times 10^5$ BMDC were infected at a 1:10 or 1:1 ratio of yeast to BMDC. After 24 or 48 hours, samples were plated on media to recover viable yeast. Data represent the mean $\pm$ SEM of 3 experiments.
Figure 17. Transfer of Ag-free BMDC impairs yeast clearance. Fungal burden in lungs and spleens 7 days post infection (A). (n=6). **p<0.001, *p<0.005. Data represent the mean ± SEM of 2 experiments.
Figure 18. The percentage of Ag-free BMDC infected with yeast is increased in comparison to Ag-BMDC early after infection. Representative FACS plots of GFP$^+$CD45.1$^+$ infected donor BMDC at days 1 and 3 post infection, (gated on CD45.1$^+$, $n=3$). *$p<0.05$. 
**Figure 19.** Transfer of Ag-free BMDC to CCR2−/− mice results in an altered distribution of yeast within phagocyte populations. Flow cytometry was performed to determine the relative percentage of lung leukocytes infected with GFP-\textit{H. capsulatum} 7 days post infection (A), percentage of CD11c+, CD11b+, I-A^bhi lung DC infected with GFP-\textit{H. capsulatum} (B), and the percentage of GFP-\textit{H. capsulatum} distributed within DC 7 days post infection (C). \((n=8-9)\). **\(p<0.001\), *\(p<0.05\) vs. WT. ##\(p<0.001\), #\(p<0.05\) vs. CCR2−/−. Data represent the mean ± SEM of 3 experiments.
Figure 20. Transfer of Ag-BMDC suppresses INF-γ and TNF-α production. Log₁₀ cytokine transcription 7 days post infection from lungs of CCR2⁻/⁻ mice immunized with BMDC (A) WT mice immunized with BMDC (B) and CCR2⁻/⁻ mice immunized with Ag (C). (n=6). *p<0.01.
Data represent the mean ± SEM of 2 experiments.
Figure 21. Depletion of CD4+ T cells but not CD8+ T cells prior to infection in CCR2−/− mice that received Ag-BMDC suppresses IL-4 and improves fungal burden. Log_{10} IL-4 transcription in lungs relative to uninfected lung levels 7 days post infection (A). (n=6). Fungal burden in lungs 7 and 14 days post infection (B). (n=6 day 7, n=4 day 14). Graph of survival post infection with 2×10^6 yeast (C). (n=12 except CCR2−/− n=6). Relative percentage and absolute number of CD8+ T cells 7 days post infection (D). (n=6). *p<0.01 vs. CCR2−/−. Data represent the mean ± SEM of 2 experiments.
Chapter 5: Summary and Future Directions

In order to resolve *H. capsulatum* infection, the host must mount a T\(_{H1}\) immune response and limit generation of T\(_{H2}\) cytokines (23, 57, 64, 159, 167). Although chemokines participate in this polarization in several infection models, their role in immunity to *H. capsulatum* infection had not been previously explored. We have demonstrated that the chemokine receptor CCR2 is required for control of yeast replication and protective immunity. CCR2-mediated signaling by the chemokine ligands CCL2 and CCL7 promoted infection resolution by limiting production of the T\(_{H2}\) cytokine IL-4. We investigated the mechanisms by which IL-4 impaired immunity in CCR2\(^{-/-}\) mice and found that elevated IL-4 was associated with alternatively activated Mφ. These Mφ exhibited increased Arg-1 which was detrimental to yeast clearance. IL-4 was produced by CD4\(^+\) T cells in lungs of mice lacking CCR2 early after infection and in addition to CD4\(^+\) T cells, Mφ and DC produced excessive amounts of the cytokine at the peak of infection. Our data provides evidence that Ag presenting DC suppress early IL-4 production by CD4\(^+\) T cells, thus it is likely that the decreased recruitment of DC to lungs of mice lacking CCR2 or both CCL2 and CCL7 contributes to dysregulated IL-4 production (Fig. 22).

Our data was the first to demonstrate a role for CCL7 in conjunction with CCL2 in regulation of IL-4. In fact, CCL7 has been previously reported to increase IL-4 levels at the site of *Leishmania* infection by the recruitment of IL-4 producing cells (104). For our studies, we investigated the impact of loss of CCL7 utilizing a neutralizing monoclonal antibody, thus it will be important to confirm our findings upon the creation of CCL2\(^{-/-}\)CCL7\(^{-/-}\) mice to ensure that IL-4 regulation was a direct result of loss of both CCR2 ligands. If IL-4 is elevated in CCL2\(^{-/-}\)CCL7\(^{-/-}\) mice as expected, we will determine if increased generation is accompanied by decreased CD8\(^+\) cDC recruitment, and if so, can adoptive transfer of Ag-BMDC limit IL-4.
Our data implicates CCL2-CCL7-CCR2 signaling in indirect regulation of IL-4 through recruitment of CD8⁺ cDC to the lung. Decreased DC recruitment is also associated with increased IL-4 in *C. neoformans* infection of CCR2⁻/⁻ mice (132). Nevertheless, CCL2 and CCL7 may also directly limit DC autocrine production of IL-4 and/or DC-induced production by CD4⁺ T cells. In support of this argument, CCR2⁻/⁻ DC have been demonstrated to be functionally different than WT in their ability to promote CD4⁺ T cell production of IL-4 *in vitro*, but the mechanism has not yet been explored (136). Conversely, CCR2⁻/⁻ Ag-BMDC could limit IL-4 production when transferred to CCR2⁻/⁻ mice arguing against a direct role for the receptor *in vivo*. To determine if CCL2 and CCL7 can restrict IL-4 production, we will treat infected-BMDC with CCL2 and CCL7. CCL2 and CCL7 will be also added to co-cultures of Ag-BMDC and Ag-specific T cell clones treated with IL-4 to determine if these cytokines reduce T cell generation of IL-4 in an environment permissive for T cell proliferation and Th2 cytokine production. In addition we can use CCL2⁻/⁻CCL7⁻/⁻ Ag-BMDC in these studies to determine if production of these cytokines by BMDC is critical for IL-4 suppression.

The detrimental impact of excess IL-4 in lungs of CCR2⁻/⁻ mice was evident since all CCR2⁻/⁻ mice in which IL-4 was neutralized survived infection despite impaired inflammatory cell recruitment. Arg-1, a downstream target of IL-4 signaling (187, 209), also reduced fungal clearance, but it remains to be explored if Arg-1 generation is mediated by IL-4 or other factors. These studies could be pursued by examination of Arg-1 in IL-4-deficient CCR2⁻/⁻ mice. If Arg-1 levels are similar to that of WT, then we would conclude that elevated Arg-1 is primarily a result of elevated IL-4. Alternatively, if IL-4-deficient CCR2⁻/⁻ mice manifest increased Arg-1, other mechanisms would need to be explored. For example, *M. tuberculosis* infection of Mφ induces Arg-1 in an IL-4-independent, TLR2-MyD88-dependent manner which promotes
pathogen survival (191). To begin to address the contribution of TLR signaling to \textit{H. capsulatum}-induced Arg-1 production, BMDM\(\phi\) from WT mice and mice lacking the TLR adapter molecule MyD88 could be generated and infected with increasing amounts of yeast. If Arg-1 transcription is reduced in MyD88\(^{-/-}\) BMDM\(\phi\) in comparison to WT, then TLR signaling, possibly due to increased fungal burden in CCR2\(^{-/-}\) mice, may explain the increased induction of Arg-1. To assess the contributions of increased fungal burden to elevated Arg-1 production, WT mice would be infected with a lethal dose of yeast which results in elevated fungal burden that is independent of IL-4.

Although our data indicate that Arg-1 is detrimental to infection when elevated and that lung M\(\phi\) and DC manifest elevated Arg-1, we have not established if production by a specific cell population contributes to impaired yeast clearance. To examine the contribution of M\(\phi\) Arg-1 to impaired fungal clearance, mice that lack Arg-1 expression in M\(\phi\) would be infected with a lethal dose of yeast and fungal burden and survival assessed. If Arg-1 production by M\(\phi\) promotes pathogen persistence, then we would expect improved fungal burden and survival. An exploration of the mechanisms by which elevated Arg-1 impairs immunity also needs to be addressed. Future experiments examining T cell proliferation, NO production by phagocytes, and apoptosis, processes which are modulated by elevated Arg-1 (183, 184, 192, 193, 209, 210), will need to be performed to understand how Arg-1 restriction promotes host immunity.

Inhibition of Arg-1 in CCR2\(^{-/-}\) mice only partially reduced fungal burden in CCR2\(^{-/-}\) mice and did not improve survival (data not shown) thus IL-4 must also impair host immunity by mechanisms independent of Arg-1. One possibility is by inhibition of autophagy. Autophagy is crucial for the clearance of intracellular pathogens and is inhibited by IL-4 (196). A role for autophagy in \textit{H. capsulatum} clearance has not yet been examined. To determine if autophagy
promotes yeast clearance, autophagy could be inhibited in infected IFN-γ-activated peritoneal Mφ (46) and GM-CSF-activated BMDMφ that restrict *H. capsulatum* growth (Winters, M.S., Deepe, G.S. unpublished results) by treatment with rapamycin (211). If yeast growth restriction is dependent on autophagy, then inhibition should promote yeast replication. Sorted Mφ from lungs of WT and CCR2<sup>−/−</sup> mice could also be analyzed for expression of autophagic markers such as LC3 and their co-localization with yeast-containing phagosomes. Another mechanism by which IL-4 may impair immunity in CCR2<sup>−/−</sup> mice is by inhibiting apoptosis of Tregs (197).

Previous studies have linked an increased number of Tregs to increased *H. capsulatum* growth (63). Experiments examining the numbers of Tregs in lungs may indicate an increased number or proportion relative to effector T cells in CCR2<sup>−/−</sup> mice.

At the peak of infection, CD4<sup>+</sup> T cells and phagocytes contributed to excess IL-4 in lungs of CCR2<sup>−/−</sup> mice. Our initial data suggested that CD4<sup>+</sup> T cell may not be a major source of IL-4 since depletion of CD4<sup>+</sup> T cells at the time of infection did not improve fungal burden, however subsequent studies revealed that depletion prior to infection suppressed IL-4 indicating that CD4<sup>+</sup> T cells produce the cytokine early during infection. We have not yet examined how increased IL-4 production by CD4<sup>+</sup> T cells is induced, although we have shown that Ag presenting DC can restrict generation. CD4<sup>+</sup> T cell secretion of IL-4 is often associated with an environment in which IL-4 is already present (144, 151); therefore IL-4 production by another cell type may occur prior to that of CD4<sup>+</sup> T cells. One possibility is that CD4<sup>+</sup> T cell interaction with CCR2<sup>−/−</sup> DC provides a positive signal that promotes IL-4 transcription. In the absence of CCR2, the DC that are present in the lung have a phenotype associated with induction of TH2 cytokines. The percentage of DC in CCR2<sup>−/−</sup> lungs expressing CD40 was decreased in comparison to WT mice which could be indicative of a DC bias to induce CD4<sup>+</sup> T cells to
produce IL-4 since CD40 expression is associated with regulation (149). To determine the impact of CD40 expression on IL-4 generation, administration of activating CD40 antibody to CCR2$^{+/}$ mice could be utilized. Generation of CCR2$^{+/}$ mice that exclusively express MHCII on CD11c cells (212) and CCR2$^{-/-}$ mice that lack MHCII expression of all cells would allow us to determine if DC are involved in early priming of CD4$^{+}$ T cells. If IL-4 production by CD4$^{+}$ T cells remains elevated in mice in which MHCII is restricted to DC and is suppressed in mice that lack MHCII on all cells, then we would conclude that DC-CD4$^{+}$ T cell interaction is necessary and sufficient for IL-4 production. If CD11c expression on DC is not sufficient to drive IL-4 production, yet MHCII expression is required, then Ag-presentation by other cellular sources will be explored. Alternatively, if MHCII expression is not required, another possibility is that NKT cells contribute to early IL-4 production. DC can interact with NKT cells to promote IL-4 production in a CD1d-dependent manner (148).

Another hypothesis is that basophils are an initial source of IL-4 since these cells store IL-4 in granules and thus can secrete IL-4 quickly without having to initiate transcription (213). Many recent studies have demonstrated that DC interaction with CD4$^{+}$ T cells are not sufficient for induction of IL-4 and that basophils are required (174, 199, 202). These same studies revealed for the first time that in addition to IL-4 production, basophils express MHCII and present Ag in an MHCII-restricted manner to CD4$^{+}$ T cells. Since basophils can drive Th2 responses, we will investigate the role of basophils in IL-4 production in CCR2$^{-/-}$ mice. If basophils induce CD4$^{+}$ T cell derived IL-4, than depletion of basophils utilizing the anti-FCεR basophil-depleting antibody MAR1 should reduce IL-4. We could further analyze the ability of sorted basophils from infected mice or WT and MHCII$^{-/-}$ BM-derived basophils (cultured with IL-3) and exposed to Ag to induce Ag-specific T cell proliferation and IL-4 production from T
cells clones. In addition, we would hypothesize that adoptive transfer of BM-derived basophils into WT or CCR2^{-/-} mice would result in increased generation of IL-4 by CD4^{+} T cells if basophils contribute to IL-4 induction.

Due to the detection limits of our IL-4 secretion assay, we were not able to quantitate the number of IL-4 secreting CD4^{+} T cells or other cellular sources of IL-4 initially after infection. Identification of IL-4 producing cells at the onset of infection will be critical for understanding the mechanism by which CD4^{+} T cells produce excessive IL-4 in CCR2^{-/-} mice. To overcome this obstacle, we are utilizing IL-4 reporter mice to generate mice that express the reporter and lack CCR2. The IL-4 reporter mice have been engineered to co-express GFP and IL-4 allowing for more sensitive detection and have been used previously to identify IL-4 producing cells in infected mice (174). Utilization of IL-4 reporter mice will also be advantageous for examining if infection of phagocytes promotes autocrine IL-4 production in vivo. Our data support the hypothesis that IL-4 production by infected phagocytes amplifies the type 2 response in lungs of CCR2^{-/-} mice since many sorted IL-4 secreting phagocytes contained yeast and infection of BMDMφ and BMDC in vitro also induced IL-4. By FACS analysis will be able to examine the number of infected IL-4-producing phagocytes over the course of infection in lungs and immunohistochemistry will allow us to examine location.

IL-4 production was increased specifically in lungs of CCR2^{-/-} mice, not spleens. Why excess IL-4 production is specific to the lungs while there is significant infection in spleens remains to be explored. Utilizing CCR2^{-/-} IL-4 reporter mice, we will examine the trafficking of IL-4 producing cells. These studies will allow us to determine if the IL-4 producing cells originate in the lung, or begin to produce IL-4 at other sites and subsequently migrate to the lung. For example, DC and basophil-mediated polarization of CD4^{+} T cells originates within draining

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lymph nodes followed by trafficking of CD4+ T cells back to the site of infection (174, 199, 201, 202, 212), thus it will be necessary to identify if the same is true in CCR2−/− mice or conversely if IL-4 production is lung specific. Possibly, the absence of IL-4 producing cells in the spleen at the peak of infection is a result of migration of IL-4 producing cells from the spleen to the lung at earlier time points. To visualize leukocyte migration in vivo, we will employ intravital microscopy. This technique allows for the visualization of fluorescently labeled cells in live mice and has been employed to previously visualize interaction and trafficking of DC and T cells in lymph nodes (214). Imaging within the thoracic region is challenging due to the movement of tissues that results from respiration and cardiac function but it can be limited (215).

CD4+ T cells play dual roles in immunity to H. capsulatum infection. On one hand, CD4+ T cells produce large quantities of IFN-γ and are consequently necessary for a protective immune response (57), but on the other hand, specific depletion of CD4+CD25+ T cells from TNF-neutralized mice rescues from mortality. A proportion of CD4+CD25+ cells express the transcription factor Foxp3 indicating that this population contains Tregs (63). Tregs dampen immunity by inhibiting expansion of T cells and by production of IL-10 (63, 118, 208). One hypothesis to explain increased control of infection in CD4-depleted CCR2−/− mice that received Ag-BMDC is that depletion of CD4+CD25+ Tregs resulted in increased Ag-BMDC-induced CD8+ T cell expansion since we did not observe increased IL-10 levels in these mice (Fig. 21A). To address this hypothesis we will need to deplete CD25+ cells in conjunction with addition of Ag-BMDC to CCR2−/− mice to determine if it is the depletion of Tregs that mediates improved immunity in this scenario. To further confirm the suppressive effects of this population we can adoptively transfer Tregs isolated from infected CCR2−/− mice to MHCII−/− mice prior to infection. If this population can suppress immunity, than MHCII−/− mice should exhibit exacerbated fungal
burden. The ability of MHCII\(^{-/-}\) mice to control infection is dependent on CD8\(^{+}\) T cell expansion and function, thus if Tregs dampen immunity it may be more evident in MHCII\(^{-/-}\) mice although we may observe an altered phenotype upon transfer to WT mice as well. We will also need to further investigate the role of CD8\(^{+}\) T cells in CCR2\(^{-/-}\) mice that received Ag-BMDC. We have demonstrated that these mice manifest an increased number of CD8\(^{+}\) T cells in lungs but we have not investigated if this is a result of increased proliferation, survival, or recruitment. We will also examine the mechanisms by which CD8\(^{+}\) T cells contribute to control of infection by analyzing granzyme B production and \textit{ex vivo} cytolytic activity against infected BMDM\(\phi\) in comparison to CD8\(^{+}\) T cells from WT and CCR2\(^{-/-}\) mice. CD8\(^{+}\) T cells have been demonstrated to cross-present \textit{H. capsulatum} Ag acquired from apoptotic M\(\phi\) to CD8\(^{+}\) T cells via MHCI receptors to activate the cytolytic activity of CD8\(^{+}\) T cells (56). We can further confirm the role of CD8\(^{+}\) T cells by depletion of both Tregs and CD8\(^{+}\) T cells in CCR2\(^{-/-}\) mice that received Ag-BMDC. We would expect that these mice would not be able to control infection.

Adoptive transfer of Ag-BMDC was critical for limiting IL-4 generation in \textit{H. capsulatum}-infected CCR2\(^{-/-}\) mice. Since we have demonstrated that CCR2\(^{-/-}\) mice manifest decreased recruitment of DC to lungs, the few DC that are recruited have decreased MHCII expression, and the DC harbor an increased number of yeast per cell, we postulate that elevated IL-4 in lungs of CCR2\(^{-/-}\) mice stems from the decreased recruitment of DC capable of degrading and presenting Ag. It will be necessary to determine if Ag presentation by DC is impaired in lungs of CCR2\(^{-/-}\) mice. \textit{Ex vivo} culture of DC from CCR2\(^{-/-}\) mice with ova-specific CD4\(^{+}\) T cells in the presence of ova or infection of isolated DC following co-culture with \textit{H. capsulatum} Ag-specific T cell clones would provide further support that Ag presentation is impaired if the DC elicit reduced Ag-dependent T cell proliferation. Our analysis of T cell proliferation in CCR2\(^{-/-}\).
mice provides further evidence that CCR2\(^{-/-}\) may exhibit defects in Ag presentation. The decreased number of CD4\(^{+}\) T cells in lungs of CCR2\(^{-/-}\) mice could be a result of decreased DC-Ag induced T cell proliferation.

Adoptively transferred BMDC limited generation of IL-4 through presentation of a component of the *H. capsulatum* cell wall or cell membrane. We did not test secreted yeast Ag or cytosolic components to determine if other Ag can limit IL-4 generation by DC when presented. Presentation of the *H. capsulatum* Ag Hsp60 was not sufficient for BMDC suppression of IL-4 although Hsp60 is critical for inducing IFN-\(\gamma\) production (157). Screening of yeast Ag involved in IL-4 regulation *in vivo* will expand our knowledge of how the host limits production of \(T_{H2}\) cytokines and identification of components of the yeast that suppress \(T_{H2}\) responses could promote vaccine-induced immunity. For example, addition of *M. tuberculosis* culture filtrate proteins to immunized mice promotes protection by inhibiting IL-4 secretion (216).

In addition to mice lacking CCR2, elevations in IL-4 arise in *H. capsulatum*-infected mice deficient in TNFR1, GM-CSF, or in which apoptosis has been inhibited (64, 65, 179). Decreased numbers of DC have not been reported in these models, but the end result is similar to that of CCR2\(^{-/-}\) mice, impaired immunity. One hypothesis is that impaired Ag presentation by DC in these other infection models results in increased production of IL-4 by CD4\(^{+}\) T cells which would indicate a central role for DC in regulation of the cytokine in *H. capsulatum* infected mice. Conversely, if elevated IL-4 is independent of DC it would still be important to identify the mechanisms responsible for the dysregulated cytokine production since they remain unexplored. It is also not known if elevations in IL-4 cause alternative MΦ activation and elevated Arg-1 in these mice that express CCR2 but exhibit impaired infection resolution.
Additional studies of the role of chemokines in mice and humans will enhance our understanding of how the host resolves acute and latent *H. capsulatum* infection. The role of chemokines in mediating protective immunity to *H. capsulatum* in humans is unknown. The importance of study of chemokines in this infectious disease is highlighted by the recent wave of cases of disseminated histoplasmosis in those receiving TNF antagonists. Since TNF can modulate chemokine generation (217), perhaps one of the underlying immune deficiencies is an alteration in chemokine generation rather than a direct effect of TNF. The role of chemokines in controlling infection begins with an understanding of the mouse. In that way, we can eventually move to ask clinically relevant questions regarding the human condition. Our studies addressing the mechanisms by which CCR2 directs immunity are the beginnings to understanding how chemokine signaling contributes to host defense against *H. capsulatum* infection.
Figure 22. Model of IL-4 regulation vs. IL-4 overproduction in lungs of WT and CCR2\(^{-/-}\) mice. We hypothesize that IL-4 regulation occurs in both the lungs and draining mediastinal (MLN) lymph nodes of WT mice whereas further studies will need to be performed to determine if increased production of IL-4 in lungs of CCR2\(^{-/-}\) mice is a result of dysregulation in lungs or trafficking of IL-4 producing cells from other tissues.
Figure 23. Proposed mechanism by which fungal burden is controlled in CD4-depleted CCR2−/− mice that received Ag-BMDC. We hypothesize that addition of Ag-BMDC in the absence of CD4+ Tregs promotes MHCI-mediated cross-presentation of fungal Ag to CD8+ T cells to induce expansion and cytolytic activity against infected target cells.
Chapter 6: Materials and Methods

Mice

Male C57BL/6 and B6.SJL-Ptprc (CD45.1) mice and breeding pairs of CCR2\(^{-/-}\), CCL2\(^{-/-}\), and IL-4\(^{-/-}\) mice on a C57BL/6 background were purchased from Jackson Laboratories (Bar Harbor, ME). CCR2\(^{-/-}\) and CCL2\(^{-/-}\) mice were backcrossed > 9 generations. Animals were housed in isolator cages and were maintained by the Department of Laboratory Animal Medicine, University of Cincinnati, which is 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* yeast strain G217B was grown for 72 h at 37\(^{\circ}\)C as previously described (156). To produce infection in mice, animals were inoculated intranasally i.n. with 2 x 10\(^{6}\) *H. capsulatum* yeast cells in a ~30 \(\mu\)L volume of Hanks Balanced Salt Solution (HBSS) (Hyclone, Logan, UT).

Organ Culture for H. capsulatum

Organs were homogenized in sterile HBSS and serially diluted and plated onto Mycosel agar (Becton Dickinson) plates containing 5% sheep blood and 5% glucose. Plates were incubated at 30\(^{\circ}\)C for 1 week. The limit of detection was 1 x 10\(^{2}\) CFU.
In vivo neutralization of IL-4, CCL7, CCL12, and CD4⁺ T or CD8⁺ T cell depletion

For neutralization of IL-4, mice were injected i.p. with 1 mg of rat anti-mouse IL-4 (clone 11B11) or 1 mg of rat IgG1 control antibody dissolved in 1 ml of HBSS at the time of infection. For survival studies, mice were injected additionally at one-week intervals. For CCL7 and CCL12 neutralization, 100 µg of polyclonal goat anti-mouse CCL7 and/or anti-CCL12 (R&D Systems, Minneapolis, MN) or goat IgG in 0.5 ml HBSS was administered i.p. at the time of infection and 3 days post infection. For CD4⁺ or CD8⁺ T cell depletion, mice were given 100 µg of anti-CD4 (clone GK1.5) or anti-CD8 (clone 2.43) in 500 µl HBSS at the time of infection for experiments in which depletion was begun prior to infection antibody was administered at 7 days, 5 days and 3 days prior to infection as well as at the time of infection. Mice that were sacrificed at time points later than day 7 were given an additional dose of mAb at day 6 or 7 post infection. Treatment resulted in > 95% depletion of CD4⁺ or CD8⁺ T cells from WT and CCR2⁻/⁻ mice at day 7.

In vivo administration of L-arginine, D-arginine, and BEC

L-arginine or D-arginine (Sigma Aldrich, St. Louis, MO) at a concentration of 8.7 mg/500 µl HBSS or 500 µl of a 30 mM BEC ((S)-(2-Boronoethyl)-L-cysteine, HCl) solution, (Calbiochem, San Diego, CA) was given i.p. to WT and CCR2⁻/⁻ mice at day 0, 1, 3, 5, and 7 post infection.

Isolation of Lung Leukocytes

Lungs were homogenized in HBSS using a gentleMACS Dissociator (Miltenyi Biotec, Auburn, CA). The solution was filtered through 60 µm nylon mesh (Spectrum Laboratories Inc., Rancho Dominguez, CA) and washed with HBSS. Leukocytes were isolated by Lympholyte M (Cederlane Laboratories, Ontario, Canada) separation.
**Flow Cytometry**

The phenotype of cells from mouse lungs was determined by incubating lung leukocytes with the indicated antibodies and CD16/32 to limit non-specific binding. Leukocytes were stained at 4°C for 15 min in HBSS containing 1% BSA and 0.01% sodium azide. Cells were stained with combinations of the following antibodies: FITC-conjugated CD4, CD8, Ly6C, Mac-3, I-A<sup>b</sup>, CD69, and NK1.1; PE-conjugated CD11c, I-A<sup>b</sup>, TR, CD40, CD80, CD86, Ly6G, CD3 and Mac3; PerCP-conjugated CD11b and CD4; and APC-conjugated CD11c, CD3, and CD25, CD45.1 from BD Biosciences (San Jose, CA). PE-conjugated Dec205 was purchased from Miltenyi Biotec (Auburn, CA). FCεRI-FITC was a kind gift from Dr. Fred Finkelman at Cincinnati Children’s Hospital. Cells were washed and resuspended in 1% paraformaldehyde to fix. Appropriate isotype controls were performed in parallel. Fluorescence intensity was assessed using a FACSCaliber (BD Biosciences) flow cytometer and analyzed using FCS Express Software. Intracellular staining for IFN-γ was performed subsequent to *ex vivo* stimulation of 5x10<sup>5</sup> cells per well in a 96 well plate with 20ng/ml PMA, 1μg/ml ionomycin, and 1μg/ml brefeldin A for 4 hours. Surface stained cells were permeabilized in Cytofix/Cytoperm (BD Biosciences) for 20 minutes, washed in Permwash buffer (BS Biosciences), and stained with PE-conjugated IFN-γ for 30 minutes.

**Histology**

Lungs were inflated, excised, fixed in 10% formalin and embedded in paraffin blocks. Sections (5μm) were stained with hematoxylin and eosin. Analysis of the sections was performed in a blinded fashion.

**FACS Sorting of Lung Leukocyte Populations**
To enrich for specific lung leukocyte populations, cells were stained with the indicated antibodies as described above and sorted in HBSS using a FACSVantage (BD Biosciences) cell sorter. Isolated cells exhibited greater than 95% purity.

**RNA Isolation and cDNA synthesis**

Total RNA from whole lungs was isolated from mouse lungs using TRIzol (Invitrogen, Carisbad, CA). To isolate RNA from sorted populations containing low cell numbers, the Micro RNAEasy kit (Qiagen, Valencia, CA) was utilized according to the manufacturer’s instructions. Oligo(dT)-primed cDNA was prepared by using the Reverse Transcriptase System (Promega, Madison, Wisconsin).

**Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)**

qRT-PCR for cytokine transcription analysis was performed using Taq-Man Master Mix and primers from Applied Biosystems (Foster City, CA). Samples were analyzed on an ABI Prism 7500 (Applied Biosystems). In each experiment, the housekeeping gene hypoxanthine phosphoribosyl transferase was used as an internal control. The conditions for amplification were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min.

**Measurement of Cytokines by ELISA**

Lungs were homogenized in 5 ml of HBSS and centrifuged. Cytokines in homogenates were quantified by ELISA. IL-4, TNF-α, IFN-γ, IL-1β and GM-CSF ELISA's were purchased from Endogen (Cambridge, MA) and IL-10 and IL-12 ELISA kits were purchased from R&D Systems (Minneapolis, MN).
Measurement of NO

Isolated lungs leukocytes from mice were plated ex vivo at 5x10^5 cells per well of a 96 well plate in DMEM supplemented with 10% FBS (Hyclone). Leukocytes were stimulated with 1μg/ml LPS and 100ng/ml IFN-γ for 24 hours. Measurement of NO reaction products nitrate and nitrite were determined by the Nitrate/Nitrite Assay (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions.

IL-4 Secretion Assay

An IL-4 secretion assay and detection kit (Miltenyi Biotec) was used according to the manufacturer's instructions. Leukocytes isolated from the lung were restimulated ex-vivo in a 96-well plate at 1.5 x 10^6 leukocytes/200 μl media for three hours in the presence of 20 ng/ml PMA and 1 μg/ml ionomycin. Cells were then incubated an additional 45 minutes with IL-4 capture antibody at 37° C. Leukocytes were incubated with anti-IL-4-PE and the indicated additional antibodies before enrichment for IL-4 secreting cells using anti-PE microbeads and an LS column (Miltenyi Biotec). Unstimulated lung leukocytes were used as negative controls. Recovered cells were analyzed using a FACSCalibur flow cytometer or sorted with a FACSVantage cell sorter as described above.

Generation of BMDMφ and BMDC

Bone marrow was isolated from the hind tibia and femurs of 5-6 week old mice by flushing with HBSS. Isolated cells were plated at 2x10^5 cells/ml in 50ml of RPMI-1640 supplemented with 10% fetal bovine serum, 0.1% gentamicin sulfate, and 0.005% 2-betamercaptoethanol. Cells were treated with 10ng/ml GM-CSF (Endogen) to stimulate differentiation and cultured at 37°C in 5% CO₂. Bone marrow cells were fed with an additional 50ml of media and 10ng/ml GM-
CSF at day 3 and day 6 after isolation. At day 8-10, the non-adherent CD11c⁺ BMDC were enriched for with CD11c⁺ microbeads (Miltenyi Biotec). Enriched cells were >80% CD11c⁺. To obtain BMDMφ, the adherent cells were treated with 5ml of trypsin-EDTA for 5-10 min at 37°C and scraped unidirectionally with a cell scraper after 6-8 days of culture.

Adoptive Transfer of BMDMφ and BMDC

BMDMφ and BMDC from WT or CCR2⁻/⁻ mice were isolated as described above. At days 7, 5, and 3 before infection 1-2 x 10⁶ BMDMφ or BMDC were administered i.n. in ~30µl of HBSS to CCR2⁻/⁻ mice. For experiments in which CCR2⁻/⁻ mice were given matured and Ag exposed BMDC or BMDMφ, the cells were treated with 5µg/ml anti-CD40 (clone 3/23, BD Pharmingen) and 1µg/ml LPS to induce maturation plus heat-killed *H. capsulatum* yeast at a ratio of 8 yeast per DC for 4 hours. For experiments in which CWM, HSP60, or ova were used as a source of Ag, a concentration of 5ug/ml was used. CWM and HSP60 from *H. capsulatum* was prepared as previously described (157, 204) and ova was purchased from Sigma Aldrich.

Statistics

ANOVA was used to compare multiple groups while student's T test was used to compare two groups. Survival was analyzed using log rank. p<0.05 was considered statistically significant.
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