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Unraveling the IL4-IL33 nexus in *Histoplasma capsulatum* infection

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THESIS ABSTRACT

Exaggerated synthesis of type II cytokines subverts host immunity in multiple intracellular infections. This scenario is effectively demonstrated in CCR2\(^{-/-}\) animals that have been exposed to the intracellular fungal pathogen *Histoplasma capsulatum*. The infected mutant mice mount an amplified and sustained IL-4 response that is associated with impaired fungal clearance. We investigated the cellular source of IL-4 and the reason for dysregulated immunity in *H. capsulatum*-challenged CCR2\(^{-/-}\) mice. Our findings reveal eosinophils were the prominent contributors of non-protective IL-4 and depleting this cell population ameliorated fungal infection in CCR2\(^{-/-}\) animals. This immune defect was a result of CCL7 engaging chemokine receptors preferentially expressed on eosinophil surface. An unexpected finding in CCR2\(^{-/-}\) mice was that elevated IL-4 prompted IL-33 generation in pulmonary macrophages. Dissection of mechanisms that promulgated the latter cytokine revealed IL-4 and *H. capsulatum* synergistically elicited an IL-33 response in macrophages via STAT6/IRF-4 and Dectin-1 pathways respectively. Furthermore, this IL-4-driven IL-33 response compromised antifungal defenses by driving an alternatively activated phenotype in phagocytes.

The molecular interaction between eosinophils and *H. capsulatum* has not been explored. We discovered that eosinophils phagocyted *H. capsulatum* yeasts and this biological process was dependent on the pattern recognition receptor CR3. Internalization of fungi induced a robust IL-4 response that was conserved in both murine and human eosinophils. These observations reveal an unappreciated attribute of eosinophil biology that could potentially trigger a collapse in antifungal immunity in the host.
In summary, the investigation in this work provides novel insights into how type II immune responses are shaped and their detrimental effects in the setting of an intracellular fungal infection.
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ABBREVIATIONS

Ag, antigen
AM, alveolar macrophages
Arg-1, arginase 1
BM, bone marrow derived macrophages
CCL, chemokine (C-C motif) ligand
CCR, chemokine receptor
CD, cluster of differentiation
CFU, colony forming unit
CR3, compliment receptor 3
DCs, dendritic cells
GFP, green fluorescent protein
GM-CSF, granulocyte macrophage-colony factor
H. capsulatum, Histoplasma capsulatum
HPRT, hypoxanthine-guanine phosphoribosyltransferase
hr, hour(s)
IFN, interferon
IL, interleukin
IL-4R, interleukin-4 receptor
i.n. intranasal(y)
i.p. intraperitoneal(ly)
MFI, mean fluorescence intensity
MHC, major histocompatibility complex
NK, natural killer
NO, nitric oxide
Ova, ovalbumin
PAMP, pathogen-associated molecular pattern
PM, peritoneal macrophages
PRR, pattern recognition receptor
RT-PCR, real time polymerase chain reaction
SEM, standard error of the mean
STAT, signal transducers and activators of transcription
TGF-β, transforming growth factor-β
TLR, toll-like receptor
WT, wild type
CHAPTER 1

Introduction
**Histoplasma capsulatum**

*Histoplasma capsulatum* is a dimorphic fungal pathogen and the etiologic agent of histoplasmosis. The fungus exists in its saprophytic mycelial state in soil enriched with bird or bat guano, and transforms into its parasitic yeast phase inside a mammalian host\(^1\). *H. capsulatum* is found worldwide but is highly prevalent in Midwestern and Southeastern USA, and several parts of Brazil. The strain variants of the fungus are *H. capsulatum*, *H. duboisii*, and *H. farciminosum* of which, *H. capsulatum* is the most prevalent in North America and therefore extensively studied\(^1\). Epidemiological data indicate that millions of individuals residing in the United States have encountered *H. capsulatum* infection at some part of their lifetime; a million new infections occur annually across the globe; over 90% of the population living in endemic areas has been exposed to the fungus\(^2\). The fungal infection is resolved spontaneously in persons that have an intact immune system, however, immunocompromised individuals often exhibit life threatening disseminated disease.

The mycelial to yeast transformation is required for successful establishment of *H. capsulatum* infection. Conversion of inhaled microconidia to pathogenic yeast is triggered in response to a shift in temperature from 25 to 37 °C. The body temperature of the mammalian host activates a number of genes required for yeast-phase growth and virulence\(^4\). The transcription factors RYP-1 (required for yeast phase), RYP-2 and RYP-3 control the molecular circuit in *H. capsulatum* that switches from filamentous form to the pathogenic yeast phase. Expression of another gene, DRK-1 (dimorphism regulating kinase-1) is critical for transforming *H. capsulatum* mycelia to virulent yeasts within the host. Mutation in either the RYP factors or DRK-1 locks the fungus in its avirulent filamentous state.
Inside the mammalian host, *H. capsulatum* yeasts are internalized by phagocytes including macrophages, neutrophils, and dendritic cells\(^3\)\(^-\)\(^5\). Phagocytosis of *H. capsulatum* is largely mediated by the pattern recognition receptor CR3\(^6\). Another innate receptor, Dectin-1 detects beta-glucan on the fungal cell wall and initiates downstream signaling events. Dectin-1, however does not participate in internalization of *H. capsulatum* yeasts\(^7\). While dendritic cells and neutrophils are efficient at arresting the growth of intracellular *H. capsulatum*, resting macrophages offer a safe niche for fungal proliferation. The pathogen employs multi-pronged strategies to survive within phagolysosomes of macrophages. These include regulating pH of the intraphagosomal compartment, modulation of phagolysosomal fusion, and iron acquisition from ferritin\(^5\),\(^8\). Infected phagocytes serve as vehicles to spread yeast cells to other peripheral organs including liver, spleen, bone marrow, and lymph nodes. Stimulation of macrophages with proinflammatory cytokines such as IFN-\(\gamma\) or GM-CSF is critical for arresting intracellular growth\(^9\),\(^10\). Eventually, activation of T\(\text{H}1\) immunity is requisite for effective resolution of infection. A hallmark of histoplasmosis is development of granulomas in infected organs. These microscopic structures presumably encompass the fungus to cease its growth and prevent further dissemination. Conversely, granulomas may be advantageous for the pathogen by serving as centers of latent infection and reactivation under immunocompromised conditions\(^11\).

**Host immunity to *H. capsulatum***

Engagement of both innate and adaptive arms of the immune system is essential for successful control of *H. capsulatum* infection. Dendritic cells, neutrophils and classically activated macrophages inhibit intracellular growth of the pathogen. These innate cells also generate IL-12,
a cytokine central for T\textsubscript{H}1 lineage commitment. Both CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells influence the outcome of \textit{H. capsulatum} infection\textsuperscript{12}. The former are a prominent source of IFN-\gamma and increased mortality is observed in mice lacking this subset of T cells during primary infection. In contrast, CD8\textsuperscript{+} T cells are a redundant source of IFN-\gamma and depletion of this cell population does not affect the survival of mice during primary infection. In secondary infection, mice that lack either CD4\textsuperscript{+} or CD8\textsuperscript{+} T cells successfully survive re-challenge, while animals depleted of both T cell subsets succumb to fungal infection. Immunoglobulins secreted by B cells serve as an additional layer to strengthen host antifungal defenses, however, absence of these lymphocytes in infection does not impact survival of animals\textsuperscript{12}.

\textbf{Cytokine responses in \textit{H. capsulatum} infection}

(i) IFN-\gamma

IFN-\gamma is the signature cytokine of T\textsubscript{H}1 immune response and is crucial for host protection in histoplasmosis. Its importance in combating \textit{H. capsulatum} infection is evident from studies indicating enhanced susceptibility to the pathogen in mice lacking IFN-\gamma\textsuperscript{13} and in a patient with genetic deletion mutation in the IFN-\gamma receptor-1 gene\textsuperscript{14}. Activation of murine macrophages with IFN-\gamma primes the cells to be more fungistatic. The cytokine is thought to inhibit intracellular growth of yeasts by stimulating the production of nitric oxide from arginine in macrophages\textsuperscript{15}. Another potential mechanism by which IFN-\gamma could regulate fungal replication is by limiting the amount of iron accessible to \textit{H. capsulatum}\textsuperscript{9}.
(ii) TNF-α

The proinflammatory cytokine TNF-α is also required for development of protective immunity to *H. capsulatum*. It shares several redundant functions with IFN-γ, including classical activation of macrophages and generation of intracellular nitric oxide to inhibit fungal growth\(^\text{16}\). TNF-α is synthesized by both hematopoietic and non-hematopoietic cells and signals via receptors TNFR-1 or TNFR-2. Signaling through both these receptors is critical for host survival during primary *H. capsulatum* infection, while only TNFR-1 activation is adequate to counter secondary infection\(^\text{17}\).

TNF-α also dictates the immune response to *H. capsulatum* by regulating the emergence of regulatory T cells (Tregs)\(^\text{18}\). Treatment of mice with TNF-α-neutralizing antibody results in elevated frequency of Tregs in the lungs in primary and secondary infection. In this setting, Tregs weaken antifungal host resistance by releasing IL-10. Clinical manifestation of this scenario is observed in patients suffering from inflammatory diseases such as rheumatoid arthritis, Crohn’s disease, psoriasis etc, who are subjected to treatment with TNF-α antagonists. An undesired consequence of this therapy is the augmented frequency and suppressor activity of Tregs that in turn have been linked to exacerbating histoplasmosis\(^\text{19}\).

(iii) GM-CSF

GM-CSF is another type I cytokine that orchestrates protective effects on the host in *H. capsulatum* infection. Neutralizing the cytokine during primary infection increases vulnerability to the disease and causes a significant proportion of animals to succumb to the pathogen\(^\text{20}\). This enhanced susceptibility to histoplasmosis is a result of depressed expression of proinflammatory cytokines IFN-γ and TNF-α, and a concomitant increase in IL-4 and IL-10. Absence of GM-
CSF during secondary exposure aggravates infection but does not alter host survival\textsuperscript{20}. The cytokine mediates its antifungal responses through sequestration of intracellular zinc from yeasts and stimulation of reactive oxygen species in macrophages\textsuperscript{21}.

(iv) IL-4

The T\textsubscript{H}2 cytokine, IL-4, is associated with weakening host resistance to \textit{H. capsulatum}. Leukocytes including eosinophils, mast cells, basophils, NKT cells, and group 2 innate lymphoid cells (ILC2s) have been implicated as important sources of innate IL-4, while T\textsubscript{H}2 cells are prominent producers of the cytokine in the adaptive immune phase\textsuperscript{22}. IL-4 signals through the IL-4R\textgreek{a} - \gamma\textsubscript{C} complex to activate downstream transcription factors including STAT6 and IRF-4. Excessive generation of this cytokine delays fungal elimination from infected tissues in mice\textsuperscript{23}. Intriguingly, overexpression of IL-4 during \textit{H. capsulatum} infection does not result in an allergic immunopathology\textsuperscript{23}.

The mechanisms by which IL-4 dampens antifungal immunity are multifactoral. In addition to disturbing the T\textsubscript{H}1/T\textsubscript{H}2 balance, IL-4 skews the macrophages to an alternatively activated phenotype. These IL-4-primed phagocytes offer a conducive environment to support intracellular fungal growth, in comparison to classically activated macrophages\textsuperscript{10}. Exposure of macrophages to IL-4 induces arginase-1, an enzyme that potentially diminishes the amount of nitric oxide required for fungicidal activity\textsuperscript{24}. Furthermore, IL-4 modulates fungal access to specific micronutrients in macrophages. The cytokine upregulates transferrin receptor (TR) on cell surface that results in enhanced iron acquisition\textsuperscript{25}. This phenomenon is believed to augment yeast growth within the cells. IL-4 also boosts intracellular zinc stores within macrophages to support \textit{H. capsulatum} proliferation\textsuperscript{10}.
Exaggerated synthesis of IL-4 subverts host resistance in other mycotic infections as well. Failure to regulate the cytokine in cryptococcosis interferes with pathogen clearance, and on rare occasions can result in a fatal outcome\textsuperscript{26,27}. In aspergillosis, undesired activation of T\(_{H2}\) responses elicits non-protective allergic inflammation in mice\textsuperscript{28}. These animals demonstrate eosinophilia, goblet cell hyperplasia, and heightened susceptibility to the pathogen. A sharp contrast is observed in pneumocystosis where IL-4 and T\(_{H2}\) responses confer protection to the host. In this setting, alternatively activated macrophages are speculated to inhibit fungal growth since these phagocytes have been shown to have fungicidal activity against \textit{Pneumocystis murina}\textsuperscript{29}.

\textbf{(v) IL-33}

IL-33 is an innate type II cytokine and member of the IL-1 family. Although the cytokine orchestrates pleotropic effects on immune cells, it is best known for instigating and shaping T\(_{H2}\) immune responses. IL-33 mediates its effects by binding to ST2L/IL-1RAcP heterodimer that is expressed on several innate and adaptive immune cells\textsuperscript{30}. Binding of the ligand to the receptor triggers recruitment of adapter molecule MyD88 to ST2L that in turn activates downstream NF-\kappaB and MAP kinase pathways. Stimulation of basophils, eosinophils, group-2 innate lymphoid cells (ILC2s), and CD4\(^+\) T cells with IL-33 promotes the generation of T\(_{H2}\) cytokines\textsuperscript{30}. In macrophages, IL-33 skews the cells to an alternatively activated phenotype\textsuperscript{31}. In addition to its extracellular stimulatory properties, IL-33 has an intracellular immunoregulatory function. The protein has been implicated in controlling expression of NF-\kappaB target genes by serving as a nuclear factor\textsuperscript{32}. 

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Several diverse functions of IL-33 in altering immune responses to microbial pathogens have been reported. The cytokine is critical for restoring tissue homeostasis in influenza A virus\textsuperscript{33} and *Pseudomonas aeruginosa* infection\textsuperscript{34}. In the former infection, IL-33 stimulates ILC2s to release anti-inflammatory cytokines to enable tissue repair, while in the latter, IL-33 shifts macrophages to an alternatively activated phenotype to promote resistance against keratitis. A unique role for IL-33 has been described in driving protective antiviral CD8\textsuperscript{+} T cell responses against lymphocytic choriomeningitis virus (LCMV)\textsuperscript{35}. In this setting, IL-33 induces clonal activation of CD8\textsuperscript{+} T cells to control viral replication. The cytokine has been shown to have beneficial effects in certain fungal infections. IL-33 increases phagocytosis and killing activity of neutrophils in candidiasis\textsuperscript{36}. In pneumocystosis, the cytokine augments fungicidal capacity of alveolar macrophages\textsuperscript{29}.

The significance of IL-33 in histoplasmosis has not been studied. Another area that lacks clarity is the mechanisms regulating the induction of IL-33 in fungal infections. It is therefore compelling to study the influence of this cytokine on host resistance against *H. capsulatum* and the regulatory check points in place to modulate IL-33 synthesis.

**Protective role of CCR2 in *H. capsulatum* infection**

Trafficking of inflammatory cells to the site of infection is controlled by several mediators including chemokines. These soluble factors are small molecules (8-12 kDa) that prompt chemotaxis, activation of leukocytes, cytokine generation, and T cell differentiation by engaging their cognate receptors. The chemokine receptor CCR2 dictates migration of myeloid lineage cells by associating with its two major ligands; CCL2, and CCL7\textsuperscript{37}. Although CCR2 is expressed
on several leukocyte populations, it is best known for driving inflammatory monocytes from bone marrow to the site of infection\textsuperscript{38}.

In histoplasmosis, a protective role for CCR2 and its ligands CCL2, and CCL7 has been elucidated\textsuperscript{39}. Animals deficient in CCR2 display dampened immunity to the pathogen and are unable to resolve infection as efficiently as WT controls. Although reduced influx of inflammatory monocytes contributes to host susceptibility, the primary immune defect in these mutant mice is an exaggerated IL-4 response generated in the lungs during infection. Neutralization of IL-4 restores protective immunity, but fails to correct the leukocyte recruitment defect in these mice. The phenotype of the CCR2\textsuperscript{−/−} mice is effectively recapitulated in mice lacking both CCL2 and CCL7. This strongly suggests that activation of CCR2 by both the chemokine ligands is essential for suppressing IL-4 responses in the host, and in turn controlling fungal infection.

The outcome of \textit{H. capsulatum} infection is largely determined by the early immune response to invasion. The amplified IL-4 response that predisposes CCR2\textsuperscript{−/−} mice to the pathogen is detected as early as day 3 of infection. This suggests involvement of innate cellular source(s) of the cytokine. Determining the identity of these innate IL-4 producing cells is critical for understanding how CCR2 exerts protective effects on the host in histoplasmosis. Additionally, this knowledge can be utilized to discern how type II immune responses are initiated and amplified.
**Eosinophils in infection**

Although eosinophils make up a minute fraction of leukocytes in the body, they are crucial for shaping immune responses to certain infections. They are instrumental in elimination of helminth parasites from the host\(^4\). Eosinophils employ multiple strategies that directly or indirectly inhibit parasite growth and contribute to resolution of infection. These granulocytes instigate T\(_{H2}\) immune responses that are decisive for antihelminth immunity. Furthermore, eosinophils release numerous factors such as toxic granule proteins, reactive oxygen species, and lipid mediators that may directly damage the parasites. Antimicrobial properties of these cells have been reported against certain bacterial pathogens. Eosinophils can phagocytose and kill *Staphylococcus aureus*, and *Escherichia coli*\(^4\). In *Pseudomonas aeruginosa* infection, release of eosinophilic granule proteins contributes to bacterial killing\(^4\).

In contradistinction, eosinophil accumulation can be debilitating for the host in certain infectious settings. Eosinophils hamper host defenses against the fungal pathogen *Cryptococcus neoformans* by sustaining T\(_{H2}\) immune responses\(^4\). IL-4 secreted by these granulocytes is believed to skew CD4\(^+\) T cells to a T\(_{H2}\) lineage and in turn heighten susceptibility to the pathogen. Animals deficient in eosinophils manifest lower fungal burden, fewer T\(_{H2}\) cells and higher expression of protective T\(_{H1}\) and T\(_{H17}\) cytokines.

The influence of eosinophils on *H. capsulatum* infection has not been elucidated. The ability of these granulocytes to instigate and/or amplify T\(_{H2}\) responses implies a possible pathologic role in histoplasmosis. Another area that requires investigation is the molecular interaction between *H. capsulatum* yeasts and eosinophils. The granulocytes express multiple pattern recognition
receptors and contain diverse array of preformed and de-novo-synthesized mediators that could potentially influence antifungal responses in the host.

Synopsis

Animals deficient in the chemokine receptor CCR2 exhibit weakened resistance to *H. capsulatum* due to an overzealous IL-4 response in the lungs. The amplified IL-4 is detected as early as day 3 of infection, raising the possibility that an innate cellular source is generating the cytokine. To comprehensively understand how CCR2 orchestrates protective effects in histoplasmosis, two key questions need to be answered, (a) what is the innate cellular source of IL-4 in CCR2−/− mice during *H. capsulatum* infection? (b) How does CCR2 signaling regulate IL-4 generation in fungal infection?

In chapter 3, we determine that eosinophils are the prominent source of non-protective IL-4 in *H. capsulatum*-infected CCR2−/− mice. We also investigate the molecular interaction between *H. capsulatum* yeasts and eosinophils that triggers the robust IL-4 response. In chapter 4, we describe a novel signaling cascade wherein IL-4 instigates IL-33 in infected CCR2−/− mice. Collectively, our findings reveal a previously unappreciated role for CCR2 in regulating a novel IL4-IL-33 nexus in intracellular fungal infection.
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Adaptive Immunity to Fungi
Adaptive Immunity to Fungi

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ABSTRACT

Life-threatening fungal infections have risen sharply in recent years due to the advances and intensity of medical care that may blunt immunity in patients. This emerging crisis has created the growing need to clarify immune defense mechanisms against fungi with the ultimate goal of therapeutic intervention. We describe recent insights in understanding the mammalian immune defenses that are deployed against pathogenic fungi. We focus on adaptive immunity to the major medically important fungi and emphasize three elements that coordinate the response: 1) dendritic cells and subsets that are mobilized against fungi in various anatomical compartments, 2) fungal molecular patterns and their corresponding receptors that signal responses and shape the differentiation of T-cell subsets and B-cells, and ultimately 3) the effector and regulatory mechanisms that eliminate these invaders while constraining collateral damage to vital tissue. These insights create a foundation for the development of new, immune-based strategies for prevention or enhanced clearance of systemic fungal diseases.
INTRODUCTION

Encounters with fungi require a coordinated host innate and adaptive immune response to successfully eradicate the fungus and promote long-lived immunological memory of the encounter. This review covers three key elements that orchestrate this coordinated response: dendritic cells (DC), pattern recognition receptors (PRR) and antigen-specific T and B cells. DCs lie at the intersection of innate and adaptive immunity. These cells are capable of taking up and processing Ag for display by major histocompatibility complex (MHC) class I or MHCII molecules to naive T cells and of mediating fungicidal activity. Surface and intracellular PRR enable DC to sense fungi. Upon fungal recognition, DC secrete cytokines and express co-stimulatory molecules that help drive naive CD4+ T cell differentiation into a T-helper (T_h) phenotype. In immune competent hosts, CD4+ T cell-mediated clearance of fungi with limited tissue damage requires a finely tuned balance between T_h1, T_h17, and T_reg subsets; in CD4-deficient hosts, CD8+ T cells may come into play. A calibrated balance of helper, regulatory, and effector T- and B-cell responses integrate optimal innate and adaptive immunity to fungi.

CHARACTERIZATION AND FUNCTION OF DC AND MONOCYTE SUBSETS

Steinman and Cohn first reported the identification of a cell with "continually elongating, retracting, and reorienting" long cytoplasmic processes in the spleen and lymph nodes of mice \(^1\). These cells, termed DC, are hematopoietic cells that serve as professional antigen (Ag) presenting cells and initiate T cell responses. When DC encounter Ag at the boundary of immunological defense sites such as the skin, airways of the lung, or draining nodes of the lymphatic system, DC amplify the innate immune response by secreting cytokines that recruit and activate other leukocytes. After uptake, processing and presentation of Ag, DC initiate and shape adaptive responses by promoting naïve T cell differentiation into effector or regulatory T cells. Since the discovery of DC, many subsets have been described based on anatomical location, function, and surface marker expression (Fig. 1).
**Plasmacytoid DC**

pDC are typified by interferon-α (IFN-α) production in response to nucleic acids sensed by endosomal Toll-like receptors, and are characterized by surface expression of sialic acid binding immunoglobulin-like lectin H (Siglec H). pDC induce IL-10 producing CD4⁺ Foxp3⁺ Treg cells, limit T₈₁ and T₈₁₇ cell polarization at mucosal sites, and activate CD8⁺ T cells. pDC control viral infection via the induction of CD8⁺ T cells, but may impair bacterial clearance and contribute to septic shock. While pDC mediate anti-viral immunity, the role of pDC in fungal infections is less clear. pDC recognize *Aspergillus fumigatus* DNA via TLR9 and inhibit *Aspergillus* growth *in vitro*. pDC accumulate in the lungs in a murine model of *Aspergillus* pulmonary infection, and their elimination enhances progression of infection, suggesting that pDC may recognize and combat fungi directly *in vivo*.

A subset of pDC exists that develops in the context of elevated IFN-α and is similar to pDC found in Peyer's patches. Uncharacteristically, this pDC subset fails to produce IFN-α after stimulation with TLR ligands, but secretes elevated levels of IL-6 and IL-23 and primes Ag specific T₈₁₇ cells *in vivo*. This finding suggests a potential role for IFN-α elicited pDC in the polarization of anti-fungal T₈₁₇ cells. Combined with the recent findings that pDC are critical mediators of Treg / T₈₁₇ balance at mucosal surfaces, recognition of fungi by pDC or IFN-α elicited pDC at mucosal surfaces may tilt the balance toward tolerance or inflammation.

**Conventional DC**

Conventional DC - or resident DC - exist in the lymphoid tissue and are comprised of two main subpopulations: CD8⁺ and CD4⁺CD8⁻ resident DC. The spleen contains a third, minor population of so-called double-negative DC, which lack CD4 and CD8 expression and appear to be largely similar in function to CD4⁺CD8⁻ DC. CD8⁺ resident DC are identified by the surface phenotype CD8⁺CD4⁻ CD11b⁻CD11c⁺MHCII⁺DEC205⁺ and are located chiefly in the T cell zone of the spleen and lymph nodes.
A major function of CD8⁺ DC is to cross-present Ag via MHCI to CD8⁺ cytotoxic T lymphocytes (CTL). CD8⁺ DC obtain Ag by engulfment of live or apoptotic cells or Ag-containing apoptotic vesicles.

DC acquire and cross-present *Histoplasma capsulatum* Ags to CTL by ingestion of live or killed yeasts or uptake of *Histoplasma*-containing apoptotic macrophages. Subcutaneous injection of apoptotic phagocytes containing CFSE-labeled, heat-killed *Histoplasma* results in the accumulation of CFSE in CD11c⁺ cells in skin draining lymph nodes and CD11c⁺-dependent CTL-mediated protection against *Histoplasma* challenge. While these studies show that fungal Ags can be acquired and presented by resident DC, the resident DC subpopulation(s) involved in vivo remain undefined.

CD4⁺CD8⁻ resident DC also may cross-present fungal Ags. In studies using an OVA-expressing strain of *Saccharomyces cerevisiae*, both CD8⁺ and CD4⁺CD8⁻ DC from mouse spleen that were primed with OVA-*S. cerevisiae*, induced robust OVA-specific CD4⁺ T cell proliferation ex vivo, however only CD4⁺CD8⁻ DC stimulated an OVA-specific CD8⁺ T cell response. Further work will be needed to unravel the relative contributions of resident DC subpopulations to CD4⁺ T cell and CTL activation and polarization in vivo.

During vaccine immunity to *Blastomyces dermatitidis*, lymph node-resident DC primed anti-fungal T cell responses after Ag transfer from other cells, although the phenotype of resident DC was not defined. DC acquisition of Ag required ferrying of yeast from the skin to the lymph node by migratory and monocyte-derived DC, after which resident DC in the skin draining, lymph nodes acquired and displayed Ag and primed Ag specific CD4⁺ T cells.

### Migratory DC

Migratory DC - or tissue DC - are immature DC located mainly in peripheral tissues such as the skin, lung, and gut. Following uptake of Ag, migratory DC exit the tissue and mature as characterized by (1) enhanced Ag processing and presentation, (2) downregulation of tissue homing receptors, (3) upregulation of CCR7 and (4) increased surface display of co-stimulatory molecules. CCR7⁺ DC migrate
to the T cell zone of lymphoid tissue where they can activate naive T cells or transfer Ag to resident DC \cite{13,14}. Migratory DC have the capacity for division and self-renewal *in situ*, while monocyte subsets also contribute to replenishment of migratory DC. Migratory DC line the surfaces of the body exposed to the environment and thus encounter fungi and other pathogens and Ags. While the migratory DC networks that line the skin, lung, and intestine share similarities, each site has functional differences that are important in anti-fungal immunity.

**Skin.** The skin contains a network of DC, which can be divided into the epidermis-associated Langerhans cell (LC) and a collection of dermis-associated dermal DC \cite{15}. In addition to their epidermal location, LC are characterized by surface expression of langerin (CD207), CD11c, and MHCII. The dermis contains LC that migrate to the draining lymph node, CD207\(^+\)CD103\(^+\) dermal DC, and a group of CD207CD103\(^-\) DC\cite{15}. Upon subcutaneous injection of *B. dermatitidis* vaccine, DEC205\(^+\) skin-derived DC migrated to the draining lymph nodes in a CCR7-dependent fashion, presented (or transferred) model Ag expressed by the yeast, and activated CD4\(^+\) T cells \cite{12}.

LC and CD207\(^+\) dermal DC were shown to have specialized Ag presentation and T cell polarization functions in a cutaneous exposure model to *Candida albicans* \cite{16}. LC were required for the generation of Ag-specific T\(_h\)17 cells via the production of elevated levels of IL-6, IL-1\(\beta\), and IL-23. While LC were not required for the generation of CTL, CD207\(^+\) dermal DC were required for CTL and also T\(_h\)1 polarization. Compared with LC, CD207\(^+\) dermal DC produced more IL-12 and IL-27, and less IL-1\(\beta\) and IL-6, and no IL-23, making them poor promoters of T\(_h\)17. CD207\(^+\) dermal DC also blunted the ability of LC and CD207\(^-\) dermal DC to promote T\(_h\)17 responses. Since IL-12 and IL-27, as well as IFN-\(\gamma\) from T\(_h\)1 cells, inhibit T\(_h\)17 differentiation and proliferation, CD207\(^+\) dermal DC likely block *Candida*-specific T\(_h\)17 cells by promoting T\(_h\)1 differentiation. Thus, exposure of LC and CD207\(^+\) dermal DC to *Candida* can promote opposing effects via elaboration of polarizing cytokines that induce development of T\(_h\)1 or T\(_h\)17 responses \cite{16}.

**Lung.** DC in the lung and airways confront constant exposure to inhaled spores and hyphal
fragments. A network of DC line the airways, sampling inhaled Ags and shuttling them to the mediastinal lymph nodes. Besides pDC, lung DC subsets include two broad subsets: CD103⁺DC and CD11b⁺ DC. CD103⁺ lung DC also express CD207, making them similar to CD207⁺CD103⁺ dermal DC. CD103⁺DC extend dendrites into the airway lumen to sample Ag without disturbing the epithelium. CD103⁺DC can acquire soluble and apoptotic cell-associated Ags from the airway and migrate to the mediastinal lymph node under steady state and inflammatory conditions. At the lymph node, CD103⁺ DC cross-present Ag and activate CTL. CD11b⁺ DC differ from monocyte-derived DC and specialize in cytokine and chemokine production as well as presenting Ag to CD4⁺ T cells in the mediastinal lymph node after migration. Rapid recruitment of Ly6C⁺ monocyte-derived DC to the lung upon inflammation has clouded the functional analysis of lung resident CD11b⁺ DC, which lack Ly6C expression. Upon lung exposure to A. fumigatus conidia, CD103⁺ DC failed to take up and transport conidia to the mediastinal lymph node, whereas CD11b⁺ DC did transport them. In this model, lung CD11b⁺ DC were reduced relative to wild type mice in CCR2⁻/⁻ mice following A. fumigatus exposure. Conversely, naive CCR2⁻/⁻ mice had similar lung CD11b⁺ DC numbers to wild type mice, suggesting that recruited monocyte-derived DC and not lung resident CD11b⁺ DC are responsible for conidial uptake and Ag presentation in the setting of A. fumigatus induced inflammation.

**Intestine.** As in the lung, DC in the intestine are situated on the basolateral side of the epithelium, and largely isolated from the gut microflora. DC in the intestine localize to the lamina propria (LP-DC) and Peyer's patch (PP-DC); both subsets in each region differentially regulate immune responses. The role of PP-DC and LP-DC in generating anti-fungal immunity in the gut is unclear and relatively unstudied. C. albicans, a gut commensal, can cause systemic infection if the gut epithelial/DC barrier is breached or in the setting of broad-spectrum antibiotic use, leading to Candida overgrowth. Strong induction of Treg cells by LP-DC in the mesenteric lymph node may highlight the critical role of limiting inflammation in the gut in order to maintain the epithelial barrier and prevent disseminated infection. Furthermore, heightened Th17 responses in the gut impair protective Th1 responses and worsen Candida infection. While bone
marrow derived DC produced IL-23 in response to *Candida in vitro* and IL-23 neutralization promoted fungal clearance *in vivo*, the identity of the DC subset recognizing and responding to the fungus in this model was not determined. Nevertheless, DC in the gut appear to tightly control tolerance and immunity to fungal organisms.

**Monocytes, monocyte-derived DC, and inflammatory DC**

Monocytes are derived from a macrophage-DC progenitor and, in the absence of inflammation, are found in the bone marrow and circulating at low levels in the blood and spleen. Two classes of CD11b⁺CD115⁺ monocytes arise from the progenitor and circulate in the blood, Ly6C⁺CCR2⁺ and Ly6C⁺CX3CR1hi monocytes. Monocytes have broad developmental plasticity, replenish subsets of DC and LC in the setting of experimental depletion, and may represent an emergency store of DC precursors that can be rapidly deployed. Under inflammatory conditions, Ly6C⁺CCR2⁺ monocytes migrate to inflammatory sites and acquire expression of the DC markers CD11c and MHCII, while losing expression of Ly6C, thus becoming "inflammatory DC". Whereas Ly6C⁺CX3CR1hi cells do not appear to be involved in innate immunity to fungi, Ly6C⁺CCR2⁺ monocytes play a critical role in responding to many medically important fungi including *A. fumigatus*, *Cryptococcus neoformans*, *H. capsulatum*, and *B. dermatitidis*.

Monocyte-derived DCs have an outsized role in anti-fungal immunity, particularly through the induction of T_h1 cells. CCR2⁻/⁻ mice exhibit skewed T_h2 responses and poorly controlled *H. capsulatum* infection compared to wild-type mice. Similar CCR2-dependent phenotypes are found in experimental infection with *A. fumigatus* or *C. neoformans*; that is, priming T_h1 cells in response to fungi requires CCR2⁺ monocyte-derived inflammatory DC. The tissue environment has a prominent role in inflammatory DC function, as the defect in CD4⁺ T cell priming by these DC during infection with *A. fumigatus* is restricted to the lung in CCR2⁻/⁻ mice and not to other lymphoid organs such as the spleen. Similarly, while Ly6C⁺CCR2⁺ monocytes play a major role in delivering *B. dermatitidis* into skin...
draining lymph nodes after subcutaneous vaccination, this shuttling function can be compensated by other skin migratory DC subsets in CCR2\(^{-/-}\) mice\(^ {12}\). Conversely, CCR2 monocytes and monocyte-derived, inflammatory DCs are essential to prime Ag-specific CD4\(^+\) T cells in the lung during infection with Blastomyces or Histoplasma infection\(^ {30}\). Thus, in the lung, which is often the primary route of infection for fungi, but not in the skin or the spleen, monocyte-derived DC appear to play a critical and indispensable role in anti-fungal immunity.

**PATTERN RECOGNITION RECEPTORS**

In mammals, fungi are detected by germline encoded pathogen recognition receptors (PRR) that are expressed by innate cells\(^ {31}\). The three major pathogen-associated molecular patterns (PAMPs) that are unique to fungi and set them apart from the mammalian host are chitin, \(\alpha\)- and \(\beta\)-glucans and mannans. The innate recognition of these fungal PAMP’s activates signaling cascades to induce the expression of MHC, co-stimulatory molecules and cytokines by antigen-presenting cells that influence the development of adaptive immunity. Since Th17 and Th1 cells are the principal T helper subsets that contribute to protective immunity to several pathogenic fungi we highlight the most recent literature on the signaling pathways and other factors that influence the differentiation of these two T helper subsets.

Among the best-characterized PRRs that recognize fungi are the Toll-like receptors (TLR) and C-type lectins (CLR) (Fig 2). TLR1-4, 6, 7 and 9 recognize a variety of fungal species through mostly undefined ligands\(^ {32-37}\). The main TLRs involved in sensing fungal ligands are TLR2, TLR4 and TLR9 that recognize zymosan, phospholipomannan, \(O\)-linked mannans, glucoronoxylomannan and fungal DNA\(^ {38-40}\). Mice lacking the signaling adaptor myeloid differentiation primary response protein 88 (Myd88) are more susceptible to infection with *C. neoformans*, *C. albicans*, *A. fumigatus*, *B. dermatitidis* and *P. brasiliensis*\(^ {36,41-44}\) emphasizing important roles for TLR signaling in antifungal immunity, but also reflecting the involvement of Myd88 in IL-1 signaling. However, in murine experimental models of
infection, there are conflicting reports on individual contributions of multiple TLRs and fungal species \cite{43,45}. In humans, a similar controversy on the role of Myd88 in mediating anti-fungal immunity has been reported. While fungal infections were not a problem for children with autosomal recessive Myd88 deficiency \cite{46}, patients with TLR1 and TLR4 polymorphisms were more susceptible to candidemia \cite{47} and invasive aspergillosis during stem cell transplantation \cite{48}.

Besides the TLRs, CLRs expressed by myeloid and mucosal epithelial cells are key PRRs for the recognition of fungi and induction of protective immunity \cite{38,39,45,49,50}. CLRs belong to a large family of proteins that recognize ligands in a calcium-dependent manner, which vary in microbes from endogenous to exogenous and are often conserved and carbohydrate-based \cite{49}. Some CLR contain cytoplasmic signaling motifs that allow direct activation of intracellular signaling cascades; others lacking these motifs make use of adaptor molecules to initiate signal transduction \cite{49}. Below, we discuss the most recent progress on the cell-associated CLRs Dectin-1, Dectin-2, Dectin-3, Mincle, and the mannose receptor.

**Dectin-1**

Dectin-1 is the archetypical and best-studied non-TLR PRR shown to link innate and adaptive immunity and instruct differentiation of Th1 and Th17 cells \cite{51,52}. Dectin-1 recognizes β-1,3-glucan from fungi, plants and some bacteria \cite{53} and unidentified T cell and mycobacterial ligands \cite{54}. Upon ligand activation, Dectin-1 signals through an immunoreceptor tyrosine-based activation-like motif (ITAM-like or HemITAM) \cite{49} to produce cytokines via multiple signaling pathways (reviewed in Vautier et al., Cytokine 2012). The best characterized Dectin-1 signaling pathway is the Syk/CARD9 pathway leading to activation of the canonical NF-κB subunits p65 and c-Rel and subsequent production of pro-IL-1β, IL-6, IL-10, IL-23 and TNF-α \cite{55}. Cytokine production can be modulated via the formation of inactive RelB-p65 dimers through the noncanonical NF-κB subunit RelB in a NIK-dependent pathway \cite{56} and the Syk-independent activation of Raf-1. The former reduces IL-1β and IL-12p40 expression, whereas the latter
increases the production of these pro Th1/Th17 cytokines. The balance toward a Th17 response is favored through the collaborative interaction of Dectin-1 with TLR-2 that leads to the production of prostaglandin E2, which will upregulate the Th17 polarizing cytokines IL-6 and IL-23.

In addition, recognition of β-glucan, A. fumigatus and C. albicans by Dectin-1 and TLR2 activates the NLRP3 inflammasome leading to the production of bioactive IL-1β thereby enhancing Th17 cell development and antifungal immunity. In a pulmonary model of A. fumigatus infection, Dectin-1 decreased the production of IL-12 and IFN-γ in innate cells, which decreased T-bet expression in A. fumigatus-specific CD4+ T cells and enabled Th17 differentiation. Thus, Dectin-1 signaling drives the production of Th17 cell promoting cytokines that yields resistance to C. albicans, A. fumigatus and P. carinii infection.

Some fungi (e.g. A. fumigatus and C. albicans) have developed mechanisms to mask their β-glucan exposure and limit detection by immune cells. β-glucan shielding can also vary among different strains within a fungal species. For example, H. capsulatum strain G186A shields its β-glucan by α-glucan so that yeast are not recognized by Dectin-1, whereas strain G217B mostly lacks α-(1,3)-glucan on the yeast surface and is recognized by Dectin-1. Thus, the induction of Th17 cells, the acquisition of vaccine-induced resistance and primary resistance to the latter strain is blunted in Dectin-1−/− vs. wild-type mice.

**Dectin-2 cluster**

The last few years have revealed exciting new insights into the function and role of the Dectin-2 cluster in mediating antifungal immunity. The Dectin-2 family is comprised of Dectin-2, Mincle, MCL, DCIR, DCAR and BDCA-2. Aside from DCIR, all the other receptors have short cytoplasmic tails that lack signaling motifs and associate with the FcRγ chain, an adaptor containing an ITAM motif. Dectin-2 recognizes C. albicans, Sacharomyces cerevisiae, Microsporum audouinii, Trichophyton rubrum, A. fumigatus, H. capsulatum, P. brasiliensis, Malassezia, B. dermatitidis and C. posadasii. Dectin-2
recognizes *C. albicans* and *Malassezia* via *N*- and *O*-linked α-mannan, respectively, on their surface. It has been proposed that an active Dectin-2 ligand could be a multivalent terminal α-1,2-mannose attached to glycans, proteins, and presumably any kind of scaffold. Dectin-2 deficient mice are more susceptible to primary *C. albicans* infection. The Dectin-2/FcRγ/Syk/Card9 signaling axis is indispensable for the development of vaccine-induced, Ag-specific Th17 cells and immunity to the systemic, endemic, dimorphic fungi *B. dermatitidis*, *H. capsulatum* and *C. posadasii*. Mincle is another FcRγ-coupled activating receptor that recognizes pathogenic fungi and *Mycobacterium*. Mincle binds glycolipids, such as trehalose-6,6-dimycolate (TDM) from *Mycobacterium tuberculosis*, and novel glyceroglycolipids from *Malassezia*. Although not shown yet for fungi, Mincle induces Th1/Th17 adaptive immunity in response to TDM and its synthetic analogue trehalose-6,6-dibehenate (TDB). Mincle-deficient mice are more susceptible to primary infection with *C. albicans* and *Malassezia*. However, Mincle is dispensable for development vaccine-induced immunity and Th17 cells against the three major systemic dimorphic fungi *B. dermatitidis*, *H. capsulatum* and *C. posadasii*. Similar to Mincle, Macrophage C-type lectin (MCL, also called Dectin-3, Clecsf8, and Clec4d) is also a FcRγ-coupled activating receptor that binds to TDM. MCL is constitutively expressed in myeloid cells whereas Mincle is barely expressed in resting cells, but inducible by TDM. In response to TDM, MCL and Mincle promote the development of Th1/Th17 cells by upregulating the co-stimulatory molecules CD80, CD86 and CD40. MCL also contributes to Th17 cell mediated EAE development. Whether MCL induces anti-fungal Th17 cell responses is unclear, but MCL-deficient mice are highly susceptible to *C. albicans* infection. The multivalent ligands on the fungal surface likely induce multimerization of monomeric and dimeric CLRs. For example, Mincle and MCL form a disulfide-linked heterodimer associated with FcRγ and Dectin-2 forms a heterodimeric PRR with Dectin-3 for sensing and mediating host defense against
The heterodimer showed higher affinity to fungal α-mannan than their respective homodimers and responded effectively to fungal infection, indicating that dimerization may provide different sensitivity and diversity for host cells to detect fungal pathogens and induce adaptive immunity. Since individual and dimeric CLR of the Dectin-2 cluster discussed here signal through the FcRγ/Syk/Card9 axis, it is noteworthy that Card9 affects the development of Th1/Th17 cells in vivo mostly at the stage of T cell differentiation and not during the activation, expansion, and survival during the contraction phase.

Mannose Receptor (MR)
The MR has a short cytoplasmic tail that lacks classical signaling motifs, and its downstream signaling pathway is unknown. The MR can induce NF-κB activation and the production of IL-12, GM-CSF, IL-8, IL-1β and IL-6. Although the MR has been reported to induce Th17 cell differentiation of human T cells in response to *C. albicans*, memory and not naïve T cells were the major source of the IL-17 produced. Since different sets of cytokines are required to prime naïve T cells and propagate already primed memory T cells, the role of the MR in inducing Th17 cell differentiation remains inconclusive. Since the TLR2/dectin-1 pathway has a secondary amplification effect on MR-induced IL-17 production, it is conceivable that *C. albicans* derived β-glucan is more important for the differentiation of naïve cells into Th17 cells, whereas the MR might play a more prominent role in triggering IL-17 production by memory cells.

T AND B CELL IMMUNITY

It is generally acknowledged that activation of the adaptive arm of the immune system is critical for resolution of fungal infection in the host. The transition from innate to adaptive immunity is facilitated primarily by DC although macrophages contribute. These phagocytes process and present fungal antigens to naïve CD4\(^+\) T cells in the context of class II MHC. This interaction initiates the commitment to effector
TH subsets. DC also activate CD8+ T cells by antigen presentation via MHC-I (Fig. 3). For antigens that enter through the exogenous pathway, engagement of CD8+ proceeds through a mechanism termed cross-presentation in which antigens are shuttled into the class I MHC pathway. In contrast to the requirement of antigen processing for activation of T cells, B cells directly react to antigens from fungal pathogens to secrete immunoglobulins that may influence the outcome of fungal infection.

TH1 immunity

The TH1 immune response is instrumental in host defense against most fungal pathogens, and its importance is well established in experimental murine models and in human infections. Following exposure, antigen presenting cells (APCs) produce IL-12 that is critical for TH1 lineage commitment. Genetic mutation in the IL-12 signaling pathway is associated with predisposition to a wide variety of fungal diseases such as cryptococcosis, candidiasis, paracoccidioidomycosis, and coccidioidomycosis. Furthermore, increased susceptibility to histoplasmosis has been observed in mice lacking IFN-γ and in a patient with a genetic deletion mutation in IFN-γ receptor-1 gene. Conversely, patients receiving adjunctive IFN-γ immunotherapy display augmented protection against aspergillosis, cryptococcosis and coccidioidomycosis.

TH1 cells orchestrate antifungal immune responses through the release of proinflammatory cytokines IFN-γ, TNF-α, and GM-CSF (Fig. 3). The signature TH1 cytokine, IFN-γ, manifests pleotropic effects on immune cells during infection. It induces classical activation of macrophages that is critical for arresting growth of intracellular fungal pathogens including H. capsulatum, B. dermatitidis, P. brasiliensis, and C. immitis. These classically activated phagocytes are speculated to mediate their fungicidal activities through the release of nitric oxide and reactive oxygen intermediates. In addition to its effects on macrophages, IFN-γ prompts antibody class switching to IgG2a in B cells (associated with antifungal effects) and enhances phagocytosis and antigen processing/presentation in APCs to combat fungal infections.
TNF-α shares multiple redundant functions with IFN-γ, including classical activation of macrophages. Animals deficient in TNF-α are highly vulnerable to a range of fungal infections. The protective effects of this cytokine during fungal diseases have also been corroborated in humans. Individuals homozygous for A/A at position -308 in the TNF-α promoter display elevated TNF-α activity and augmented resistance to aspergillosis. In contrast, patients given TNF-α blockers to treat inflammatory diseases suffer from a myriad of fungal infections. The importance of GM-CSF in fungal diseases has been elucidated in murine model of pulmonary histoplasmosis. Administration of anti-GM-CSF antibody reduced protective immunity to *H. capsulatum*. A recent report has indicated that GM-CSF facilitates its anti-fungal responses through sequestration of zinc from intracellular yeasts and stimulation of reactive oxygen species in macrophages.

**T<sub>H</sub>2 immunity**

For the vast majority of fungal infections, T<sub>H</sub>2 immunity manifests a detrimental influence on the host. These T<sub>H</sub>2 responses are comprised of CD4<sup>+</sup> T cell-derived cytokines IL-4, IL-5 and IL-13 and B cell secreted IgE. Exaggerated synthesis of these soluble factors in most mycotic diseases interferes with pathogen clearance, and on rare occasions, failure to regulate them can result in a fatal outcome. For example, in mice lacking the chemokine receptor CCR2, infection with *H. capsulatum* or *C. neoformans* induces a dominant IL-4 response that is associated with impaired host resistance. In pulmonary aspergillosis and cryptococcosis, undesired activation of T<sub>H</sub>2 response leads to non-protective allergic inflammation in mice. These animals demonstrate signs of eosinophilia, goblet cell hyperplasia, and heightened susceptibility to the pathogen. In addition to these experimental findings, a single nucleotide polymorphism in the IL-4 promoter region that is linked with amplified IL-4 production occurs with increased frequency in women with recurrent vulvovaginal candidiasis. This genetic defect is hypothesized to predispose the individuals to *C. albicans* by suppressing the fungicidal activity of macrophages encountering *C. albicans* yeasts.
The mechanisms by which T\textsubscript{H}2 cytokines dampen host immunity are multifactoral. Both IL-4 and IL-13 drive alternative activation of macrophages that is associated with uncontrolled fungal growth. *C. neoformans* and *H. capsulatum* proliferate robustly in macrophages primed with IL-4, as opposed to the ones that are classically activated\textsuperscript{124,125}. These alternatively activated phagocytes display amplified levels of arginase-1, an enzyme that potentially diminishes the amount of nitric oxide required for fungicidal activity\textsuperscript{126}. Additionally, IL-4 modulates fungal access to specific micronutrients in macrophages. This cytokine upregulates the transferrin receptor\textsuperscript{127} on cell surface that results in enhanced iron acquisition. This phenomenon is believed to augment fungal growth within the cells. In addition, IL-4 alters intracellular survival by increasing zinc content that supports the growth of fungal pathogens\textsuperscript{124}.

In sharp contrast to these findings, T\textsubscript{H}2 responses bestow protection to the host in pneumocystosis. A recent report suggested that alternatively activated macrophages driven by IL-13 exhibit increased fungicidal capacity in *P. murina* infection, as opposed to classically activated cells\textsuperscript{128}. Antibody class switching to a more protective IgG subclass induced by T\textsubscript{H}2 cytokines also might contribute to the heightened protection against *P. murina* since patients with this defect are vulnerable to *Pneumocystis* pneumonia\textsuperscript{129,130}. Our understanding of why T\textsubscript{H}2 cytokines are protective in pneumocystis but not other mycotic diseases is incomplete. A probable explanation could be that *Pneumocystis* behaves similar to helminth parasites by tightly adhering to pulmonary epithelial cells to establish infection\textsuperscript{131}. In such a setting, release of T\textsubscript{H}2 cytokines by effector cells is critical for minimizing the virulence of helminths. Although T\textsubscript{H}2 immunity significantly contributes to host defense against pneumocystosis, it cannot compensate for the loss of other T\textsubscript{H} subsets. Patients with autosomal dominant hyper IgE syndrome (HIES) exhibit defects in generation of T\textsubscript{H}17 responses (but not T\textsubscript{H}2 responses) and are found to be progressively more susceptible to *Pneumocystis* pneumonia\textsuperscript{132}. Thus, in summary, resolution of pneumocystosis depends on a coordinated action of T\textsubscript{H}2 arm of the immune system and other T\textsubscript{H} subsets.
**T_{\text{H}}17 immunity**

T_{\text{H}}17 cells are a subset of CD4\(^+\) T cells that are developmentally distinct from T_{\text{H}}1 and T_{\text{H}}2 cells and are identified by the expression of cytokines IL-17A, IL-17F and IL-22. The differentiation of this T cell lineage requires various cytokines and transcription factors. TGF-\(\beta\) and IL-6 prime the initial differentiation of naïve CD4\(^+\) T cells to T_{\text{H}}17 cells and IL-23 is critical for maintenance and expansion of these cells\(^{133}\). Activation of STAT3 by IL-6 is indispensable for this process since the former directly regulates the transcription of ROR-\(\gamma\)t, the master transcription factor controlling T_{\text{H}}17 lineage commitment. The influence of T_{\text{H}}17 immunity in bolstering host defenses against fungal pathogens has been well substantiated. Indeed, humans with genetic defects in IL-17 signaling axis are severely compromised in their ability to counter mycoses. Individuals with HIES exhibit dominant negative mutation in STAT3\(^{134}\). Consequently, these individuals are vulnerable to a spectrum of fungal infections\(^{132}\). Another rare genetic disorder called autoimmune polyendocrinopathy with candidiasis and ectodermal dystrophy (APECED) is linked with chronic and recurrent mucocutaneous candidiasis\(^{135}\). This defect is characterized by mutations in the autoimmune regulator (AIRE) gene that result in generation of autoantibodies directed against T_{\text{H}}17 cytokines. Furthermore, hereditary mutations in the Dectin-1 signaling that shapes T_{\text{H}}17 immunity have been identified in patients with chronic mucocutaneous candidiasis\(^{136}\).

The importance of IL-17 responses in antifungal immunity is best studied in diverse models of experimental candidiasis. Mice deficient in IL-17 receptor fail to generate functional T_{\text{H}}17 cells and are vulnerable to systemic and oral candidiasis\(^{137}\). The IL-23-IL-17 axis is necessary for development of optimal immunity against *C. albicans*. In models of dermal and oral infection, IL-23-deficient animals develop progressively worse disease\(^{137,138}\). Likewise, in vulvovaginal *C. albicans* infection, impairing T_{\text{H}}17 cell differentiation leads to heightened fungal burden at the site of challenge\(^{139}\).
Two disparate mechanisms have been described through which Th17 cells manifest antifungal responses. In systemic challenge models, these cells recruit neutrophils by prompting the release of CXC chemokines. The neutrophils, in turn, exhibit potent anti-candidacidal activity and clear the pathogen. In mucosal infection models, IL-17 prompts keratinocytes and epithelial cells to release antimicrobial peptides (AMPs) such as S100A proteins, β-defensins, and histatins that exhibit direct killing activity. To overcome the antifungal effects of IL-17 pathway, pathogens have evolved to subvert the activity of this cytokine. Live Candida secretes an uncharacterized soluble factor that diminishes the production of IL-17 in human peripheral blood mononuclear cells by inhibiting indoleamine 2,3-dioxygenase (IDO) expression. IDO is an enzyme that catalyzes the breakdown of tryptophan, thereby depriving microbes of this essential amino acid. Furthermore, IL-17 prompts morphological changes in C. albicans and A. fumigatus. This cytokine directly binds to the outer surface of these two pathogens and induces transcriptional changes that are associated with augmented hyphal growth and enhanced resistance to host antifungal defenses.

Other less appreciated Th17 cytokines IL-17F and IL-22 may be involved in anti-Candida responses. The latter is fundamental for antifungal resistance in gastric and systemic candidiasis. IL-22 facilitates its protective effects through the elicitation of AMPs and by maintaining the integrity of the mucosal barrier to prevent dissemination of the disease. However, contradictory findings have emerged wherein IL-22 is dispensable in murine models of oral and dermal candidiasis. Much less is known about IL-17F and its role in fungal infections. During systemic Candida infection, deficiency of this cytokine does not impact host resistance. Despite the limited or conflicting literature on IL-17F and IL-22, there is clinical evidence to support their protective effects in mycoses. Self-reacting antibodies against IL-17F and IL-22 are detected in APECED patients, who are vulnerable to mucocutaneous candidiasis. Moreover, patients with dominant negative mutations in IL-17F are at an increased risk of developing chronic mucocutaneous candidiasis.
In other experimental models of mycoses, \( \text{T} \text{H} \text{17} \) cells offer protection against several fungal infections, but they are not requisite for host survival. Neutralization of IL-17 during \( C.\) neoformans, \( P.\) carinii, or \( H.\) capsulatum infection results in a delayed clearance of disease but does not impact the lifespan of animals \(^{149-151}\). In contrast, the \( \text{T} \text{H} \text{17} \) arm is critical for vaccine-induced immunity against the dimorphic fungal pathogens \( B.\) dermatitidis, \( C.\) posadasii, and \( H.\) capsulatum \(^{152}\). In this setting, IL-17 producing cells compensate for the loss of \( \text{T} \text{H} \text{1} \) cells to counter the disease. The proposed mechanism by which these cells orchestrate antifungal activity is through recruitment and activation of neutrophils and macrophages in the lungs.

Contradictory to the above findings, unfavorable aspects of \( \text{T} \text{H} \text{17} \) cells in fungal diseases have been discovered. In mice with gastric candidiasis, IL-23 induces exaggerated activation of \( \text{T} \text{H} \text{17} \) pathway that is linked with deterioration of the disease \(^{153}\). The mice demonstrate heightened tissue damage and defective antifungal activity in neutrophils. Animals deficient in the transmembrane protein Toll IL-1R8 mount amplified \( \text{T} \text{H} \text{17} \) responses and are highly susceptible to candidiasis \(^{154}\). Parallel findings are reported in murine aspergillosis in which \( \text{T} \text{H} \text{17} \) cells hamper the outcome of infection by impairing the effector functions of neutrophils and exacerbating inflammatory pathology \(^{153}\). In these scenarios, neutralization of \( \text{T} \text{H} \text{17} \) cytokines enhances resistance to the pathogen. To date, no definite mechanism to clarify the antagonizing function of \( \text{T} \text{H} \text{17} \) cells in different fungal infections has been described.

**Regulatory T Cells**

Appropriate regulation of proinflammatory immune responses generated against invading infectious agents is necessary to limit collateral damage to the host. Regulatory T cells (Treg cells) contribute significantly to this task. This subset of T cells dampens the immune responses through a multitude of suppressive mechanisms that include secretion of inhibitory cytokines IL-10, TGF-\( \beta \) or IL-35, repression of IL-2 release, perforin/granzyme dependent cytosis of APCs, synthesis of immunosuppressive
adenosine, and through contact dependent down-modulation of APC functions via cytotoxic T-lymphocyte-associated protein 4 (CTLA4) and lymphocyte-activation gene 3 (LAG3) 155.

In murine models of fungal infections, accelerated clearance of infection is achieved by altering the Treg cell activity. In candidiasis and paracoccidioidomycosis, signaling through the toll like receptor, TLR2 and its downstream molecule, MyD88 is critical for prolonging survival of Treg cells 156,157. TLR2−/− mice express fewer Treg cells under homeostasis and disease state. Moreover, the infected mutant mice exhibit a concomitant increase in T_{H}17 cells. As a consequence, TLR2−/− mice resolve C. albicans and P. brasiliensis more efficiently than wild type controls. Similarly, the chemokine receptor CCR5 regulates the equilibrium between T_{H}17 cells and Treg cells. In the absence of this chemokine receptor, mice display reduced influx of the latter to the site of infection and enhanced resistance to P. brasiliensis and H. capsulatum 158,159.

A common approach to treat inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease, and psoriasis is administration of TNF-α antagonists. However, an undesirable consequence of this therapy is the enhanced frequency and suppressor activity of Treg cells that in turn have been linked with exacerbating fungal diseases 116. These clinical findings are corroborated by experimental evidence. Animals given TNF-α neutralizing antibody exhibit elevated numbers of IL-10+ Treg cells and are highly susceptible to the disease when infected with H. capsulatum 160. Elimination of Treg cells enhances survival of TNF-α-neutralized mice; and conversely, adoptive transfer of these cells exacerbates infection. Thus, the immunosuppressive functions of Treg cells are deleterious for host immunity in this scenario.

Despite their potent immunosuppressive functions, Treg cells impart a positive impact on host defense by promoting durable antifungal immunity. In murine gastric candidasis, these cells prevent excessive inflammation and allow the fungus to persist in the gastrointestinal tract, thereby generating an effective secondary immune response 161. A similar mechanism is in effect in A. fumigatus infection
wherein Treg cells shape immune responses to be adequate to confer protection against the pathogen, but not cause unwarranted damage to the host. In addition to immunosuppressive functions, a remarkable component of Treg cell biology is their ability to promote \( T_{\text{H}}17 \) cell differentiation and contribute to host defenses against \( C. \text{ albicans} \). Treg cells manifest such an effect by sequestration of IL-2, a cytokine that inhibits \( T_{\text{H}}17 \) cell differentiation. As a consequence, the robust \( T_{\text{H}}17 \) response generated aids in resolution of oral candidiasis. Intriguingly, humans with a genetic defect in genesis of Treg cells are also predisposed to chronic mucocutaneous candidiasis. Thus, Treg cells represent a double edged sword that are crucial for subduing inflammatory responses, but their suppressive functions may be undesirable in certain settings of mycoses.

**CD8\(^+\) T cells**

CD8\(^+\) T cells are vital for protection against viral pathogens and tumors, however, their relative contribution in host immunity against fungal infections is not as comprehensively understood as CD4\(^+\) T cells. Multiple experimental models suggest that the former share redundant functions with the latter and confer protection in the setting of CD4\(^+\) T cell deficiency. In mice deficient in major histocompatibility complex II (MHC class II), CD8\(^+\) T cell suppress \( H. \text{ capsulatum} \) infection by targeting macrophages laden with yeasts. These cytotoxic CD8\(^+\) T cells are primed by dendritic cells through the process of cross presentation (presentation of exogenous antigens on MHC class I). Furthermore, this lineage of T cells is critical for vaccine-induced immunity against \( A. \text{ fumigatus}, B. \text{ dermatitidis}, \) and \( H. \text{ capsulatum} \). The most likely mechanisms through which memory CD8\(^+\) T cells coordinate resolution of pathogen in these models is by the release of IFN-\( \gamma \) and IL-17, and cytotoxic effects on infected cells. Therefore, vaccines that elicit a robust CD8\(^+\) T cell response can potentially be used as an alternative strategy to prevent fatal mycoses in immunodeficient patients.
Humoral immunity

Although recent advances have been made in our understanding of the contribution of humoral immunity to host defense against fungal infections, contrasting opinions on its involvement still exist. Much of the confusion stems from earlier studies that concluded antibodies were dispensable for resolution of fungal infections. However, the advent of monoclonal antibody technology made it possible to identify the protective effects of immunoglobulins against fungi. Since then, the impact of immunoglobulins and B cells secreting them has been well scrutinized in C. neoformans, C. albicans, H. capsulatum, and Pneumocystis sp infections. The clinical importance of immunoglobulins in mycoses is evident from reports that patients with B cell defects including X-linked hyperIgM, hypogammaglobulinemia, and IgG2 deficiency are susceptible to cryptococcosis. Additionally, pneumocystosis was reported in a patient with hypergammaglobulinemia who displayed inability to class switch from IgM to a more specific IgG subclass.

Immunoglobulins elicit protective immune responses in the host by predominantly targeting antigens in the fungal cell wall such as β glucan (A. fumigatus, C. albicans, and C. neoformans), agglutinin like sequence 3 (C. albicans), glucuronoxylomannan (C. neoformans) and heat shock protein 60 (H. capsulatum). The mechanisms by which these antibodies mediate protection in the host are broadly classified into direct and indirect mechanisms. By definition, direct mechanisms are those that result in inhibition of growth or microbicidal activity when immunoglobulins bind to the pathogen. The fungicidal activity of certain monoclonal antibodies is well illustrated against C. albicans, C. neoformans, P. brasiliensis, and A. fumigatus. Additionally, antibody binding to the outer fungal surface prompts alteration in gene expression and fungal metabolism that ultimately suppress virulence of the pathogen.

Indirect mechanisms comprise immunoglobulin-mediated resolution of infection by enhancing the microbicidal potential of effector cells. Opsonization, activation of complement pathway, and antibody...
directed cell toxicity (ADCC) are conventionally associated with indirect effects of antibodies during infection and form integral components of host defense against fungal pathogens such as *C. albicans*, *C. neoformans*, and *H. capsulatum*. ADCC induces active lysis of cells decorated with antibodies, while the former two actions trigger innate defense mechanisms including phagocytosis. In murine cryptococcosis, administration of an anti-capsular IgG1 monoclonal antibody is associated with heightened production of IL-10 in the lungs and diminished levels of IFN-γ. In this setting, dampening of the proinflammatory response is speculated to contribute to accelerated resolution of infection and improved survival in mice. Our knowledge of why the IgG1 monoclonal antibody elicits an anti-inflammatory response is incomplete, but a probable mechanism could be the suppression of the complement pathway facilitated by highly galactosylated antibodies. N-glycan galactosylation of IgG1 engages the inhibitory IgG receptor FcγRIIB and Dectin-1, which in turn suppress C5a receptor functions. In sharp contrast, administration of monoclonal antibody to *H. capsulatum* histone like protein, H2B improves the outcome of infection by inducing Th1 cytokines and by enhancing the fungicidal activity of macrophages. Thus, antibodies shape the inflammatory response in fungal infections.

Fungal infections represent a serious threat in immunocompromised patients such as those suffering from AIDS. The majority of these affected individuals have severe defects in cell mediated immunity. Thus, there is an urgent requirement for immunotherapy that bypasses the need for CD4+ T cells and combats fungal infections efficaciously. One such strategy is DNA vaccination with *Pneumocystis* antigen, kexin linked to CD40 ligand. This vaccination approach induces a robust antibody response in mice and confers protection during *Pneumocystis* pneumonia. Other fungal antigens that elicit a vigorous humoral response in experimental models have been reported. Thus, immunoglobulin therapy represents a promising approach that could be utilized to treat mycoses in individuals with immunosuppression. However, an important consideration while administering these monoclonal antibodies to the host must be their dose. At a very high concentration, certain immunoglobulins exhibit prozone-like effects and can be non-protective or even deleterious during the course of the disease.
CONCLUDING REMARKS

We have highlighted above the major advances in knowledge concerning the development, maintenance, and function of the adaptive immune response to medically important fungi. We have tried to emphasize common pathways, mechanisms, and themes. The benefits of unearthing the pathways leading to adaptive immunity and the functions of this response are that 1) vaccines may be created that prevent or treat fungal diseases; 2) predictions may be made who will be at risk for serious fungal infections, and 3) biological agents may be developed that bolster immunity in the face of severe and life-threatening infections that are being diagnosed worldwide in increasingly large numbers of patients.
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FIGURE LEGENDS

Figure 1. Dendritic cells and priming of adaptive immunity to fungi.

There are at least 5 subsets of DCs that participate in priming T-cells during fungal infection. Lung DCs can be divided into those that are CD11b+ and CD11b-. CD103+ resident cDCs are important in response to viruses, whereas inflammatory DCs participate in response to several fungal pathogens, and plasmacytoid DCs are vital in immunity to *Aspergillus*. Inflammatory monocyte-derived DCs (moDCs) are CD11b+ and Ly6Chigh. These cells express the chemokine receptor CCR2, which mediates egress from the marrow chiefly in response to the chemokines CCL2 and CCL7. In the absence of CCR2 (CCR2-/− mice), animals evince a skewed Th response in the lung, dominated by Th2 cytokines. Inflammatory monocyte-derived DCs also deliver subcutaneously injected vaccine yeast into draining lymph nodes, where they collaborate with migratory dermal and Langerhans DCs in priming CD4 T-cells upon antigen transfer into resident lymph node DCs. Dermal DCs elaborate IL-12 and IL-27 and efficiently prime Th1 cells, whereas Langerhans DCs elaborate IL-1β, IL-6 and IL-23 and skew the response toward Th17. *Candida*, a commensal of the intestinal tract, and other yet unidentified fungi make up the “mycobiome” and modulate host physiology through interaction with C-type lectins such as Dectin-1 likely displayed on intestinal DCs. DCs in the lamina propria (LP-DC) influence the development of Treg, whereas those in the Pyer’s patch (PP-DC) have not been investigated with respect to fungi.
Recognition of fungal pathogen-associated molecular patterns (PAMPs) is mediated by TLRs and CLR. The binding of fungi or β-glucan to dectin-1 recruits SYK to the two phosphorylated receptors, which leads to the formation of a complex involving Card9, BCL10 and MALT1 (BCM). This results in the release of NF-κB consisting of either p65-p50 or REL-p50 dimers into the nucleus. Syk activation also induces the noncanonical NF-κB pathway mediated by NF-κB inducing kinase (NIK) and the nuclear translocation of p52-RELB dimers. Dectin-1 enhances TLR2 and TLR4-induced cytokines in a Syk-independent manner through the serine/threonine protein kinase RAF1 by Ras proteins, which leads to the phosphorylation of p65. Among other cytokines, these pathways lead to the production of IL-6, IL-23 and IL-12 that induce Th17 and Th1 cells, respectively. Dectin-1 recognition of *C. albicans* can also activate the NLRP3 inflammasome through a mechanism that involves Syk, ROS and potassium efflux. Fungus induced pro-IL-1β is cleaved by active caspase-1 to bioactive IL-1β to favor Th17 development. Dectin-2 activation leads to FcRγ dependent recruitment and phosphorylation of Syk and activated NF-κB and MAPKs (p38, JNK and Erk). Card9 is required for the activation of NF-κB and production of cytokines that lead to Th17 cell differentiation.

Recognition of α-mannose in *Malassezia* species by Mincle activates the FcRγ-Syk-Card9 pathway and translocates NF-κB into the nucleus to induce the activation of pro-inflammatory cytokines. Although fungal PAMPs have yet to show the ability to induce a distinct T helper subset by this pathway, the mycobacterial cord factor and its synthetic analog are potent adjuvants for the differentiation of Mincle-induced Th1 and Th17 cells. Dectin-3 (MCL, Clec4d or Clecsf8) also recognizes α-mannan from *C. albicans* and TDM from *M. tuberculosis*. Dectin-3 can dimerize with Dectin-2 and Mincle. It is unclear whether the induction of Th17 and Th1 cells requires recognition of fungal PAMPs by homo- vs. hetero-dimers of Dectin-2. The MR lacks a classical signaling motif in its short cytoplasmic
tail, but it induces pro-inflammatory cytokines that have been implicated in Th17 and Th1 differentiation. Although MR dependent triggering of human memory T cells produced IL-17, further studies with naïve T cells will be needed to establish the role of the MR in Th17 cell differentiation. Myd88 is critical for the signaling of TLR2 and TLR4. Phospholipomannans and O-linked mannans are recognized by TLRs at the plasma membrane, whereas fungal nucleic acids are sensed by endosomal TLRs and induce NF-κB-, MAPK- and IRF-dependent cytokine production. TLR2 signaling is thought to generate weaker pro-inflammatory signals, but induce strong stimulation of TGF-β and IL-10 that induces Treg cells.

Figure 3. Schematic illustration of different subsets of T lymphocytes and their roles in combating different fungal pathogens.

T cells form an integral part of adaptive immunity in vertebrates. They are classified into 2 distinct lineages; CD4⁺ (also known as T\(_\text{H}_1\) cells) and CD8⁺ cells. The former are further subdivided into T\(_\text{H}_1\), T\(_\text{H}_2\), T\(_\text{H}_17\), and Treg cells based on their effector functions. T\(_\text{H}_1\) cells are the primary source of IFN-γ and are critical for host defense against a majority of fungal pathogens. Similarly, T\(_\text{H}_17\) cells orchestrate potent anti-candidacidal activity by secreting IL-17A, IL-17F and/or IL-22. In contradistinction, T\(_\text{H}_2\) cell-derived cytokines are associated with exacerbation of most fungal infections, with the exception of pneumocystosis. Treg cells prevent excessive damage to the host during infection through various soluble mediators and contact dependent mechanisms. CD8⁺ T cells represent an additional line of defense to combat fungal infections. Two distinct lineages namely Tc1 (IFN-γ producing CD8⁺ T cells) and Tc17 (IL-17 producing CD8⁺ T cells) may exist to bolster T\(_\text{H}_1\) and T\(_\text{H}_17\) immunity respectively.
On encountering fungal pathogens, B cells undergo clonal expansion and antibody class switching. Depicted below are the five major classes of immunoglobulins secreted by B cells, namely, IgG, IgM, IgA, IgE, and IgD. While specific roles of the former three have been reported in fungal diseases, the impact of IgE in this setting remains unclear. IgD has the ability to bind to outer surfaces of certain bacterial pathogens in the respiratory mucosa \(^{214}\), however, its importance in fungal infections has not been studied.
Figure 2
Figure 3

T Cell

CD 8+
- Critical for vaccine induced immunity against:
  - A. fumigatus
  - B. dermatitidis
  - H. capsulatum

CD 4+

Th1
- IFN-γ
- TNF-α
- GM-CSF
- Protective against:
  - C. neoformans
  - C. albicans
  - H. capsulatum
  - B. dermatitidis
  - P. brasiliensis
  - A. fumigatus

Th2
- IL-4
- IL-5
- IL-13
- Protective against:
  - Pneumocystis sp.

Th17
- IL-17A
- IL-17F
- IL-22
- Protective against:
  - C. albicans
  - C. neoformans
  - H. capsulatum
  - B. dermatitidis
  - C. posadasi
  - P. murina

Tregs
- IL-10
- TGF-β
- Immunoregulatory functions in most fungal infections
Figure 4

Different subsets with varying degrees of protection against *C. neoformans*, *C. albicans*, *A. fumigatus*, *H. capsulatum*, *P. brasiliensis*

Protective against *C. neoformans*, *C. albicans*, *H. capsulatum*, *P. murina*, *P. brasiliensis*

Protective against *C. albicans*

Hallmark of T<sub>2</sub> immune response; uncharacterized function against fungal pathogens

Unknown function in mycoses

Participates in opsonization, antibody-directed cellular toxicity, modification of the inflammatory response, and direct fungicidal activity

Participates in opsonization, complement activation, and direct fungicidal activity

Direct fungicidal activity demonstrated in systemic candidiasis. Possible role in mucosal immunity against *Candida*
REFERENCES


CHAPTER 3

Eosinophils Subvert Host Resistance to an Intracellular Fungal Pathogen by Instigating Non-Protective IL-4
Eosinophils Subvert Host Resistance to an Intracellular Fungal Pathogen by Instigating Non-Protective IL-4

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Running Title:
Type II cytokine-driven IL-33 response in mycoses
Abstract

Eosinophils shape type II immune responses in helminth infections and allergic diseases, however, their influence on fungal infections is unknown. We previously reported that CCR2−/− mice exposed to the fungal pathogen *Histoplasma capsulatum* exhibit dampened immunity due to an exaggerated IL-4 response. We sought to identify the cellular source promulgating IL-4 in infected mutant animals. Eosinophils were found to be the principal instigators of non-protective IL-4 and depleting this granulocyte population improved fungal clearance in CCR2−/− animals. The undesired accumulation of eosinophils in the lungs of mutant mice was due to CCL7 engaging chemokine receptors selectively expressed on these cells. The deleterious impact of eosinophilia on mycosis was also recapitulated in transgenic animals overexpressing eosinophils. Mechanistic examination of IL-4 induction revealed that phagocytosis of *H. capsulatum* via the pattern recognition receptor CR3 triggered the heightened cytokine response in murine eosinophils. This phenomenon was also conserved in human eosinophils, since exposure of cells to the fungal pathogen elicited a robust IL-4 response. Thus, our findings elucidate a detrimental attribute of eosinophil biology in fungal infections that could potentially trigger a collapse in host immunity.
**Introduction**

Type II immune responses represent an effective strategy developed by the host to combat helminth parasites \(^1\). Several effector functions associated with anti-helminth immunity are mediated by IL-4 \(^2,3\). Conversely, this cytokine exhibits a pathologic role in the scenario of intracellular infections \(^4,5\) and allergic diseases such as asthma and eczema \(^6,7\). A long standing interest in the field has been to identify initial cellular sources of IL-4 that trigger type II immune responses. Leukocytes including eosinophils, mast cells, basophils, NKT cells, and the recently described group 2 innate lymphoid cells (ILC2s) have been implicated as important sources of innate IL-4 \(in\;vivo\) \(^8-12\). Eosinophils, in particular, store preformed IL-4 within intracellular crystalloid granules that are rapidly secreted upon cell activation \(^13\). This eosinophil-derived IL-4 contributes to the development of T\(\text{H}2\) cells in allergic disorders \(^14\) and regulates metabolic homeostasis through maintenance of alternatively activated macrophages in adipose tissues \(^15\).

*Histoplasma capsulatum* is an intracellular fungal pathogen and the etiologic agent of histoplasmosis. The fungus is endemic to the Midwestern regions of the United States, and immunocompromised patients (such as those suffering from AIDS) inhabiting these areas often contract this fungal disease \(^16\). An estimated 25,000 life threatening *H. capsulatum* infections are reported every year in the US \(^17\). In contrast, infections in immunocompetent individuals are generally asymptomatic and efficiently resolved.

The successful clearance of *H. capsulatum* is dependent on the coordinated action of innate and adaptive immune responses \(^18\). In the environment, the pathogen gains entry into the host through the pulmonary route, wherein it is internalized by phagocytes. Ingestion of the organism \(via\) the pattern recognition receptor, CR3 triggers innate responses that consequently shape T\(\text{H}1\)
Proinflammatory cytokines IFN-γ, and GM-CSF activate macrophages to inhibit the growth of *H. capsulatum* [20,21]. In contradistinction, T<sub>H</sub>2 cytokines polarize macrophages to an alternatively activated phenotype, thus providing favorable environment for *H. capsulatum* to proliferate, and ultimately increase vulnerability to the disease [5,22].

We have reported that enhanced susceptibility of CCR2<sup>−/−</sup> mice to *H. capsulatum* infection is primarily attributed to an exaggerated IL-4 response generated in the lungs [5]. Here, we show that eosinophils were the primary drivers of the heightened IL-4 response in infected mutant mice and depletion of these granulocytes improved fungal clearance. Undesired accrual of eosinophils in infected CCR2<sup>−/−</sup> mice was a result of CCL7 engaging chemokine receptors preferentially expressed on the granulocytes. The pathologic role of eosinophils in subverting antifungal immunity was further evidenced in animals overexpressing these cells. Weakening of host defenses against *H. capsulatum* was due to phagocytosis of the fungal yeasts by eosinophils that prompted a robust non-protective IL-4 response. Finally, this phenomenon was also found to be conserved in human eosinophils as they internalized *H. capsulatum* and mounted an amplified IL-4 response in comparison to uninfected cells.
Results

Identification of IL-4+ cells in CCR2−/− mice during fungal infection

CCR2−/− mice manifest an augmented fungal burden and exaggerated IL-4 in the lungs in histoplasmosis. In order to identify the source of IL-4 in infected mutant animals, we generated CCR2−/−.IL-4 reporter mice (designated as CCR2−/−.4get mice) by crossing CCR2−/− and 4get mice on a C57BL/6 background. Analogous to CCR2−/− mice, the transgenic reporter mice exhibited a heightened pulmonary fungal burden in comparison to the controls at day 7 of H. capsulatum infection. The Mean ± SEM log_{10}CFU in CCR2−/−.4get mice - 7.29±0.11 exceeded that of WT.4get mice- 6.13±0.15, n=6, P<0.01. Previous observations have indicated that CCR2−/− mice mount an elevated IL-4 response as early as day-3 of infection. Concordantly, we found CCR2−/−.4get animals expressed higher percentage and absolute number of IL-4+ cells in the lungs in comparison to WT controls at day 3 p.i (Fig 1A). Gating on the IL-4+ cell population revealed that the majority of those cells were eosinophils (defined as SSCFceRI−SiglecF+CD11b+) (Fig 1B). Elevated IL-4+ cells in the lungs of CCR2−/−.4get mice were not a result of a preexisting bias towards an IL-4 response. Both the WT and CCR2−/− reporter mice expressed similar frequency of IL-4+ cells prior to infection (Fig 1C). To verify if CCR2−/− mice displayed increased accumulation of eosinophils during fungal infection, we analyzed these leukocytes in the lungs at day-3 p.i. Similar to the transgenic reporter mice, we observed a greater number and percentage of eosinophils (but not other cell populations) in CCR2−/− mice, in comparison to controls (Fig 1D & E). Together, these data suggested that eosinophils were the innate source of IL-4 in infected CCR2−/− animals.

Eosinophil depletion in CCR2−/− mice results in decreased IL-4 and fungal burden
To determine if eosinophils were the chief contributors to the exaggerated IL-4 response in CCR2\(^{-/-}\) mice, we depleted this cell population using the IL-5 neutralizing antibody. We observed specific diminution of the eosinophil population in the lungs using this monoclonal antibody (Fig 2A). Consequently, the αIL-5-treated CCR2\(^{-/-}\) mice expressed a reduced percentage and number of IL-4\(^{+}\) cells as opposed to control IgG-treated group after 3 days of infection (Fig 2B-D). To substantiate that eosinophils were the causative agent of the dominant IL-4 response in CCR2\(^{-/-}\) mice, the quantity of this cytokine was analyzed in the lungs of mice 7 days after they were challenged with *H. capsulatum*. A marked decrease in pulmonary IL-4 protein was noted following eosinophil depletion (Fig 2E). Furthermore, we detected diminished transcription of IL-4-regulated genes including Arg-1 and YM-1 in the lungs of αIL-5-treated group (Fig 2F & G). Finally, a significant lowering in fungal burden (at one week of infection) was observed in CCR2\(^{-/-}\) mice that received IL-5 neutralizing antibody (Fig 2H). Treatment of the WT mice with αIL-5 antibody did not diminish the pulmonary fungal burden (Fig 2H). These findings establish that eosinophils are the key instigators of the dominant IL-4 response in CCR2\(^{-/-}\) mice that augments their susceptibility to *H. capsulatum*.

**In the absence of CCR2, CCL7 directs recruitment of eosinophils to the site of infection**

We investigated the reason for increased influx of eosinophils to the lungs of CCR2\(^{-/-}\) mice during *H. capsulatum* infection. CCL7 is a major ligand of CCR2 and mice deficient in the latter exhibit elevated protein levels of the ligand during infection (data not shown). CCL7 has also been reported to bind to CCR3, a chemokine receptor highly expressed on eosinophils. Thus, we hypothesized that in the CCR2\(^{-/-}\) mice, CCL7 would recruit eosinophils to the site of infection by engaging CCR3. To test the hypothesis, we neutralized CCL7 in infected mutant mice and determined eosinophil count in the lungs 3 days after *H. capsulatum* challenge. A decrement in
eosinophil population was noted in anti-CCL7-treated group as opposed to control IgG-treated group (Fig 3A).

**IL-5 transgenic animals manifest enhanced susceptibility to *H. capsulatum* infection**

To test if an increased number of eosinophils subvert host defense against *H. capsulatum* in an additional experimental model, we utilized the IL-5 transgenic mice. These animals exhibit constitutive eosinophilia in their blood and peripheral organs including the lungs (Fig 4A). Although the transgenic mice are on a Balb/C background, we have previously reported similar *H. capsulatum* burden in C57BL/6 and Balb/C strains. In comparison to WT controls, an elevated fungal burden was discerned in the IL-5 transgenic mice after 7 days of *H. capsulatum* challenge (Fig 4B). Furthermore, the infected transgenic mice expressed higher amount of IL-4 in the lungs (Fig 4C). No noteworthy differences were observed in pulmonary IL-4 concentration between the WT and IL-5 transgenic mice prior to infection. These data reinforce the deleterious role of eosinophils during histoplasmosis.

**Phagocytosis of *H. capsulatum* by murine eosinophils results in IL-4 secretion**

A prior report has indicated that eosinophils display phagocytic activity against the pathogenic fungus *Cryptococcus neoformans*. To test if these granulocytes phagocytose *H. capsulatum* yeasts, we infected murine bone marrow derived eosinophils with GFP-labeled *H. capsulatum*. Flow cytometric analysis revealed distinct association of yeasts with eosinophils at 1 and 5 multiplicity of infection (MOI) (Fig 5A & B). To investigate if *H. capsulatum* yeasts localized within the eosinophils, we employed confocal microscopy. Examination of Z-stacked images of infected cells demonstrated that they had completely internalized GFP- *H. capsulatum* (Supp Fig...
1). Furthermore, in three experiments, extended exposure (24 h) to *H. capsulatum* induced cell death in >80% of the eosinophils (as measured by trypan blue exclusion).

Our *in vivo* findings suggested that eosinophils were a prominent source of IL-4 in fungal infection. We therefore measured the concentration of this cytokine in culture supernatants of *H. capsulatum*-infected eosinophils. We noted robust expression of IL-4 protein that increased with the duration and magnitude of infection (Fig 5C & D). These findings signify the phagocytic capacity of eosinophils against *H. capsulatum* and also that they are a potent source of IL-4 in fungal infection.

**H. capsulatum uptake by eosinophils is dependent on CR3**

Previous studies have reported the involvement of CR3 complex (CD11b/CD18) in uptake of non-opsonized *H. capsulatum* yeasts by phagocytes including macrophages and neutrophils. To test if engagement of the same pattern recognition receptor was requisite for fungal uptake by eosinophils, we blocked the CD18 subunit on the cells. Eosinophils pre-treated with αCD18 antibody displayed a marked decrease in phagocytosis of non-opsonized yeasts as compared to control IgG treated cells (Fig 6A). The former group also manifested a diminished IL-4 response following infection (Fig 6B). In these experiments, we used a monoclonal antibody directed against CD18 (clone GAME-46). Others have reported this antibody displays similar efficacy in blocking phagocytosis of *H. capsulatum* as the anti-CR3 antibody, suggesting the two can be used interchangeably. Inhibition of signaling through Syk kinase (downstream of CR3) has been shown to disrupt phagocytosis. To further substantiate the contribution of CR3 in eosinophilic phagocytosis of fungal yeasts, we treated cells with the Syk-inhibitor, Piceatannol and exposed
them to *H. capsulatum*. We detected a pronounced decrement in phagocytosis and IL-4 release by eosinophils (Fig 6C & D).

**Uptake of *H. capsulatum* by murine eosinophils *in vivo***

We investigated if murine eosinophils phagocytosed *H. capsulatum in vivo*. We utilized the CCR2<sup>−/−</sup> model since these animals mount an eosinophilic response in the lungs upon fungal challenge (as indicated above). We infected the mutant mice with GFP-labeled *H. capsulatum* and analyzed the leukocyte population 3 days later. The mice were infected with a high infectious dose (2x10<sup>7</sup> yeasts) to increase the probability of detecting *H. capsulatum*-associated eosinophils. We observed a small fraction of GFP<sup>+</sup> eosinophils (SSC<sup>hi</sup> FceRI<sup>+</sup> SiglecF<sup>+</sup> CD11b<sup>+</sup>) in the lungs of CCR2<sup>−/−</sup> mice (Fig 7A). To confirm these cells were eosinophils, we FACS-sorted the cell population and stained them with hematoxylin & eosin (H&E). Microscopic analysis revealed these cells to be eosinophils. We observed intracellular yeasts within eosinophils (Fig 7B). Thus, phagocytosis of *H. capsulatum* by eosinophils is not restricted to *in vitro* conditions but can occur *in vivo* as well.

**H. capsulatum** infection induces an IL-4 response in human eosinophils

We examined if human eosinophils manifest similar properties as their murine counterparts. Eosinophils were purified from human peripheral blood and infected with GFP<sup>+</sup> *H. capsulatum* for 4 hrs. Flow cytometric analysis indicated a clear association between these granulocytes and the yeasts (Fig 8A). These results were confirmed by H&E staining of infected cells (Fig 8B). To determine if *H. capsulatum* infection promulgated an IL-4 response in eosinophils, we measured the cytokine in cell culture supernatants. No detectable IL-4 could be discerned (IL-4 < 12 pg/ml). However, we did observe amplification in IL-4 protein by intracellular staining of
infected eosinophils as opposed to uninfected cells (Fig 8C). Thus, these data demonstrate that human eosinophils manifest heightened IL-4 response following phagocytosis of *H. capsulatum.*
Discussion

Animals deficient in the chemokine receptor CCR2 exhibit dampened immunity to *H. capsulatum* due to exaggerated IL-4 in the lungs. This dominant IL-4 response is detected at day 3 of fungal infection, raising the possibility that an innate cellular source is generating IL-4. We found eosinophils to be the primary initiators of non-protective IL-4 as depletion of this granulocyte population abrogated the IL-4 response and improved the outcome of infection in CCR2\(^{-/-}\) animals. The adverse impact of eosinophilia on histoplasmosis was also demonstrated in IL-5 transgenic animals that constitutively express high numbers of eosinophils. These mice manifested elevated pulmonary fungal burden that was accompanied by increased IL-4 protein concentrations. Mechanistic examination of IL-4 induction revealed that phagocytosis of *H. capsulatum* yeasts prompted the heightened cytokine response in murine and human eosinophils. Our findings reveal an unappreciated attribute of eosinophil biology that could potentially be detrimental to the host in intracellular fungal infections.

Overproduction of IL-4 enhances susceptibility to intracellular microbial pathogens by disturbing the T\(_{H1}\)/T\(_{H2}\) balance in the host\(^{4,28}\). This scenario is effectively demonstrated in CCR2\(^{-/-}\) animals challenged with fungal pathogens *H. capsulatum* or *Cryptococcus neoformans* \(^{5,29}\). In cryptococcosis, T\(_{H2}\) cells are implicated as the source of IL-4 in these mutant mice \(^{30}\), while the cellular source or sources of this cytokine are unclear in *H. capsulatum*-challenged CCR2\(^{-/-}\) mice. Identification of the origins of IL-4 will aid in designing better therapeutic strategies to counter mycoses and also enhance our understanding of how type II immune responses are initiated and sustained. Accordingly, we sought to determine the nature of the cells instigating IL-4 in CCR2\(^{-/-}\) mice exposed to *H. capsulatum*. We chose to focus at day 3 of fungal infection since our previous data indicates the dominant IL-4 response is initiated at this particular time point.
Several innate cell populations including basophils, mast cells, NKT cells, and type II innate lymphoid cells have been shown to prompt IL-4 generation, however, we observed comparable percentages and numbers of these leukocytes in infected WT controls and CCR2\(^{-/-}\) animals. Thus it was unlikely these cells made a significant contribution to the amplified IL-4 response in infected mutant mice. In contrast, we observed an increased pulmonary eosinophilic response in CCR2\(^{-/-}\) mice that suggested a possible involvement of these granulocytes in triggering the elevated IL-4 in \textit{H. capsulatum} infection. Indeed, depletion of eosinophils greatly diminished IL-4 in infected CCR2\(^{-/-}\) mice. T\(_{H2}\) cells did not contribute to this heightened IL-4 response since we have demonstrated earlier that loss of CD4\(^{+}\) T cells does not impact pulmonary IL-4 concentrations in CCR2\(^{-/-}\) mice\(^5\).

Defective regulation of IL-4 responses during histoplasmosis augments vulnerability to the disease\(^{31}\). This cytokine induces alternative activation of macrophages that is associated with uncontrolled \textit{H. capsulatum} growth. Mechanistically, IL-4 drives the expression of arginase-1, an enzyme that diminishes the amount nitric oxide required for fungicidal activity in macrophages\(^{32}\). In addition, alternatively activated macrophages exhibit augmented intracellular stores of zinc required for \textit{H. capsulatum} proliferation\(^{33}\). We observed increased expression of markers associated with alternative macrophage activation in addition to elevated fungal burden in infected CCR2\(^{-/-}\) mice, thus indicating detrimental effects of eosinophil-derived IL-4 on the host. In another experimental model of IL-5 transgenic mice, higher numbers of eosinophils were found to subvert antifungal immunity against \textit{H. capsulatum}. Heightened fungal burden was accompanied by amplified IL-4 concentrations in the lungs of these transgenic animals in comparison to infected controls, further emphasizing the adverse impact of IL-4 producing eosinophils on \textit{H. capsulatum} infection.
In CCR2\textsuperscript{−/−} animals, eosinophil accumulation at the site of infection was a result of CCL7 engaging chemokine receptors preferentially on these granulocytes. Although CCL7 is a primary ligand for CCR2, the former has been shown to associate with CCR3\textsuperscript{24}. Other CCR3 ligands, eotaxin-1 and eotaxin-2 have been described as potent chemoattractants of eosinophils to the lungs\textsuperscript{34}. However, we noted comparable concentrations of both chemokines between WT and CCR2\textsuperscript{−/−} animals during \textit{H. capsulatum} infection, suggesting the non-involvement of eotaxins in this setting. Thus, mutant mice deficient in CCR2 represent a unique scenario where undesired recruitment of eosinophils to the site of infection is directed by CCL7.

Another unexpected finding was that murine eosinophils phagocytosed fungal yeasts upon contact in \textit{in vitro} and \textit{in vivo} settings. Besides secreting IL-4, these cells were inefficient at fungal killing since we observed comparable \textit{H. capsulatum} growth between eosinophil culture and control media (data not shown). In fact, prolonged exposure to \textit{H. capsulatum} induced cell death in majority of the eosinophils. These observations are in stark contrast to reports describing antimicrobial activity of eosinophils against \textit{Cryptococcus neoformans}, \textit{Staphylococcus aureus}, and \textit{Escherichia coli}\textsuperscript{35,36}.

CR3 is the major innate receptor that participates in detection and uptake of non-opsonized \textit{H. capsulatum} by phagocytes\textsuperscript{19,37}. Eosinophils were found to engage the same receptor in phagocytosis of fungal yeasts. Inhibiting internalization of fungi also disrupted the ensuing IL-4 response in infected eosinophils. These observations are in agreement with a recent report describing the involvement of CR3 signaling in phagocytosis and downstream cytokine responses in \textit{H. capsulatum}-infected macrophages\textsuperscript{19}. Although phagocytosis and activation of CR3 signaling are two independent biological events, they cannot be uncoupled in \textit{H. capsulatum} infection\textsuperscript{38}. This is because blockade of one disrupts the other and \textit{vice versa}. 
Hence, we were unable to determine if activation of CR3 pathway without switching on the phagocytic machinery of eosinophils would prompt a strong IL-4 response.

In concordance with our findings in murine eosinophils, human eosinophils phagocytosed *H. capsulatum* yeasts and mounted an amplified IL-4 response. We failed to detect IL-4 protein in cell culture supernatants, rather, augmented intracellular protein content of the cytokine in infected cells was noted by flow cytometry. Others have reported that human eosinophils are not adept at secreting IL-4 in *in vitro* culture conditions despite possessing intracellular stores of the cytokine. Failure to detect IL-4 in *H. capsulatum*-infected human eosinophil cultures could likely be due to the limitations of an *in vitro* system.

Prior data suggest that a subset of patients with disseminated histoplasmosis may manifest a type II immune response phenotype. Based on our findings, it is possible that eosinophils are the origin of type II cytokines in that subgroup. Several case reports document the presence of eosinophilia in patients with disseminated histoplasmosis. These patients exhibited >4% eosinophilia coincident with the diagnosis of histoplasmosis. Moreover, eosinophilia has also been described in other *H. capsulatum*-infected mammals including canines. Apart from histoplasmosis, high numbers of eosinophils have been observed in individuals suffering from other fungal diseases such as coccidioidomycosis and paracoccidioidomycosis. In such reports, it is difficult to discern if the eosinophilia was present prior to the onset of clinical symptomatology or was manifest only at the time of presentation. Regardless, the heightened number of eosinophils in these subjects may provide a clue that the balance between type I and type II immunity is perturbed.
In summary, we present compelling evidence that highlights the detrimental attribute of eosinophils in impairing immunity against an intracellular fungal pathogen, *H. capsulatum*. Although this cell population is critical for anti-helminth immunity, it enhances susceptibility to *H. capsulatum* by driving a non-protective IL-4 response. Specific depletion of these granulocytes in eosinophilic patients suffering from histoplasmosis or other mycotic infections represents a novel and exciting therapeutic approach that could potentially be used to improve the outcome of the diseases.
Materials and Methods

Mice

Male C57BL/6 and breeding pairs of CCR2−/− (C57BL/6 background) mice were purchased from The Jackson Laboratory, Bar Harbor, ME. 4get mice (generously provided by Drs. Fred Finkelman and Richard Locksley of Cincinnati Childrens’ Hospital Medical Center and the University of California San Francisco, respectively) were backcrossed to C57BL/6 background (>10 generations). CCR2−/−.4get mice were generated by crossing 4get mice with CCR2−/− animals. Male IL-5 transgenic mice (on Balb/C background) and sex matched BALB/c WT controls were provided by Dr. Marc Rothenberg (Cincinnati Childrens’ Hospital Medical Center). Animals were housed in isolator cages and were maintained by the Department of Laboratory Animal Medicine (University of Cincinnati, Cincinnati, OH), 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 and green fluorescent expressing yeast were grown for 72 hours at 37°C as previously described 50. To produce infection in mice, 6-8 week old animals were inoculated intranasally (i.n.) with 2 x 10⁶ yeast cells in a ~30μl volume of HBSS (HyClone, Logan, UT).

Organ culture for H. capsulatum
Organs were homogenized in sterile HBSS and serially diluted and plated onto mycosel-agar plates containing 5% sheep blood and 5% glucose. Plates were incubated at 30°C for 8 days. The limit of detection was $10^2$ CFU.

**In vivo neutralization experiments**

To deplete eosinophils, mice were injected intraperitoneally (i.p.) with 25 μg of anti-human/mouse IL-5 monoclonal antibody (TRFK5 clone; R&D Systems, Minneapolis, MN) or isotype control antibody (purchased from Bio X Cell, Lebanon, NH) on days -1, 2, and 5 days of infection. For CCL7-neutralization, 100 μg of polyclonal goat anti-mouse CCL7 (R&D Systems) or goat IgG in 0.5 ml HBSS was administered i.p on days -1 and 2 of infection.

**Isolation of lung leukocytes**

Lungs were homogenized with the gentleMACS™ dissociator (Miltenyi Biotec, Auburn, CA) in 5 ml of HBSS with 2 mg/ml of collagenase D (Roche, Mannheim, Germany) and 40 U/ml of DNase I (Roche) for 30 min at 37°C. Following treatment, the homogenate was percolated through a 60μm nylon mesh (Spectrum Laboratories, Rancho Dominguez, CA) and washed 3 times with HBSS. Leukocytes were isolated by separation on Lympholyte M (Cedarlane Laboratories, Burlington, ON).

**Flow cytometry and cell sorting**

The phenotype of cells from mouse lungs was determined by incubating lung leukocytes with the indicated antibodies and CD16/32 to limit nonspecific binding. Leukocytes were stained at 4°C for 15 min in PBS containing 1% BSA and 0.01% sodium azide. Cells were stained with combinations of the following antibodies: PE-conjugated CD3, FcεRI, and SiglecF; PerCP-conjugated cKit, and Streptavidin; Biotinylated-FcεRI; and APC-conjugated NK1.1, CD49b, and
CD11b from BD Biosciences (San Diego, CA). PE-conjugated Lineage cocktail was purchased from BioLegend, San Diego, CA. Human cells were stained with Alexa Fluor® 647-conjugated CD193 from BD Biosciences. For intracellular IL-4 staining, human eosinophils were incubated with Cytofix/Cytoperm (BD Biosciences), washed in Permeabilization Buffer (BD Biosciences), and stained for 60 min with PE-conjugated anti-human IL-4 (MyBioSource, San Diego, CA). Cells were washed and resuspended in 1% paraformaldehyde to fix. Appropriate isotype controls were performed in parallel. Data was acquired using a BD Accuri™ C6 (BD Biosciences) flow cytometer and analyzed using FCS Express 4.0 Software. For cell sorting experiments, SSChi SiglecF+ FcεRI+ CD11b+ leukocytes from the lungs of CCR2−/− mice were isolated at day 3 p.i. using 5-laser FACS Aria II (BD Biosciences) in a BSL-II facility at Cincinnati Childrens’ Hospital.

**Generation of bone marrow derived eosinophils**

Bone marrow cells were isolated from the hind tibia and femurs of 6-10 week old mice by flushing with HBSS, and erythrocytes were lysed using RBC lysis buffer. Following a density gradient of Histopaque 1083 (Sigma-Aldrich, St. Louis, MO), the low density bone marrow cells were collected and plated at 1 x 10^6 cells/ml in Iscove’s Modified Dulbecco’s Medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum, 0.1% gentamycin sulfate, 200mM L-glutamine (Life Technologies), and 55μM 2-mercaptoethanol. During the first 4 days, the medium also contained stem cell factor (PeproTech, Rocky Hill, NJ) and Fms-like tyrosine kinase 3 ligand (PeproTech) at 100 ng/ml each. From day-4 to day-14, the cells were cultured in medium containing 10 ng/ml IL-5 (PeproTech). The medium was changed every 2 days until day 14. On the final day of the culture, differentiated eosinophils were collected, pooled, and plated for at least 1 hour in a tissue culture dish to remove any contaminating cells.
such as stromal cells or macrophages. Finally, the non-adherent cells were collected, washed, counted, and incubated with different treatments, according to the experiments.

**Isolation of human eosinophils from peripheral blood**

Human blood was purchased from the Hoxworth Blood Center, Cincinnati, OH, and eosinophils were isolated using EasySep™ Human Eosinophil Enrichment Kit (Stemcell Technologies, Vancouver, BC) as per the manufacturer’s protocol. The cell purity was >95% in all experiments (determined by flow cytometry and H&E staining).

**H. capsulatum association with eosinophils**

For *in vitro* murine eosinophil experiments, cells were cultured in IMDM in the presence of recombinant murine IL-5 (10 ng/ml) before being exposed to GFP⁺ *H. capsulatum* yeasts. Fungal association with eosinophils was quantified by flow cytometry. To inhibit *H. capsulatum* internalization, eosinophils were pretreated with either anti-CD18 (clone GAME-46; BD Biosciences) or Piceatannol (TOCRIS, Bristol, UK), 90 min prior to infection. Human eosinophils were cultured in RPMI medium with recombinant human IL-5 (10 ng/ml) and were subjected to infection with GFP⁺ *H. capsulatum*.

**Microscopy**

Eosinophils were infected with GFP⁺ *H. capsulatum* for 6 hours. Cells were then washed with PBS containing 1% BSA and mounted on glass slides using Fluromount-G (SouthernBiotech, Birmingham, AL). Images were acquired on a Zeiss LSM710 confocal microscope and analyzed with ImageJ software.

**RNA Isolation, cDNA synthesis, and quantitative real time reverse transcription PCR (qRT-PCR)**
Total RNA from whole lungs of mice was isolated using TRIzol (Invitrogen). Oligo(dT)-primed cDNA was prepared by using the reverse transcriptase system (Promega, Madison, WI) according to the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) for analysis of gene transcription was performed using TaqMan master mixture and primers obtained from Applied Biosystems (Foster City, CA). Samples were analyzed with ABI Prism 7500 (Applied Biosystems). In each experiment, the hypoxanthine phosphoribosyl transferase (HPRT) housekeeping gene was used as an internal control. The conditions used for amplification were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

**Measurement of IL-4 by ELISA**

IL-4 protein concentration was quantified in lung homogenates (dissolved in 5 ml HBSS) and eosinophil-culture supernatants by using an enzyme-linked immunosorbent assay (ELISA) kit that was purchased from R&D Systems.

**Statistics**

Statistics were performed using the Student's t test or one-way ANOVA with Boneferroni’s correction. P value of < 0.05 was considered statistically significant. For all graphs, * P=0.01-0.05, ** P=0.005-0.01, and *** P<0.005.
Figure Legends

Figure 1

Identification of IL-4+ cells in CCR2−/− mice during *H. capsulatum* infection.

(A) Quantitative analysis of IL4-eGFP+ cells in the lungs of WT and CCR2−/− mice at day 3 p.i by flow cytometry. (B) Identification of IL4-eGFP+ eosinophils at day 3 p.i. Eosinophils defined as SSChi SiglecF+ FceRI+ CD11b+. (C) Comparison of IL4-eGFP+ cells in the lungs of WT and CCR2−/− mice prior to infection. (D and E) Analysis of leukocytes associated with innate IL-4 production in WT and CCR2−/− (non-reporter) mice at day 3 p.i. All FACS plots are representative of 1 of 8 mice from two independent experiments. Graphical data are depicted as mean ± SEM, n=8 from 2 independent experiments.

Figure 2

Consequence of eosinophil depletion in CCR2−/− mice.

(A) FACS analysis of eosinophils in the lungs of CCR2−/−.4get mice treated with anti-IL5 or isotype control antibody at day 3 p.i. (B, C, and D) FACS analysis of pulmonary IL4-eGFP+ cells in control IgG or anti-IL5-treated CCR2−/−.4get mice at day 7 p.i. (E) Measurement of IL-4 protein concentration in lung homogenates of control IgG or anti-IL5-treated CCR2−/− mice at day 7 p.i by ELISA. (F and G) qRT-PCR analysis of IL-4-dependent genes following eosinophil depletion (day 7 p.i). (H) Pulmonary fungal burden represented as log_{10}CFU in control IgG or anti-IL5-treated CCR2−/− mice at day 7 p.i. All FACS plots are representative of 1 of 7-8 mice from two independent experiments. Graphical data are depicted as mean ± SEM, n=7-8 from 2 independent experiments.
Figure 3

Neutralization of CCL7 in *H. capsulatum*-infected CCR2−/− mice.

(A) FACS analysis of eosinophils in the lungs of CCR2−/− mice treated with anti-CCL7 or isotype control antibody at day 3 p.i. Plots representative of 1 of 6 mice from two independent experiments. Graphical values are mean ± SEM, n=6 from 2 independent experiments. (B) Transwell assay legend.

Figure 4

*H. capsulatum* infection in IL-5 transgenic mice.

(A) Eosinophil influx in lungs of WT and IL5-transgenic mice prior to infection, as analyzed by flow cytometry. FACS plots representative of 1 of 6 mice from two independent experiments. Fungal burden depicted in log_{10}CFU (B), and IL-4 protein concentration (C) in lungs of WT and IL-5 transgenic animals after 7 days of *H. capsulatum* challenge. Values are represented as mean ± SEM, n=7-8 from 2 independent experiments.

Figure 5

Phagocytosis of *H. capsulatum* by eosinophils results in IL-4 secretion.

(A and B) FACS analysis of bone marrow derived eosinophils challenged with 1 or 5 MOI GFP⁺ *H. capsulatum* yeasts for 6 hour. Plots representative of 1 of 5 independent experiments. (C and D) Measurement of IL-4 protein concentration in culture supernatants of eosinophils infected with different MOI of *H. capsulatum* yeasts for varying time points as indicated. Values are mean ± SEM from at least 3 independent experiments.
Figure 6

Eosinophil uptake of *H. capsulatum* is dependent on CR3.

FACS analysis (A) and measurement of IL-4 protein in culture supernatants (B) of infected-bone marrow derived eosinophils that were pre-incubated with control IgG or CD18 blocking antibody (5μg/ml) for 90 minutes prior to infection, following which they were then infected with 5 MOI GFP+ *H. capsulatum* yeasts for 6 hours. FACS analysis (C) and measurement of IL-4 protein in culture supernatants (D) of infected-bone marrow derived eosinophils pre-incubated with DMSO or Syk inhibitor (50μM) for 90 minutes prior to infection. FACS plots are representative of 1 of 3 independent experiments, and graphical values are mean ± SEM from 4-5 independent experiments.

Figure 7

Association of *H. capsulatum* with eosinophils *in vivo*

(A) FACS analysis of *H. capsulatum*-infected eosinophils *in vivo*. CCR2− mice were infected with 1x10⁷ GFP+ *H. capsulatum* yeasts and GFP+ eosinophils in the lungs were analyzed by flow cytometry 3 days p.i. Plot representative of 1 of 6 mice from two independent experiments. (B) Microscopic analysis of infected eosinophils FACS-sorted from lungs of CCR2− mice, 3 days after challenge. Sorted cells were stained with H&E and analyzed at 1000X magnification. Representative image from 1 of 2 independent experiments.

Figure 8

*H. capsulatum* infection and IL-4 response in human eosinophils.
(A) FACS analysis of human eosinophils incubated without or with 5 MOI GFP$^+$ *H. capsulatum* yeasts for 6 hours. (B) H&E stained image of infected human eosinophils at 1000X magnification. (C) FACS analysis of IL-4 MFI in infected and uninfected human eosinophils. Plots and images are representative of 1 of 3 independent experiments.

**Supplementary Figure 1.**

Phagocytosis of *H. capsulatum* yeasts by murine eosinophils.

*Z* stacked images of an eosinophil infected with 5 MOI of *H. capsulatum*. The images were collected at 0.66μm intervals. Representative image of 1 of 3 independent experiments.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8
Eosinophil Phagocytosis (Supp Fig)

Supplemental Figure 1
References


CHAPTER 4

Type II Cytokines Impair Host Defense Against Intracellular Fungal Pathogen by Amplifying Macrophage Generation of IL-33
Type II Cytokines Impair Host Defense Against Intracellular Fungal Pathogen by Amplifying Macrophage Generation of IL-33

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Running Title:
Type II cytokine-driven IL-33 response in mycoses

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Abstract

IL-4 subverts protective immunity to multiple intracellular pathogens including the fungus *Histoplasma capsulatum*. Previously, we reported that *H. capsulatum*-challenged CCR2<sup>-/-</sup> mice manifest elevated pulmonary fungal burden due to exaggerated IL-4. Paradoxical to our anticipation in IL-33 driving IL-4, we discovered the latter prompted IL-33 in mutant mice. In infected CCR2<sup>-/-</sup> animals, amplified IL-33 succeeded the heightened IL-4 response and inhibition of IL-4 signaling decreased IL-33. Moreover, macrophages, but not epithelial cells or dendritic cells from these mice expressed higher IL-33 in comparison to controls. Dissection of mechanisms that promulgated IL-33 revealed type-II cytokines and *H. capsulatum* synergistically elicited an IL-33 response in macrophages via STAT6/IRF-4 and Dectin-1 pathways respectively. Neutralizing IL-33 in CCR2<sup>-/-</sup> animals, but not controls, enhanced their resistance to histoplasmosis. Thus, we describe a previously unrecognized role for IL-4 in instigating IL-33 in macrophages. Furthermore, in presence of intracellular fungal pathogens, the type-II cytokine-driven IL-33 response impairs immunity.
Introduction

The prototypical Th2 cytokine, IL-4, is vital for host immunity against helminth infections\(^1\) and for wound healing and tissue repair\(^2\). Conversely, the cytokine has deleterious effects in the setting of intracellular infections\(^3,4\) and allergic disorders like asthma and atopic dermatitis\(^5,6\). IL-4 polarizes macrophages to an alternatively activated phenotype, thereby providing a congenial environment for intracellular pathogens to proliferate freely\(^7\). Another key attribute of IL-4 is to differentiate naïve CD4\(^+\) T cells to the Th2 lineage; however, undesired activation of Th2 cells during certain microbial infections suppresses Th1 immune responses\(^8\).

Recently, considerable interest has been generated in the epithelial derived cytokines IL-25, IL-33, and thymic stromal lymphopoietin (TSLP) that are important in initiating IL-4\(^9\). IL-33, in particular, acts on a variety of innate and adaptive immune cell populations including basophils, dendritic cells (DCs), macrophages and CD4\(^+\) T cells via its transmembrane receptor ST2 to induce Th2 immune responses\(^10\).

*H. capsulatum* is a dimorphic intracellular fungal pathogen and the etiologic agent of histoplasmosis. It is found worldwide but there are regions of notably high incidence of infections, such as the Ohio and Mississippi River Valleys in the USA and regions of South America\(^11\). Each year, in the US alone, it is estimated that up to 25,000 people develop life threatening infections in endemic regions and 10-fold more individuals acquire mild or asymptomatic disease\(^12\). To establish extensive infection in humans, *H. capsulatum* transforms into its unicellular yeast phase and disseminates to other organs using macrophages as vehicles. Acute fungal infection in immunocompetent hosts is usually efficiently resolved; however, the more severe symptomatic infections occur in the setting of immunosuppression including patients with AIDS and individuals chronically receiving immunosuppressive drugs\(^13\).
The successful clearance of *H. capsulatum* infection is largely based on the coordinated action of innate and adaptive arm of the immune system. The pathogen initially triggers a host response by activating innate immunity through pattern recognition receptors such as CR3 and Dectin-1 expressed on phagocytes.\(^{14,15}\) This is followed by a robust Th1 immune response characterized by proinflammatory cytokines IFN-γ, TNF-α, and GM-CSF and classical activation of macrophages.\(^{16}\) In contrast, a defective immune response or an overproduction of Th2 cytokines leads to alternative macrophage activation and ultimately increased susceptibility to *H. capsulatum*\(^2\).

In previous work, we reported that in the absence of chemokine receptor 2 (CCR2), mice exhibit enhanced susceptibility to *H. capsulatum* in comparison to wild-type (WT) controls.\(^3\) The major defect in immunity in these mutant mice is heightened production of IL-4 in the lungs that starts as early as day 3 of infection. In this manuscript, we report that CCR2\(^{-/-}\) mice mounted an elevated IL-33 response after initiation of the IL-4 response. Paradoxical to the function of IL-33 in initiating a Th2 immune response, we discovered that IL-4 induced it in the lungs of infected CCR2\(^{-/-}\) mice. Moreover, macrophages were the central source of IL-33 in these mice. Dissection of the molecular mechanism revealed that IL-4 and *H. capsulatum* synergistically evoked IL-33 in macrophages and the signaling pathway was dependent on STAT6/IRF-4 and Dectin-1 respectively. Finally, IL-33 exhibited a deleterious role in *H. capsulatum* infection by inducing alternatively activated phenotype in macrophages, and neutralizing its effects in the CCR2\(^{-/-}\) mice resulted in decreased fungal burden in the lungs. Thus, we report IL-4 and *H. capsulatum* are inducers of IL-33 in macrophages, and the IL-4 driven IL-33 response during fungal infection orchestrates detrimental effects in the host that in turn leads to a collapse in immunity to the disease.
Results

Induction of IL-33 by IL-4 in vivo

*H. capsulatum*-infected CCR2−/− mice display exaggerated levels of IL-4 in the lungs in beginning as early as day 3. Using these mutant mice as a model for dysregulated IL-4 in an intracellular infection, we explored the cytokines associated with regulation of IL-4. TSLP, IL-25 and IL-33 have been implicated in initiating a type II immune response. To investigate their contribution in evoking the dominant IL-4 response in CCR2−/− mice, we measured concentrations in lung homogenates between days 0 and 3 of infection. This particular time frame was chosen because CCR2−/− mice exhibit elevated IL-4 beginning as early as day 3 of *H. capsulatum* infection. No differences were observed in protein concentrations of IL-25 (< 50 pg/ml) and TSLP (< 25 pg/ml) between days 0-3 in WT and CCR2−/− mice. Thus, it was unlikely that these cytokines instigated the exaggerated IL-4 response. We did observe slightly upregulated IL-33 transcripts in lungs of CCR2−/− mice at day 3 of infection (3.2±0.34 fold vs 1.9±0.18 fold in WT mice; P<0.05), but protein concentrations were similar between the two groups (Fig 1A).

To examine if IL-33 triggered IL-4 in CCR2−/− mice, we administered ST2 blocking antibody. Similar pulmonary IL-4 protein concentration following anti-ST2 or control IgG antibody treatment led us to conclude that IL-33 did not evoke the amplified IL-4 response in mutant mice (Fig 1B). Serendipitously, we discovered augmented IL-33 protein in the lungs of CCR2−/− mice at days 7 and 14 post-infection that succeeded, rather than preceded the exaggerated IL-4 response (Fig 1A). The unexpected kinetics of IL-4 and IL-33 prompted us to determine if IL-4 elicited an IL-33 response. We treated CCR2−/− mice with IL-4Rα blocking antibody prior to
infection and analyzed pulmonary IL-33 concentration 7 days later. Although using anti-IL4-Rα antibody disrupts IL-4 and IL-13 signaling, IL-13 is not upregulated in H. capsulatum-infected CCR2−/− mice. Inhibition of IL-4 engagement with its receptor decreased IL-33 in the lungs (Fig 1C).

IL-33 is generated by lung epithelial cells in response to helminth and fungal infections. To investigate the cellular source of this cytokine in the lungs, its expression in pulmonary epithelial cells was analyzed by flow cytometry. WT and CCR2−/− mice displayed similar percentages and mean fluorescence intensity (MFI) of IL-33+ epithelial cells (CD45− MHCII− EpCAM+) at 7 days of infection (Fig S1A). Although pulmonary epithelial cells are a source of IL-33, leukocytes synthesize it. IL-33 was upregulated in leukocytes at 7 and 14 days after infection in CCR2−/− mice (Fig 1D). Among leukocytes, macrophages and DCs are associated with IL-33 production in lungs. Intracellular staining revealed that WT and CCR2−/− mice displayed similar percentages of IL-33+ macrophages but the latter expressed a higher IL-33 MFI (Supp Fig 1B and Fig 1E-G). To corroborate this finding, we sorted F4/80+ macrophages from the lungs of infected WT and mutant mice and measured IL-33 expression. Macrophages isolated from CCR2−/− mice exhibited increased IL-33 mRNA (Fig 1H). No differences in the total percentage and MFI of IL-33+ DCs were observed (Fig 1F & G). These findings strongly suggested that IL-4 induces IL-33 in macrophages during H. capsulatum infection in vivo.

**IL-4 and H. capsulatum instigate a strong synergistic IL-33 response in macrophages in vitro**

To dissect the signaling pathway of how IL-4 drives IL-33, an in vitro system was established to study IL-33 expression in macrophages following IL-4 exposure. Stimulation of bone marrow-
derived macrophages with IL-4 or infection with *H. capsulatum* yeasts resulted in a modest induction of IL-33 mRNA. IL-4-treated infected macrophages manifested a synergistic IL-33 mRNA response (Fig 2A). Similarly, peritoneal macrophages and alveolar macrophages synthesized IL-33 when exposed to IL-4 or *H. capsulatum* or both (Fig 2B and C). CCR2−/− macrophages generated an IL-33 response comparable to WT cells following incubation with IL-4 or *H. capsulatum* or both (Fig S2A). IL-33 in the lungs of WT and CCR2−/− mice was similar prior to infection (Fig 1B). These findings demonstrated that the exaggerated IL-33 response in CCR2−/− animals following infection was a result of heightened production of IL-4 rather than an intrinsic defect caused by the lack of CCR2. Thus, we utilized WT macrophages for the subsequent experiments. To examine if this response was restricted to *H. capsulatum*, macrophages were infected with a phylogenetically related fungus, *Blastomyces dermatitidis*. An analogous IL-33 expression profile was noted when macrophages were infected with *B. dermatitidis* alone or with IL-4 (Fig S2B).

Similar to other members in the IL-1 family, IL-33 lacks a classical secretory leader sequence that prevents its active release from the cells. We did not detect IL-33 in culture supernatants. To ascertain if protein was produced, we lysed cells with water and assessed the IL-33 concentrations. The intracellular protein concentration mirrored the mRNA response when macrophages were incubated with IL-4 or *H. capsulatum* or both (Fig 2D). There was no secretory defect in these infected macrophages since IL-1β and TNF-α were detected in culture supernatants. These cytokines were readily detected 24 h after infection (IL-1β- 75±4 pg/ml, and TNF-α- 174±44 pg/ml; n=5).

We investigated the kinetics of IL-33 transcription by IL-4. Macrophages required treatment with IL-4 for at least 48h to produce a maximal IL-33 response (Fig S2C). The magnitude of the
response directly correlated with the quantity of IL-4 added to cultures (Fig S2D). Infecting macrophages with an increasing multiplicity of infection (MOI) of \textit{H. capsulatum} yeasts augmented IL-33 mRNA (Fig S2E). Thus, IL-4 or \textit{H. capsulatum} alone elicits modest upregulation of IL-33 in macrophages; however when both are present, macrophages mount a strong synergistic IL-33 response.

**IL-4-induced IL-33 response in macrophages is dependent on STAT6 and IRF-4**

IL-4 mediates its downstream effects through the transcription factor STAT6\textsuperscript{21}. To determine if this factor was necessary for elicitation of IL-33, STAT6\textsuperscript{−/−} macrophages were subjected to IL-4 or IL-4 plus \textit{H. capsulatum}. The IL-33 response was abolished in these macrophages when stimulated with IL-4 (Fig 3A). The synergistic IL-33 response in STAT6\textsuperscript{−/−} cells was lost when they were exposed to a combination of IL-4 and \textit{H. capsulatum}. Treatment of cells with IL-13, another cytokine that activates STAT6, produced effects analogous to IL-4 (Fig 3B).

IRF-4 is a STAT6-inducible transcription factor that is rapidly transcribed when macrophages are stimulated with IL-4\textsuperscript{22}. To determine if IRF-4 was requisite for the enhanced IL-33 response, IRF-4\textsuperscript{−/−} macrophages were exposed to IL-4 or \textit{H. capsulatum} or both. IL-33 expression in these cells was comparable to STAT6\textsuperscript{−/−} macrophages (Fig 3A). Hence, the induction of IL-33 is dependent on an IL-4/STAT6/IRF-4 signaling cascade in macrophages.

**Synergistic induction of IL-33 is not a result of increased expression of IL-4Rα or autocrine IL-4 and IL-13 generated by macrophages during infection**

The intracellular pathogen \textit{Mycobacterium tuberculosis} induces the expression of IL4-Rα in macrophages\textsuperscript{23}. A possible explanation for the synergistic elicitation of IL-33 was that \textit{H. capsulatum} upregulated the expression of IL4-Rα on the macrophage surface, which in turn
potentiated reactivity to IL-4. To test this, expression of IL-4Rα was analyzed in macrophages infected with *H. capsulatum*. Exposure to the fungus did not significantly upregulate IL-4Rα mRNA compared to controls (Fig 4A). Another possibility was that infected macrophages secreted IL-4 and/or IL-13 that acted in an autocrine manner to drive IL-33. We assessed IL-4 and IL-13 in culture supernatants of infected macrophages, but did not detect either (IL-4 and IL-13 < 2 pg/ml). Western blot analysis of infected cell lysates indicated that STAT6 was not phosphorylated during the course of infection (Fig 4B). We exposed IL-4−/− macrophages to IL-4 or *H. capsulatum* or both and observed an analogous IL-33 expression profile as WT cells (Fig 4C). To demonstrate that the synergistic evocation of IL-33 was not a result of autocrine IL-4 or IL-13, IL4-Rα−/− macrophages were utilized. These did not mount an IL-33 response when stimulated with IL-4 alone but had a similar induction of IL-33 as the WT macrophages upon infection with *H. capsulatum* (Fig 4D). The synergistic elicitation of IL-33 was not a result of increased expression of IL-4Rα or autocrine IL-4/IL-13 produced by infected macrophages.

**H. capsulatum-induced IL-33 response in macrophages is dependent on Dectin-1 signaling pathway**

Dectin-1 exerts an auxiliary role in triggering innate responses when macrophages interact with *H. capsulatum* yeasts *in vitro*\(^5\). The significance of Dectin-1 signaling pathway in the inception of IL-33 response was examined following *H. capsulatum* exposure. We infected Dectin-1−/− macrophages treated with IL-4 or vehicle for 24 h and analyzed the expression of IL-33. The synergistic IL-33 response in Dectin-1−/− macrophages was greatly diminished compared to WT cells (Fig 5A). A potential caveat with this experiment was an attenuation in binding of *H. capsulatum* yeasts to Dectin-1−/− macrophages and hence a subsequent decrease in phagocytosis. We quantified the association index of green fluorescent protein (GFP)-expressing *H. capsulatum* yeasts to Dectin-1−/− macrophages and hence a subsequent decrease in phagocytosis.
capsulatum to Dectin-1−/− macrophages. No differences were observed in binding of H. capsulatum yeasts to either WT or mutant macrophages at 5 or 10 MOI (Fig 5B). To substantiate the importance of Dectin-1 in IL-33 generation, macrophages were stimulated with the Dectin-1 agonist, curdlan. Treatment of WT cells with curdlan resulted in modest induction of IL-33 (4 hours post treatment) and a vigorous synergistic IL-33 response in the presence of IL-4 (24 hours post treatment) (Fig 5C).

To demonstrate the involvement of Dectin-1 signaling in the H. capsulatum-induced IL-33 response, we treated macrophages with the Syk-inhibitor, piceatannol and incubated them with H. capsulatum alone or IL-4 and H. capsulatum. A marked decrease in evocation of IL-33 was observed when cells were incubated with H. capsulatum or IL-4 plus H. capsulatum (Fig 5D). Treatment with this agent did not influence IL-4-induced IL-33 transcription. The Syk inhibitor at a concentration greater than 20μM retards phagocytosis of H. capsulatum yeast particles15. We used the inhibitor at a concentration that did not interfere with uptake of yeasts by macrophages (data not shown). Thus, these findings strongly suggest that Dectin-1 signaling is crucial for the H. capsulatum induced IL-33 response in macrophages.

Blocking IL-33 signaling decreases fungal burden in vivo

To determine the impact of IL-33 during H. capsulatum infection in CCR2−/− mice, we administered αST2 antibody to one group of mice, while the other received isotype control antibody. A significant lowering of fungal burden was noted in CCR2−/− mice that received ST2 blocking antibody at day 7 post infection (Fig 6). Infected WT mice do not mount an exaggerated IL-33 response and treating them with αST2 antibody did not lower their pulmonary fungal burden. We investigated the mechanism by which IL-33 exacerbated infection in the host.
IL-33 has been shown to induce genes associated with alternative activation in macrophages\textsuperscript{24}. We observed a similar upregulation in \textit{Arg-1}, \textit{Chil-3} and \textit{Retnla} in macrophages stimulated with IL-33 for 24 hrs (Fig 6B). Moreover, infected-CCR2\textsuperscript{−/−} mice treated with αST2 antibody exhibited reduced mRNA expression of alternative activation markers, in comparison to isotype control-treated mutant animals (Fig 6C). The IL-33 primed macrophages were found to be more permissive to intracellular fungal growth, in comparison to resting macrophages (Fig 6D). Thus, our data indicate the detrimental attribute of IL-33 in driving an alternatively activated phenotype in phagocytes that in turn, enhances susceptibility to \textit{H. capsulatum} in CCR2\textsuperscript{−/−} mice.
Discussion

*H. capsulatum*-infected CCR2<sup>−/−</sup> mice mount an exaggerated and sustained IL-4 response that is localized to the lungs. The elevated IL-4 is associated with impaired host resistance and results in the death of mice from a progressive infection<sup>3</sup>. In this study, we discovered a new facet of IL-4 biology using CCR2<sup>−/−</sup> mice infected with *H. capsulatum*. In a search for an inducer of IL-4 in these mutant mice, we found that IL-4 was not a target of IL-33 but rather the converse. The latter is firmly established as a potent initiator of IL-4 synthesis in various cell populations such as basophils, NKT cells and CD4<sup>+</sup> T cells<sup>10</sup>. Examination of the molecular mechanism revealed that an exaggerated IL-33 response was kindled only when macrophages were exposed to IL-4 and an intracellular pathogen. The generation of IL-33 exerted a deleterious role in pulmonary mycosis as evidenced by decreased fungal burden in CCR2<sup>−/−</sup> mice treated with ST2 blocking antibody.

Given our previous findings in CCR2<sup>−/−</sup> mice and the knowledge concerning IL-33 initiating Th2 immunity, we initially sought to determine if IL-33 instigated the exaggerated IL-4 response in CCR2<sup>−/−</sup> mice. To study the *in vivo* function of IL-33, we blocked interaction of this cytokine with its receptor ST2. Although antibodies may act as agonists or antagonists, this monoclonal antibody has been used to block ST2 engagement in diverse experimental models<sup>19,25-27</sup>. In each of these studies, the antibody does not trigger an agonistic effect on the receptor ST2. Much to our surprise, generation of IL-33 in the lungs of these mice was actually observed during the onset of the adaptive phase of the immune response to *H. capsulatum* rather than the innate immune phase. Even more surprising was the fact that it was macrophages that differentially produced this cytokine and not pulmonary epithelial cells. Equally peculiar, there was at least a 4 day hiatus between the time when IL-4 was upregulated in the lungs of CCR2<sup>−/−</sup> mice (day 3) and
the detection of enhanced IL-33 (day 7). This finding implies that the exaggerated IL-33 response by macrophages must be shaped by a cascade of interactions rather than a constitutive process. This delay was mimicked partially in vitro in which we did not observe peak IL-33 transcription until 48 hours following exposure to IL-4.

The unexpected inverse relationship between IL-4 and IL-33 prompted us to investigate the possibility that the former triggered an IL-33 response. Indeed, interruption of IL-4 signaling using anti-IL-4Rα antibody diminished pulmonary IL-33 protein content in the lungs of infected CCR2−/− mice. Although antibody to this receptor blocks both IL-4 and IL-13, the latter is not altered in the lungs of infected CCR2−/− mice3. Thus, the impact of receptor blockade is strictly on IL-4. Disrupting the same signaling pathway in WT controls did not affect IL-33 since these mice inherently mount a weaker IL-4 response in infection as compared to the mutant mice. This finding suggests that there exists a threshold for elicitation of IL-33 by IL-4. IL-4 has been reported to evoke IL-33 in murine splenocytes and peritoneal exudate cells in vitro, but the precise mechanism was not identified28. In concordance, we did detect a modest IL-33 response to IL-4 in uninfected macrophages in vitro that was magnified upon infection. Yet, to the best of our knowledge, IL-4-driven IL-33 in vivo has not been described.

Although epithelial cells and DCs represent potential sources of IL-33 in lungs, the quantity of cytokine in these cell populations was comparable between WT and CCR2−/− mice. On the other hand, macrophages were the prominent producers of IL-33 in the lungs of mutant animals. An explanation for upregulation of IL-33 in the macrophage population is that alternatively activated macrophages exhibit enhanced expression and function of Dectin-129. Activation of both the Dectin-1 and IL-4 signaling pathway were critical for triggering the synergistic IL-33 response. We detected intracellular IL-33 (but no extracellular release) protein that mirrored its
transcription when cells were exposed to IL-4 or *H. capsulatum* or both. Failure to detect IL-33 in the culture supernatants was not a result of defective secretory machinery as evidenced by detecting TNF-α and IL-1β in culture supernatants, but most likely a result of the lack of leader peptide sequence required for secretion of IL-33 from cells. Others have reported release of this cytokine from cells in response to external stimuli including ATP or lysophosphatidylcholine (LPC)\textsuperscript{18,30}. We did not detect release of IL-33 from macrophages following similar treatments (data not shown). A probable consideration for the contradictory findings is that the prior studies used cell lines.

IRF-4 is a transcription factor that is engaged following activation of STAT6 and controls the expression of genes linked with alternative activation in macrophages\textsuperscript{22}. Activation of STAT6 and IRF-4 were crucial for the IL-4-prompted IL-33 response. A recent report has indicated that IRF-4 directly binds to the IL-33 locus to activate the gene\textsuperscript{31}. A similar mechanism might be in effect in macrophages stimulated with IL-4. However, an indirect role for IRF-4 in regulating IL-33 expression cannot be excluded.

Apart from its function in triggering cytokine responses to *H. capsulatum* yeast cells, Dectin-1, has been linked with recognition and innate immune responses to other fungal pathogens such as *Candida, Coccidioides* and *Aspergillus*\textsuperscript{32-34}. Macrophages deficient in Dectin-1 had a marked reduction in IL-33 when infected with *H. capsulatum* yeasts. This finding is congruent with a recent report implicating Dectin-1 in regulating IL-33 in an allergic model of chronic exposure to the fungus *Aspergillus fumigatus*\textsuperscript{35}. This study did not address the cellular source of IL-33 during the allergic phase, but its results suggest that engagement of Dectin-1 by β-glucan on the *A. fumigatus* surface is critical for evoking IL-33 in the lungs. β-glucan is a vital component of *H. capsulatum* cell wall in several of the North American isolates, and engagement of this
constituent by Dectin-1 activates the receptor to transduce signals that lead to IL-33 in macrophages.

*H. capsulatum*-prompted IL-33 is reliant on Dectin-1/Syk signaling pathway. In human neutrophils and peripheral blood mononuclear cells, engagement of IL-4 with its receptor IL4-Rα activates Syk kinase that ultimately augments cell adhesion and delays apoptosis. Thus, there is a possibility that IL-4 initiated IL-33 transcription partially depends on Syk kinase. This consideration is unlikely since IL-4 stimulation of macrophages that had been previously treated with the Syk inhibitor yielded an intact IL-33 response. The synergistic elicitation of IL-33 was significantly ablated when either the IL-4 or Dectin-1 signaling pathway was disrupted. These findings strongly support that both the signaling pathways act autonomously to evoke IL-33 in a robust manner.

We demonstrated the deleterious aspect of IL-33 during intracellular fungal infection and provide proof that it contributes to host susceptibility. Others have reported adverse effects of this cytokine but they have largely been a consequence of inducing T_{H2} cytokines. In experimental cryptococcal infection or in allergic sensitization with *Aspergillus* or *Alternaria*, IL-33 is detrimental to the host by skewing the immune response to a T_{H2} phenotype. However, IL-33 did not contribute to the instigation of IL-4 response in *H. capsulatum* infection. Rather, the latter evoked IL-33 in the lungs to aggravate *H. capsulatum* infection in CCR2^{-/-} mice.

One mechanism by which IL-33 predisposed these mutant mice to histoplasmosis was by triggering an alternatively activated phenotype in macrophages. Alternative activation was demonstrated previously in macrophages from infected CCR2^{-/-} mice at 7 days p.i. coincident
with elevated IL-33\(^3\). Exposure of macrophages to IL-4 induces arginase-1, an enzyme that diminishes nitric oxide required for fungicidal activity\(^{39}\). Moreover, IL-4 blocks the activating properties of GM-CSF and enhances intracellular zinc that supports the fungal growth\(^{40,41}\). In addition to serving as an extracellular cytokine, IL-33 might function as a nuclear factor to downregulate proinflammatory genes and in turn, augment susceptibility to intracellular infections\(^{10}\).

Given previous data regarding the influence of IL-4 in causing the demise of CCR2\(^{+/−}\) mice, a reexamination of that work indicates that the likely cause of the collapse of immunity is attributable to IL-33. The reason why neutralization of IL-4 restored immunity was a consequence of reducing IL-33, rather than exerting an IL-33-independent effect. This finding represents a novel paradigm in T\(_H2\) immunity since IL-33 is principally an initiator of IL-4 and other T\(_H2\) cytokines. The work establishes the existence of a novel positive feedback loop between two key type II immunity inducing cytokines. The knowledge from this study can be extrapolated to other intracellular infections in which IL-4 dampens protective immunity. Indeed, an IL-4/IL-33 axis could exist that exacerbates infection, and IL-33 may be target for interdiction in progressive intracellular infections. Our findings are important for non-infectious T\(_H2\) diseases including allergic asthma and eczema where a similar IL4/IL33 coupling might be in effect. Our work highlights the detrimental effect of IL-33 in host defenses to an intracellular pathogen. Comprehensive understanding of how exaggerated amounts of IL-33 condition the host to be more susceptible to \textit{H. capsulatum} or other intracellular pathogens is a necessary pursuit.
Methods

Mice

Male C57BL/6 and breeding pairs of CCR2−/− (C57BL/6 background) mice were purchased from The Jackson Laboratory, Bar Harbor, ME. Bone marrow cells from IL-4−/−, IL4Ra−/− and STAT6−/− mice (C57BL/6 background) were generously provided by Senad Divanovic (Cincinnati Childrens’ Hospital Medical Center). Bone marrow cells from Dectin-1−/− and IRF-4−/− mice were a gift from Stuart Levitz at University of Massachusetts, Worcester, MA and Lu Runqing at University of Nebraska, Omaha, NE respectively. Animals were housed in isolator cages and maintained by the Department of Laboratory Animal Medicine, 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 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 and GFP+ yeasts were grown for 72h at 37°C as described42. To infect mice, 6-8 week old animals were inoculated intranasally with 2x10^6 yeasts in ~30μl of HBSS (HyClone, Logan, UT).

Organ culture for H. capsulatum

Organs were homogenized in sterile HBSS and serially diluted and plated onto mycosel-agar plates containing 5% sheep blood and 5% glucose. Plates were incubated at 30°C. The limit of detection was 10^2 CFU.

In vivo blocking of IL4-Ra and T1/ST2
For blocking IL-4Rα, mice were injected intraperitoneally with 500 μg of rat anti-mouse IL4-Rα (AMGEN, Thousand Oaks, CA) or control antibody (Bio X Cell, Lebanon, NH) on day 0. For disrupting IL-33 signaling, mice were injected with 300μg of rat anti-mouse T1/ST2 antibody (AMGEN) on day 0 and day 3 of infection.

**Isolation of lung leukocytes**

Lungs were homogenized with the gentleMACS™ dissociator (Miltenyi Biotec, Auburn, CA) in 5ml of HBSS with 2mg/ml of collagenase D (Roche, Mannheim, Germany) and 40U/ml of DNase I (Roche) for 30min at 37° C. The homogenate was percolated through a 60μm nylon mesh (Spectrum Laboratories, Rancho Dominguez, CA) and washed 3 times with HBSS. Leukocytes were isolated by separation on Lympholyte M (Cedarlane Laboratories, Burlington, ON).

**Flow cytometry and cell sorting**

The phenotype of cells from mouse lungs was determined by incubating lung leukocytes with the indicated antibodies and CD16/32 to limit nonspecific binding. Leukocytes were stained at 4°C for 15 min in PBS containing 1%BSA and 0.01% sodium azide. Cells were stained with combinations of the following antibodies: FITC-conjugated I-A^b^; PE-conjugated CD11c; PerCP-conjugated CD45; and APC-conjugated F4/80 and EpCAM from BD Biosciences. For intracellular IL-33 staining, cells were incubated with Cytofix/Cytoperm (BD Biosciences, San Diego, CA), washed in Permeabilization Buffer (BD Biosciences), and stained for 30 min with PE-conjugated IL-33 (R&D systems, Minneapolis, MN). Cells were washed and resuspended in 1% paraformaldehyde. Isotype controls were used. Data was acquired using BD Accuri™ C6 cytometer and analyzed using FCS Express 4.0 Software. For cell sorting experiments, F4/80^+
leukocytes from the lungs of WT and CCR2−/− mice were isolated at day-7 p.i. using 5-laser FACS Aria II (BD Biosciences).

**Generation of bone marrow derived, peritoneal, and alveolar macrophages**

Bone marrow was isolated from the tibia and femurs of 6-10 week old mice by flushing with HBSS. Cells were dispensed into tissue culture flasks at a density of 2×10^5 cells/ml of RPMI-1640 supplemented with 10% fetal bovine serum, 0.1% gentamicin sulfate, 5×10^{-5}M 2-mercaptoethanol, and 10ng/ml of mouse granulocyte macrophage colony-stimulating factor (GM-CSF) (Peprotech, Rocky Hill, NJ). Flasks were incubated at 37°C in 5% CO₂. Macrophages were harvested at day 7. Non-adherent cells were removed and trypsin-EDTA was added for 10min at 37°C. Cells were collected, washed with HBSS and dispensed into culture dishes. Resident peritoneal macrophages from mice were isolated and adhered overnight. The following day, non-adherent cells were removed. Alveolar macrophages were isolated by lavaging the lungs of uninfected WT mice with PBS. Non adherent cells were washed off and adherent macrophages were used.

**Binding of H. capsulatum yeast to macrophages**

Bone marrow derived macrophages were seeded in 12-well plates and cultured overnight. The following day, plates were cooled on ice for 20min before the addition of non-opsonized GFP⁺ *H. capsulatum* to the wells at final yeast: macrophage ratio of 5:1 or 10:1. The plates were placed on a shaker at a rotation speed of 150rpm for 2min and left on ice for another 60min. This process allowed GFP-labeled yeasts to settle on and contact with macrophages before phagocytosis took place. The plates were then placed in a CO₂ incubator to allow phagocytosis to have a synchronous start. After 60min of incubation at 37°C, cells were washed twice in warm HBSS. Macrophages were then detached by treatment with cell dissociation buffer (Invitrogen,
Carlsbad, CA) and fixed in 1% paraformaldehyde. Finally, to determine the Association Index (AI), percentages of GFP⁺ cells were quantified by flow cytometry.

**In vitro culture conditions**

For most experiments, bone marrow derived, peritoneal or alveolar macrophages (except the control or *H. capsulatum* only group) were primed with 10ng/ml of IL-4 (Peprotech) for 24h. Subsequently, fresh media was replenished and macrophages were subjected to IL-4 or *H. capsulatum* or both for 24h. For inhibition studies, Piceatannol (TOCRIS, Bristol, UK) was added to macrophages 90min before infection.

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

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

**Growth inhibition assays**

100,000 bone marrow derived macrophages were plated on a 96-well in culture media with or without IL-33 (10ng/ml) for 24h. The following day, cells were infected with 0.1 MOI of yeasts. Fungal growth was assayed 24h after infection. Macrophages were lysed and yeasts plated. Colonies were counted 10 days later.
**Measurement of IL-33 by ELISA**

IL-33 protein was quantified in lung homogenates and macrophage-cell lysates (lysed using DI water) by using an ELISA kit (R&D Systems).

**Statistics**

Analysis of variance (ANOVA) with Dunn’s test was used to compare multiple groups, while Student's $t$ test was used to compare two groups. $P$ value of < 0.05 was considered significant. For all graphs, * $P=0.01-0.05$, ** $P=0.005-0.01$, and *** $P<0.005$. 
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Figure Legends

Figure 1

IL-4 prompts an IL-33 response in macrophages in vivo.

(A) IL-4 mRNA expression in whole lung homogenates of mice treated with anti-ST2 or isotype control antibody. Transcription is expressed as $\log_{10}$ relative quantification (RQ) normalized to uninfected WT lung. (B) IL-33 protein concentration measured by ELISA in lung homogenates of WT and CCR2$^{-/-}$ mice at indicated time points. (C) IL-33 protein concentration measured at day-7 p.i in lung homogenates of mice treated with anti-IL-4Rα or isotype control antibody. (D) IL-33 mRNA expression in isolated lung leukocytes from WT and CCR2$^{-/-}$ mice at indicated time points. Data normalized to lung leukocytes from uninfected WT animals. (E) Intracellular staining of IL-33 in pulmonary macrophages from WT and CCR2$^{-/-}$ mice at 7 days p.i (gating strategy depicted in Fig S1B). Representative plot of one of eight mice from 2 independent experiments. (F & G) Analysis of IL-33 expression and mean fluorescence intensity by flow cytometry in macrophages and DCs at day-7 p.i. (H) IL-33 mRNA expression in sorted F4/80$^+$ macrophages from lungs of WT and CCR2$^{-/-}$ mice at 7 days p.i. Data normalized to uninfected WT macrophages. Pooled values of 8-12 mice from 1 of 2 similar experiments. For all other in vivo experiments, data are represented as mean ± SEM, n=6-8 from 2 independent experiments.

Figure 2

Synergistic induction of IL-33 by IL-4 and H. capsulatum (Hc) in vitro.

(A) IL-33 mRNA expression measured by qRT-PCR in WT bone marrow derived macrophages (BM) that were exposed to IL-4 (10 ng/ml), or H. capsulatum (5 MOI), or both for 24 hrs. All macrophages exposed to IL-4 or IL-4+H. capsulatum were initially primed with IL-4 (10 ng/ml) for 24 hrs. Values normalized to untreated macrophages. All treatment groups are significantly
different (P<0.05) from each other. (B and C) IL-33 mRNA expression in resident macrophages (PM) and alveolar macrophages (AM) isolated from WT mice that were exposed to IL-4 (10 ng/ml), or *H. capsulatum* (5 MOI), or both *in vitro* for 24 hrs. All treatment groups are significantly different (P<0.05) from each other. (D) Intracellular IL-33 protein content in whole cell lysates (cells lysed with deionized water) measured by ELISA after 24 hrs of exposure to the stimulus. All treatment groups are significantly different (P<0.05) from each other. Values depicted in all the above experiments are mean ± SEM from at least 5 independent experiments.

**Figure 3**

IL-4-induced IL-33 response is dependent on STAT6 and IRF-4.

(A) IL-33 mRNA expression in bone marrow derived macrophages from WT, STAT6<sup>−/−</sup> and IRF-4<sup>−/−</sup> animals that were subjected to IL-4 treatment or *H. capsulatum* infection or both *in vitro* for 24 hrs. Values normalized to untreated WT macrophages. Data are mean ± SEM from at least 3 independent experiments. (B) IL-33 transcription measured in WT macrophages exposed to IL-13 (10 ng/ml) or IL-13 plus *H. capsulatum* (5 MOI) for 24 hrs. Data are mean ± SEM from 5 independent experiments.

**Figure 4**

Synergistic induction of IL-33 is not a result of increased expression of IL-4Rα or autocrine IL-4/IL-13.

(A) IL-4Rα mRNA expression in macrophages quantified by qRT-PCR after 24 hrs of *H. capsulatum* infection (5 MOI). Data are mean ± SEM from 3 independent experiments. (B) Immunoblot of phospho-STAT6 and total STAT6 from whole cell lysates of macrophages infected with *H. capsulatum* for the indicated time. Macrophages stimulated with IL-4 were used as positive control. Representative blot of 3 similar experiments. (C & D) IL-33 mRNA
expression in IL-4−/− and IL-4Rα−/− macrophages exposed to IL-4 or *H. capsulatum* or both for 24 hrs. Values normalized to unstimulated WT macrophages. Data are mean ± SEM from 3 independent experiments. All treatment groups of IL-4−/− macrophages are significantly different (P<0.05) from each other.

**Figure 5**

*H. capsulatum*-induced IL-33 response is dependent on Dectin-1 signaling.

Effect of *H. capsulatum* (5 MOI) or IL-4 plus *H. capsulatum* on IL-33 transcription in Dectin-1−/− macrophages after 24 hrs of challenge. Data normalized to uninfected WT macrophages. (B) FACS analysis quantifying the uptake of GFP+ yeasts (5 or 10 MOI) by WT and Dectin-1−/− macrophages 1 hr after infection. (C) IL-33 mRNA expression in WT macrophages stimulated with curdlan (100 μg/ml) or IL-4 plus curdlan for 24 hrs. (D) IL-33 mRNA expression in macrophages pre-treated with DMSO or Syk inhibitor (100 nM) for 90 minutes before exposure to *H. capsulatum* or IL-4 plus *H. capsulatum*. Values depicted in all the above experiments are mean ± SEM from 3-5 independent experiments.

**Figure 6**

Detrimental effect of IL-33 during *H. capsulatum* infection.

(A) Infected WT and CCR2−/− mice were i.p treated with ST2 blocking or isotype control antibody. Animals were euthanized on day 7 of infection and pulmonary fungal burden was quantified. Values are represented as log_{10} CFU from 7-8 mice/group from two independent experiments. (B) mRNA expression of genes associated with alternatively activated phenotype in bone marrow derived macrophages stimulated with IL-33 (10 ng/ml) for 24 hrs. Data are mean ± SEM from 3 independent experiments; normalized to unstimulated macrophages. (C) qRT-PCR analysis of genes associated with alternative activation. Analysis done on mRNA from whole
lungs homogenates of control IgG or αST2-treated CCR2−/− mice after 7 days of infection, N=7-8 from two independent experiments. (D) Percent of fungal growth inhibition in unstimulated or IL-33-stimulated macrophages 24 hrs after *H. capsulatum* challenge (0.1 MOI). Data normalized to fungal growth in unstimulated macrophages. IFN-γ-primed macrophages used as positive control for the growth inhibition study. Data compiled from values of 3 independent experiments.
Figure 3

A

![Graph showing IL-33 mRNA expression in different conditions.](image)

B

![Graph showing IL-33 mRNA expression in different conditions.](image)
Figure 4

A: IL4Rα expression

B: IL-4 (2hrs)

C: IL4−/− Mφ

D: IL4Ra−/− Mφ
Figure 5

A

B

C

D

IL-33 mRNA expression

WT
Dec1 ΔT-/-

Percent of GFP+ Mφ

5 MOI
10 MOI

350
300
250
200
150
100
50
0

Ctrl
BM+Hc
BM+IL4+Hc

BM Ctrl
BM+Hc
BM+IL4+Hc

IL-33 mRNA expression

BM Ctrl
BM+Curdian
BM+IL4+Curdian

BM Ctrl
BM+Hc
BM+IL4+Hc

BM Ctrl
BM+Hc
BM+IL4+Hc

DNDSO
Sylkinib

*  **  ***
Inventory of Supplemental Figures

**Figure S1:** Identification of IL-33+ cells during *H. capsulatum* infection. Related to Figure 1.

**Figure S2:** Induction of IL-33 by intracellular fungal pathogens and IL-4 in macrophages. Related to Figure 2.
Supplemental Information

Figure S1
Identification of IL-33\(^+\) cells during *H. capsulatum* infection.
FACS analysis of IL-33\(^+\) epithelial cells (A) and macrophages (B) in lungs of WT and CCR2\(^{-/-}\) mice at 7 days p.i. Plot representative of 1 of 8 mice from two independent experiments.

Figure S2
Induction of IL-33 by intracellular fungal pathogens and IL-4 in macrophages.
(A) IL-33 mRNA expression in CCR2\(^{-/-}\) macrophages exposed to IL-4 or *H. capsulatum* or both for 24 hrs. (B) IL-33 mRNA expression in macrophages exposed to *B. dermatitidis* (1 MOI) or a combination of IL-4 and *B. dermatitidis* for 24 hrs. (C & D) Analysis of IL-33 transcription in macrophages stimulated with IL-4 for the indicated time and concentration. (E) IL-33 transcription measured by qRT-PCR in untreated or IL-4-primed macrophages challenged with increasing MOI of *H. capsulatum* for 24 hrs. All treatment groups are significantly different (P<0.05) from each other. All Values are normalized to uninfected WT macrophages and represent mean ± SEM from at least 3 independent experiments.
Supplemental Figures

Supplemental Figure 1

A

B

Figure S1: Identification of IL-33$^+$ cells during *H. capsulatum* infection
Figure S2: Induction of IL-33 by intracellular fungal pathogens and IL-4 in macrophages
References


CHAPTER 5

Discussion and Future Directions


**Discussion & Future Directions**

The successful resolution of *H. capsulatum* infection requires coordinated action of multiple cell populations and soluble mediators. The roles of several proinflammatory cytokines, including IFN-γ, TNF-α, GM-CSF, and IL-1β have been comprehensively studied in the setting of *H. capsulatum* infection. In contrast, the significance of chemokines and chemokine receptors facilitating recruitment of leukocytes to the site of infection remain unclear. Our previous findings indicate the chemokine receptor CCR2 is critical for promoting antifungal immunity in the host\(^1\). Animals deficient in this chemokine receptor demonstrate enhanced susceptibility to *H. capsulatum* due to an exaggerated IL-4 response generated in the lungs. The finding prompted us to investigate the dysregulated nature of the immune response in these infected mutant mice. Two major questions that arose from the work were (a) What is the cellular source of IL-4 in *H. capsulatum*-infected CCR2\(^{-}\) mice, and (b) What factor(s) instigates the amplified IL-4 response in mutant mice during fungal infection? The section below summarizes our novel findings presented in the thesis, as well as potential future directions based on the results described in Chapters 3 and 4.

**CCR2 regulates IL4-IL33 axis in *H. capsulatum* infection**

The functional importance of CCR2 has been best studied in chemotaxis of inflammatory monocytes. The chemokine receptor is heavily expressed on this subpopulation of monocytes and is critical for their egress out of the bone marrow and recruitment to the site of infection\(^2\). CCR2 has also been shown to control trafficking of conventional dendritic cells to infected tissues\(^3\). Animals deficient in CCR2 are vulnerable to a multitude of microbial infections.
including histoplasmosis\textsuperscript{1}, listeriosis\textsuperscript{4}, cryptococcosis\textsuperscript{5}, and toxoplasmosis\textsuperscript{6}. In majority of these experimental models, dampened antimicrobial immunity is attributed to defective recruitment of inflammatory monocytes and dendritic cells. However, in CCR2\textsuperscript{−/−} mice challenged with \textit{H. capsulatum}, the enhanced susceptibility to pathogen results from an over-exuberant IL-4 response generated in the lungs. Accordingly, we sought to identify the cells instigating the exaggerated IL-4 response in infected mutant mice. Eosinophils were found to be the primary source of non-protective IL-4 and depleting this granulocyte population improved the outcome of fungal infection in CCR2\textsuperscript{−/−} animals. The abnormal accumulation of eosinophils in the lungs of the mutant mice was likely due to CCL7 engaging CCRs selectively expressed on these granulocytes.

We investigated the factors associated with initiating the dominant IL-4 response in CCR2\textsuperscript{−/−} mice during \textit{H. capsulatum} infection. Given the knowledge concerning IL-33 influencing type II immune responses, we postulated that this innate cytokine might be triggering the amplified IL-4 response observed in CCR2\textsuperscript{−/−} mice. However, to our surprise, elevated concentrations of IL-33 succeeded the IL-4 response and were detected in the adaptive immune phase. The inverse relationship in the kinetics of IL-4 and IL-33 prompted us to inspect the possibility that the former triggered an IL-33 response. This was found to be true since blockade of IL-4 signaling diminished pulmonary IL-33 in infected CCR2\textsuperscript{−/−} mice. Depletion of eosinophils that instigated the IL-4 response resulted in decreased IL-33 concentration as well (Appendix Fig 3). We also discovered that macrophages were the prominent source of IL-33 in infected mutant animals. Finally, IL-33 compromised antifungal defenses in CCR2\textsuperscript{−/−} mice by driving an alternatively activated phenotype in macrophages.
In chapter 3, we have established that eosinophils are the primary instigators of the non-protective IL-4 response in *H. capsulatum*-infected CCR2\(^{-/-}\) mice, however, the reason for their accrual in the lungs of mutant animals remains to be determined. Preliminary data from our lab suggests a possible role for CCL7 in attracting eosinophils to the site of infection in CCR2\(^{-/-}\) mice. Although CCL7 is a major ligand for CCR2, it has the capacity to associate with CCR3, a chemokine receptor heavily expressed on eosinophils. In fact, CCL7 binds to CCR3 with a much lower affinity (\(K_d \sim 9.7\mu\text{M}\)) than CCR2\(^{30,31}\). Thus, we postulate that in the absence of CCR2, CCL7 engages CCR3 expressed on eosinophils to direct the recruitment of these cells to the lungs. To test this hypothesis, we will block CCR3 in infected mutant mice using CCR3 antagonist and analyze the influx of eosinophils in the lungs. To further ascertain the involvement of this CCL7-CCR3 axis, we will also perform chemotaxis assays where migration of CCR2\(^{-/-}\) eosinophils will be measured in response to recombinant CCL7. We anticipate that CCL7 will elicit a chemotactic response in eosinophils by engaging CCR3. An alternative explanation could be that CCL7 directs recruitment of eosinophils by engaging CCR1, another receptor that has the capacity to bind this chemokine ligand.

Our findings aid in understanding the protective role exerted by CCR2 in *H. capsulatum* infection. However the knowledge of how IL-33 exacerbates fungal infection in these mutant animals is still incomplete. Our current data indicate that IL-33 manifests deleterious effects on the host by acting as an extracellular cytokine. Reports have described that IL-33 can also function as a nuclear factor and is capable of suppressing proinflammatory genes including TNF-\(\alpha\)^{7,8}. To comprehensively understand how this biomolecule subverts antifungal immunity in the host, IL-33\(^{-/-}\) mice will be used. These mutant animals will serve as an effective tool to determine the extracellular and intracellular effects of IL-33. The mice will be infected intranasally with *H.*
capsulatum yeasts and their rate of clearance of fungal infection will be analyzed in lungs and spleen. Other immune parameters such as proinflammatory cytokines, anti-inflammatory cytokines, and markers associated with alternative activation will also be measured. We predict that animals lacking endogenous IL-33 will display accelerated fungal clearance and a more profound proinflammatory cytokine response, in comparison to WT controls. A possibility is that IL-33−/− mice might suffer from excessive tissue damage resulting from an overzealous type I immune response. To determine the effect of IL-33 in CCR2−/− mice, IL-33−/−CCR2−/− double knockout mice will be generated and challenged with a sublethal dose of H. capsulatum yeasts. Fungal clearance and cytokine responses will be monitored in infected mice. We anticipate restoration of protective immunity in infected double knockout animals.

In certain infectious settings, IL-33 boosts host resistance to microbial pathogens. For instance, IL-33 promotes antiviral CD8+ T cell responses against lymphocytic choriomeningitis virus (LCMV)9. The cytokine also augments antibacterial defenses in the host against Staphylococcus aureus10. In contradistinction, our findings reveal a non protective role for the cytokine during intracellular fungal infection. IL-33 has also been reported to increase susceptibility to the fungal pathogen C. neoformans by triggering an allergic pattern of inflammation in the lungs during infection11. Thus, the cytokine appears to have strikingly different functions under diverse infectious scenarios. While adjunctive immunotherapy with recombinant IL-33 might be beneficial in certain bacterial and viral infections, targeting this cytokine or its receptor in intracellular fungal infections could potentially bolster antifungal immunity.
IL-33, a novel downstream target of IL-4 in *H. capsulatum* infection

An unexpected observation while studying the dysregulated immune response to *H. capsulatum* in CCR2<sup>−/−</sup> mice was the IL-4 generation preceded the IL-33 response. Additionally, disruption of IL-4 signaling diminished pulmonary IL-33 in infected mutant mice. Another peculiar observation was that macrophages but not epithelial cells were the prominent source of IL-33 in the lungs of CCR2<sup>−/−</sup> mice. The IL-4-kindled IL-33 was recapitulated in another *in vivo* scenario. Intraperitoneal administration of recombinant IL-4 followed by *H. capsulatum* infection elicited heightened IL-33 response in peritoneal macrophages of WT mice (Appendix Fig 1). Together, these data strongly suggested that IL-4 instigated IL-33 in macrophages.

To dissect the signaling mechanism of how IL-4 drives IL-33, we established a reductionist *in vitro* system where macrophages were exposed to IL-4 or *H. capsulatum* or both. We observed a modest induction of IL-33 when macrophages were stimulated with IL-4 or challenged with *H. capsulatum* alone. In presence of both stimuli (IL-4+*H. capsulatum*), the IL-33 response was exponentially magnified in the cells. The IL-4-driven IL-33 response was found to be dependent on STAT6 and IRF-4, while the *H. capsulatum*-elicited IL-33 was reliant on Dectin-1 signaling pathway. Thus, IL-4 and *H. capsulatum* triggered synergistic expression of IL-33 in macrophages.

Data presented in chapter-4 describe a novel signaling mechanism of how activation of STAT6 and IRF-4 by type II cytokines kindles IL-33 in macrophages. However, our understanding of these signaling events is still incomplete. A recent report indicated that IRF-4 directly binds to the IL-33 promoter region to activate the gene and prompt its transcription<sup>12</sup>. To determine if a similar molecular mechanism is in effect in IL-4-primed macrophages, we will employ
Chromatin Immunoprecipitation (ChIP) assay. Pull down will be carried out using αIRF-4 monoclonal antibody and PCR will be performed on the isolated DNA fragments. Another possibility is that IRF-4 drives IL-33 transcription through secondary mediators. In such an event, a systems biology approach will be utilized to identify genes responsible for directly activating IL-33. Putative binding sites of IRF-4-inducible genes in the IL-33 promoter region will be determined. Subsequently, knock down of suspect gene(s) will be performed to examine its role in driving IL-33 transcription.

While the pleotropic effects of IL-33 as a cytokine have been extensively studied, there is paucity of literature on its role as a nuclear factor. Intriguingly, IL-33 was first discovered as a nuclear protein differentially expressed in vasopastic arteries of canines. Subsequent reports have indicated a role for IL-33 (as a nuclear factor) in suppressing proinflammatory genes. We hypothesize a similar role for IL-33 in downregulating proinflammatory genes in macrophages during *H. capsulatum* infection. To test this theory, WT and IL-33−/− macrophages will be challenged with *H. capsulatum* yeasts and the resulting proinflammatory cytokines including TNF-α, IL-1β, IL-1α, IL-6 will be measured at transcriptional and protein levels. We expect a robust proinflammatory cytokine response in IL-33−/− macrophages, in comparison to infected WT controls. To further understand the genes under transcriptional control of IL-33, RNA sequencing will be performed on mRNA isolated from infected WT and IL-33−/− macrophages. Finally, to examine if transcriptional repression of proinflammatory genes by IL-33 conditions the macrophages to be more permissive to intracellular fungal proliferation, growth inhibition studies will be accomplished in WT and IL-33−/− macrophages. We anticipate IL-33−/− macrophages will display heightened fungicidal activity and hence will be able to better control
*H. capsulatum* replication. Thus, results from these studies will aid in better understanding of the intracellular immunoregulatory functions of IL-33 in fungal infections.

As an extension to the proposed experiments, we will determine the functions of intracellular IL-33 promulgated by IL-4 in macrophages. A similar approach as mentioned above will be adopted wherein mRNA from IL-4 primed WT and IL-33\(^{-/-}\) macrophages will be sequenced to identify genes under transcriptional regulation of IL-33 in alternatively activated phagocytes. Recognition of these gene targets is pivotal to discern the complex gene regulatory networks controlling alternative macrophage activation. The findings will also enhance our understanding of how alternatively activated macrophages mediate tissue repair and control metabolic homeostasis in the adipose tissue. Intracellular IL-33 may also alter the transcriptional profile of other leukocytes such as dendritic cells or CD4\(^{+}\) T lymphocytes. In such a scenario, effects of cell-specific deletion of IL-33 in these populations will be investigated.

Stimulation of macrophages with IL-4 primes the cells to be more permissive to intracellular *H. capsulatum* growth by augmenting phagolysosomal stores of zinc (trace metal required for fungal growth)\(^{14}\). Although the precise molecular mechanism of intracellular zinc release is not clear, unpublished data from our lab suggest that IL-4 prompts degradation of Metallothionein 3 (a protein responsible for sequestering intracellular zinc away from fungal pathogens) to discharge sequestered zinc within the phagolysosome. Recent reports have indicated that IL-4 activates Cathepsins, a family of cysteine proteases that degrade a myriad of cellular proteins\(^{15,16}\). A possible link exists that IL-4 enhances Cathepsin activity in the phagolysosome that in turn degrade Metallothionein 3 to release bound zinc. Since we have found that IL-4 kindles IL-33, and the latter has intracellular immunomodulatory effects, we will explore the possibility that proteolytic activity of Cathespins may be regulated by IL-33. Activation of
Cathepsin genes will be measured in IL-4 primed WT and IL-33/− macrophages. We expect diminution in Cathepsin activity and lower intracellular zinc levels in phagolysosomes of mutant macrophages.

**Eosinophils subvert antifungal immunity in the host by triggering non-protective IL-4**

We identified eosinophils were the primary contributors to the exaggerated IL-4 response in CCR2−/− mice during *H. capsulatum* infection. These granulocytes were also associated with augmented IL-4 observed in infected IL-5 transgenic mice. To investigate if eosinophils were a direct source of IL-4 in *H. capsulatum* infection, molecular interaction of these cells with the yeasts was examined. We discovered that phagocytosis of *H. capsulatum* prompted a vigorous IL-4 response in both murine and human eosinophils. This was an unexpected finding since phagocytosis of *H. capsulatum* is a cardinal feature of macrophages, neutrophils and dendritic cells. The eosinophilic phagocytosis of yeasts was dependent on the pattern recognition receptor CR3. In summary, our data reveal an unappreciated trait of eosinophil biology that could potentially trigger a collapse in antifungal immunity in the host.

Activated eosinophils are capable of secreting a multitude of proinflammatory cytokines (TNF-α, IL-12, IL-18), chemokines (CCL-5 and eotaxin-1), and lipid mediators (platelet-activating factor and leukotriene C4). Additionally, these cells are a potent source of toxic granular proteins such as major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN), and eosinophil peroxidase (EPO). In the setting of bacterial infections, eosinophils manifest protective effects. These granulocytes have been shown to phagocytose and kill *Staphylococcus aureus* and *Escherichia coli*. Toxic granule proteins secreted by
Eosinophils display potent bactericidal activity against *Pseudomonas aeruginosa*, *S. aureus*, and *E. coli*\(^{22-24}\). Hence it was surprising to discover that eosinophils were inefficient at killing *H. capsulatum* yeasts. In fact, prolonged exposure to the fungus induced cell death in majority of the granulocytes. We wish to determine if this phenomenon can be reversed to direct the eosinophils into being more fungicidal. The cytokine GM-CSF augments antifungal responses in macrophages through sequestration of zinc away from intracellular yeasts and stimulation of reactive oxygen species. This cytokine is also required for development and activation of eosinophils\(^{20}\). We postulate that GM-CSF will arbitrate similar effects on eosinophils to enhance their fungal killing capabilities. To test this, murine eosinophils will be activated with GM-CSF and subjected to *H. capsulatum* infection for 24 hrs. Subsequently, fungal growth inhibition will be measured by plating yeasts and counting colony forming units in eosinophil cultures. Cytokines including TNF-α, IL-4, IL-13 will also be measured in culture supernatants. We expect increased fungal growth inhibition by GM-CSF-activated eosinophils, in comparison to unstimulated eosinophils. *In vivo* studies will also be performed to examine if protective immunity to *H. capsulatum* can be restored in IL-5 transgenic mice and CCR2\(^{-/-}\) mice by administration of recombinant GM-CSF. A potential caveat is that GM-CSF might activate monocytes/macrophages in these mutant mice and override the immune dampening effects of eosinophils. In such a scenario, we will adoptively transfer GM-CSF-activated or unstimulated eosinophils to infected IL-5 transgenic and CCR2\(^{-/-}\) animals and measure fungal burden and IL-4 levels in the lungs. We predict that transfer of activated eosinophils will bolster immunity in mice and accelerate the clearance rate of fungal infection, in comparison to controls. Thus, GM-CSF might strengthen antifungal defenses in the host by a two-pronged mechanism, by activating macrophages and the other by stimulating eosinophils to be more fungicidal.
Numerous cases documenting the presence of eosinophilia in patients with disseminated histoplasmosis have been reported. These clinical observations imply that an eosinophil-rich immune response may perturb the T_{H1}/T_{H2} cytokine balance and in turn, increase susceptibility to *H. capsulatum*. As an extension to these findings, we will determine if eosinophilia weakens host resistance to other fungal pathogens such as *Paracoccidioides brasiliensis* and *Coccidioides immitis*. Interestingly, high numbers of eosinophils have been observed in individuals suffering from coccidioidomycosis\textsuperscript{25} and paracoccidioidomycosis\textsuperscript{26}. We anticipate that eosinophils will manifest deleterious role in other fungal infections as well. Thus, depleting eosinophils in patients suffering from mycotic infections represents a novel and promising therapy that could potentially be used to improve outcome of the diseases.

**Despite a heightened eosinophil response, asthmatics do not display increased predisposition to *H. capsulatum***

Eosinophilia is a key feature of allergic asthma. Surprisingly, we do not observe a significant number of asthmatics with *H. capsulatum* infection in the clinical setting. In agreement, resolution of *H. capsulatum* infection and host survival is comparable between animals with ovalbumin allergen-induced asthma and WT controls (unpublished data). We wish to understand the conundrum as to why asthmatics are not predisposed to histoplasmosis despite having a high frequency of eosinophils.

Asthma is a complex allergen induced disease that manifests from undesired recruitment and activation of eosinophils, and other cell populations such as neutrophils, T_{H2}, and T_{H17} cells in the lungs\textsuperscript{27,28}. A multitude of cytokines including IL-4, IL-5, IL-9, IL-13, and IL-17 also
contribute to the etiology of the disease. Contrasting, in *H. capsulatum* infection, IL-17 mediates protective effects by promoting the homing and effector functions of neutrophils and macrophages. Given these findings, we hypothesize that IL-17 may be beneficial in asthmatic individuals that encounter *H. capsulatum*. To verify the hypothesis, we will neutralize the effects of IL-17 in *H. capsulatum*-infected asthmatic mice. Ovalbumin challenge model will be utilized to simulate an asthma-like phenotype in WT mice (C57Bl/6 background). Subsequently, animals will be infected with a non-lethal dose of *H. capsulatum* and fungal resolution will be monitored. In the test group, IL-17 signaling will be disrupted by administering αIL-17 monoclonal antibody. IL-17RA−/− animals will also be used in supporting experiments. We predict that asthmatic mice with disrupted IL-17 signaling will be more susceptible to *H. capsulatum* infection, in comparison to control asthmatic mice. A likely mechanism is that IL-17 elicits protective immunity through recruitment of neutrophils to the site of infection. These granulocytes are adept at phagocytosis and fungal killing. Experimental data from this study will help solve the conundrum of why asthmatics do not exhibit a predisposition towards *H. capsulatum* infection.

In summary, our future directions will uncover several fascinating aspects of type II immunity and its influence on intracellular infections. Proposed experiments in section-1 will determine the impact of IL-33 on fungal infections, while the second segment will shed light on how the molecule regulates fundamental properties of alternatively activated macrophages. The latter two sections will help in discerning the function of eosinophils in intracellular fungal infections. Knowledge from these studies will provide a platform for developing novel therapeutics to counter mycoses in eosinophilic individuals.
References


Appendix
Figure 1. IL-4 induces IL-33 in another in vivo scenario

WT mice were administered recombinant IL-4 (0.5μg) daily for 6 days i.p. One set of mice were challenged i.p with 2x10⁶ H. capsulatum while the other remained unchallenged. For controls, animals were administered HBSS daily for 6 days; amongst these, one group was exposed to H. capsulatum, while the other was not. Animals were euthanized 2 days after infection and peritoneal macrophages isolated. IL-33 expression in these cells was measured using RT-PCR. Data are mean ± SEM from 1 experiment (4 animals/group).
Figure 2. *H. capsulatum*-driven IL-33 response is dependent on canonical and non-canonical Dectin-1 signaling.

IL-33 mRNA expression in macrophages pre-treated with DMSO or Syk inhibitor (0.1μM) or Raf-1 inhibitor (0.5μM) for 90 minutes before exposure to *H. capsulatum* or IL-4 plus *H. capsulatum*. Values depicted are mean ± SEM from 3-5 independent experiments.
Figure 3. Depletion of eosinophils in *H. capsulatum*-infected CCR2−/− mice results in diminished pulmonary IL-33 response.

CCR2−/− mice were i.p administered αIL-5 (25 μg) or isotype control antibody on days -1, 2, and 5 p.i. IL-33 concentration in whole lung homogenates was analyzed by ELISA after 7 days of infection. Data are mean ± SEM from 1 of 3 similar experiments (3 animals/group).