Classical versus alternative macrophage activation: the Ying and the Yang in host defense against pulmonary fungal infections

CM Leopold Wager[1,2] and FL Wormley Jr[1,2]

Macrophages are innate immune cells that possess unique abilities to polarize toward different phenotypes. Classically activated macrophages are known to have major roles in host defense against various microbial pathogens, including fungi, while alternatively activated macrophages are instrumental in immune-regulation and wound healing. Macrophages in the lungs are often the first responders to pulmonary fungal pathogens, and the macrophage polarization state has the potential to be a deciding factor in disease progression or resolution. This review discusses the distinct macrophage polarization states and their roles during pulmonary fungal infection. We focus primarily on *Cryptococcus neoformans* and *Pneumocystis* model systems as disease resolution of these two opportunistic fungal pathogens is linked to classically or alternatively activated macrophages, respectively. Further research considering macrophage polarization states that result in anti-fungal activity has the potential to provide a novel approach for the treatment of fungal infections.

INTRODUCTION

In the past few decades, the prevalence of fungal disease has increased. Multiple medically important respiratory fungal infections begin with the inhalation of the infectious propagule into the lungs where, in immunocompetent individuals, the fungus is often cleared or controlled by the host's immune system.[1] However, infection with several fungal pathogens, including *Histoplasma capsulatum*, *Blastomyces dermatitidis*, and *Cryptococcus neoformans*, can cause potentially fatal disease in immunocompromised individuals, including those with AIDS and those undergoing immunosuppressive therapies.[2–5] Therefore, the ability of the host's immune system to eradicate or limit the spread of fungal pathogens from the lungs is vitally important.

Macrophages are innate immune cells that have critical roles in protection against pulmonary fungal pathogens, including *C. neoformans*, *Aspergillus fumigatus*, *Pneumocystis*, *B. dermatitidis*, and *Paracoccidioides brasiliensis*.[6–13] Although patients infected with what are classically considered opportunistic infections, such as *C. neoformans*, *A. fumigatus*, and *Pneumocystis*, rely more heavily on innate immunity when they are T celll deficient, studies suggest that protective responses against the primary pulmonary fungal pathogens in immunocompetent individuals also depend on innate immunity.[14] Macrophages are a unique cell type as they can differentiate into multiple phenotypes with different roles: wound healing, immune regulation, and host defense.[15,16] The purpose of this review is to demonstrate the critical role classically and alternatively activated macrophages have during respiratory fungal infections and how the outcome of infection can be determined by the macrophages' ability to polarize in a way that supports resolution and not exacerbation of disease.

TISSUE-RESIDENT AND INFILTRATING MACROPHAGES

Resident alveolar macrophages reside within the lung alveolar airspaces and are often the first line of defense against pulmonary pathogens, making them critical to host defense in pulmonary tissues.[17] Resident murine alveolar macrophages are classified as $CD45^+/CD68^{hi}/F4/80^+/CD11b^-/CD11c^+/Gr1^-$ while lung interstitial macrophages are categorized as $CD45^+/CD68^{lo}/CD11b^+/Gr1^-$ and express CD11c and low concentrations of F4/80.[18] Alveolar macrophages also express

[1]Department of Biology, The University of Texas at San Antonio, San Antonio, Texas, USA and [2]The South Texas Center for Emerging Infectious Diseases, The University of Texas at San Antonio, San Antonio, Texas, USA. Correspondence: FL Wormley (floyd.wormley@utsa.edu)

Received 11 March 2014; accepted 21 June 2014; published online 30 July 2014. doi:10.1038/mi.2014.65

DEC205 and Siglec-F; however, these markers are not exclusive to macrophages and have not been detected on macrophages from other tissues.[19–21] (An in-depth discussion of the characteristics and plasticity of alveolar macrophages is reviewed elsewhere.[19]) Infiltrating macrophages express high levels of CD11b and Ly6C, are F4/80$^+$/CD11c$^-$, and produce pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ).[22,23] Recent fate mapping studies have revealed that tissue-resident lung macrophages and other tissue-resident macrophages, including liver Kupffer cells, splenic, and peritoneal macrophages, have origins established before the birth of the organism.[24] Tissue-resident lung macrophages do appear to be of mixed origin. For example, F4/80^{hi} macrophages, which have a gene signature common to yolk sac macrophages, can persist in adult mice independently of hematopoietic stem cells and transcription factor *Myb*; however, CD11b^{hi}/F4/80^{lo} and some F4/80^{hi} macrophages are continuously replaced by *Myb*-dependent bone marrow precursors.[25] A variety of tissue-resident macrophages, including microglia, lung, and peritoneal macrophages, have been shown to self-renew throughout the adult life of mice during steady state in a macrophage colony-stimulating factor (M-CSF/CSF-1) and granulocyte-macrophage (GM)-CSF (CSF-2)-dependent manner.[26]

Following the release of cytokines by resident alveolar macrophages, circulating mononuclear phagocytes infiltrate into the lungs.[27] Recruited monocytes are generally divided into two main subsets: Ly6C^{hi}/CC-chemokine receptor 2 (CCR2)$^+$ and Ly6C^{lo}/CX3C-chemokine receptor 1 (CX3CR1)^{hi} (reviewed in Mosser and Edwards,[15] Shi and Pamer,[23] and Gordon and Taylor[28]). CCR2, macrophage chemoattractant protein-1 (MCP-1)/CCL2, and MCP-3/CCL7 are critical for the egress of monocytes from the bone marrow and maintenance of normal blood monocyte counts during steady state and infection.[29–31] Deficiency of CCR2 in mice during pulmonary infection with *C. neoformans* results in decreased monocyte recruitment and subsequent differentiation into macrophages in the lungs and the development of a non-protective Th2-type immune response in the chronic infection model, thereby demonstrating the importance of infiltrating monocytes/macrophages for protection.[32] In a disease setting, it is difficult to distinguish between tissue-resident lung macrophages and infiltrating macrophages; therefore further study is necessary to effectively differentiate between these subsets of macrophages. Determination of the roles played by each subset during the stages of a protective immune response, including the initiation of inflammation and the return to steady state, could facilitate the development of novel therapies that take advantage of the intrinsic roles of these cells.

MACROPHAGE ACTIVATION

Alveolar and infiltrating macrophages are able to phagocytize and kill invading pathogens and present antigen to activated T cells to further stimulate an adaptive immune response in immunocompetent individuals. A primary role of macrophages is to clear the cellular debris generated during tissue remodeling and apoptosis. During these activities, macrophages can detect danger signals through their pattern-recognition receptors, such as Toll-like receptors (TLRs) and C-type lectin receptors (CLRs). These danger signals include pathogen-associated molecular patterns on the invading organisms and damage-associated molecular patterns, which occur as the infection progresses and cells are damaged.[14,33,34] Macrophages have a plasticity that allows them to respond to changes in their microenvironment by altering their activation phenotype resulting in the broad classification of classical (M1) or alternative (M2) macrophage activation.[15,35,36] Following TLR and CLR recognition of invading microorganisms, immune cells respond by secreting inflammatory cytokines, including IFN-γ, resulting in macrophage polarization toward an M1 phenotype (**Figure 1**).[15,16,19,37] Macrophage activation phenotypes are identified by hallmark markers, including cytokines and chemokines secreted by the activated macrophages that can induce leukocyte recruitment and aid in the resolution of infection.[15,16] Many M1 markers are induced/upregulated by IFN-γ, which is secreted by innate and adaptive immune cells, including natural killer (NK) cells, CD8$^+$ T cells, and CD4$^+$ T helper type 1 (Th1)-type T cells.[15] Monocytes/macrophages stimulated with IFN-γ and lipopolysaccharide or lipopolysaccharide alone in humans or IL-12 and IL-18 in mice are subsequently capable of producing IFN-γ.[38,39] Studies in our laboratory examining M1-polarized macrophages have also led to the observation of IFN-γ gene expression in murine lung macrophages during anti-cryptococcal immune responses.[6]

The markers commonly used for identification of M1 macrophages include inducible nitric oxide synthase (iNOS) or nitric oxide synthase 2, chemokine (C-X-C motif) ligand 9 (CXCL9), CXCL10, CXCL11, IL-12, and suppressor of cytokine signaling 3 (SOCS3).[15,40] CXCL9, CXCL10, and CXCL11 are chemokines that bind to a common receptor, CXCR3, and this binding results in leukocyte trafficking of principally CD4$^+$ Th1-type T cells, CD8$^+$ T cells, and NK cells.[41] SOCS3 restricts responsiveness to IL-4, resulting in downregulation of M2 macrophage activation markers, and promotes pro-inflammatory responses.[42,43]

M1 macrophages mediate host defense against foreign pathogens by microbicidal activity, including the generation of reactive oxygen and nitrogen species (ROS and RNS, respectively).[44] The enzyme iNOS acts on the substrate L-arginine to produce nitric oxide (NO), which has anti-bacterial and anti-fungal properties. Molecules common to fungal organisms—β-glucan in fungal cell walls and zymosan, a β-glucan-rich cell wall particle prepared from *Saccharomyces cerevisiae*—can stimulate the production of ROS in macrophages via Dectin-1, a CLR that recognizes β-glucans, with and without the collaboration of TLRs.[45,46] Dectin-1, along with Dectin-2, a CLR capable of α-mannan recognition, helps mediate protection against multiple fungal infections, including *Coccidioides*,[47,48] *Pneumocystis*,[49] and *Candida*.[45,50] These receptors are found on various immune cells, including monocytes/macrophages, and are important for the recognition of fungal pathogens.[46,48,50,51] Recognition of fungi by Dectin-1 on macrophages leads to

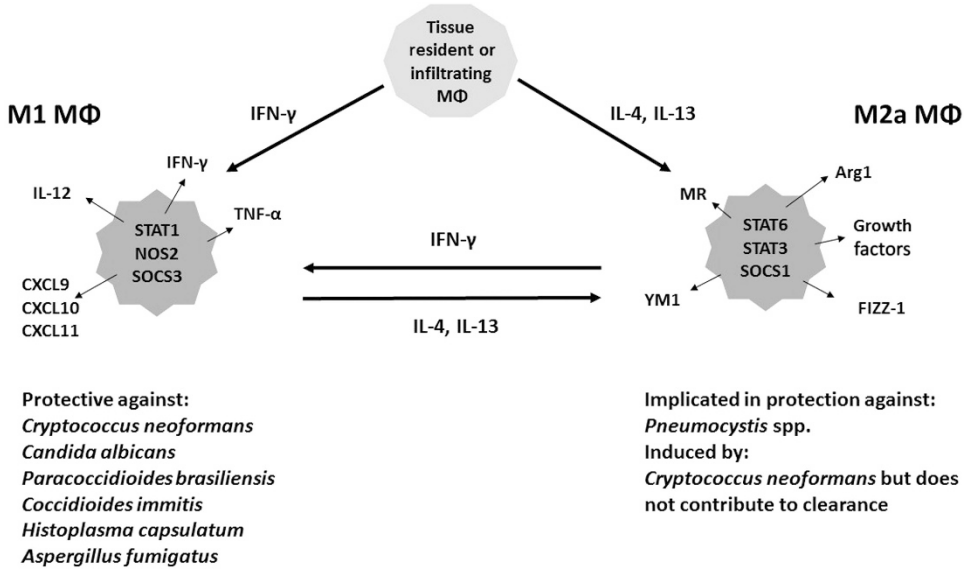

Figure 1 Predominant macrophage activation during pulmonary fungal infections includes M1 and M2a phenotypes. M1 macrophages arise in the presence of interferon (IFN)-γ produced by T helper type 1 (Th1)-type T cells, natural killer cells and activated antigen-presenting cells (not shown). These macrophages are generally microbicidal via the production of reactive oxygen and nitrogen species and promote the generation of a Th1-type immune response. M2a macrophages arise in the presence of cytokines interleukin (IL)-4 and/or IL-13 and are generally not efficient killers of invading pathogens. Instead, M2a macrophages produce growth factors and components of the extracellular matrix and promote the generation/maintenance of Th2-type immune responses. Macrophages have a unique plasticity as polarized macrophages are capable of changing their functional phenotype as the cytokine microenvironment changes. Arg1, arginase 1; CXCL, C-X-C motif chemokine ligand; FIZZ-1, found in inflammatory zone 1; MR, mannose receptor; NOS2, nitric oxide synthase 2; SOCS, suppressor of cytokine signaling; STAT, signal transducer and activator of transcription; TNF, tumor necrosis factor.

Dectin-1-dependent ROS production,[46] and Dectin-2 is linked to the promotion of protective Th17 cytokine responses against *Candida*.[50,52] (The complex roles of Th17 immunity and Dectin-1/Dectin-2 have been reviewed elsewhere.[14,53–59])

During the damage response, anti-inflammatory cytokines, including IL-4 and IL-13, increase, and this increase induces the polarization of macrophages toward an M2 phenotype (**Figure 1**).[15,58,60] M2 macrophages contribute to the suppression/regulation of inflammatory responses and have a pivotal role in wound healing.[15,37,40,58] M2 macrophages have been further subcategorized into three functional categories.[15,16] Macrophages activated by IL-4 or IL-13 are termed M2a macrophages, which secrete components of the extracellular matrix that are important for wound healing; exposure to immune complexes and IL-1R or TLR agonists induces M2b macrophages, which exert immune-regulatory functions and, along with M2a macrophages, drive Th2-type responses; and M2c macrophages are induced by IL-10 and glucocorticoid hormones and have roles in immune-regulation and tissue remodeling.[15,16] A recent review by Martinez and Gordon[61] proposes that macrophages do not form stable subsets but instead respond to a combination of factors present in tissues where different cellular pathways interact forming complex phenotypes. Thus, the traditional M1/M2 paradigm needs to be reassessed to better understand the complexities of macrophage activation. The current review focuses on M1 macrophages and M2a macrophages, both of which are associated with antifungal responses[6,62] (**Figure 1**). IL-4 and IL-13 are secreted by immune cells, such as Th2-type $CD4^+$ T cells, basophils, eosinophils, and mast cells.[37,40] These cytokines can affect macrophages by inhibiting autophagy, hampering NO production, increasing arginase activity, and deterring macrophage-mediated pathogen killing.[37] Other sources of IL-13 include the group 2 innate lymphoid cells (ILCs; ILC2s), which aid in the promotion of Th2-type immune responses.[63,64] Recent studies showed that exposure of epithelial cells to chitin, an integral part of the cell wall of numerous fungi, led to the production of IL-25, IL-33, and thymic stromal lymphopoietin.[65] In response to these cytokines, ILC2s expressed IL-5 and IL-13 resulting in increased eosinophil and M2a macrophages.[65] Chitin also triggers IL-4 expression by innate immune cells such as eosinophils and basophils, leading to induction of M2 macrophages.[65,66] Stimulation with IL-4 induces the expression of mediators used for tissue remodeling, fibroblast accumulation, and collagen deposition—components which are important for wound healing, but do not generally assist in antimicrobial activities.[15,16,37,40] The expression of IL-4 results in increased expression of mannose receptor (MR or CD206), the first described M2 macrophage activation-associated marker.[67] Historically, the general markers of M2 macrophage activation include arginase1 (Arg1), found in inflammatory zone 1 (FIZZ-1, also known as resistin-like α or Relm-α), and chitinase and chitinase-like molecules, such as YM1 (Chi3l3) and YM2.[15,40] Arg1 has a role in the production of polyamines and prolines, which control cell proliferation and collagen production, respectively, thus indicating the essential role of M2 macrophages

in tissue remodeling and wound healing.[68,69] FIZZ-1 is not only expressed by M2 macrophages but is also highly induced in type II alveolar epithelial cells when stimulated by Th2-associated cytokines IL-4 and IL-13, and it is associated with the pathogenesis of lung fibrosis.[70] Therefore, when evaluating FIZZ-1 and the expression of other M2 macrophage markers *in vivo*, it is critical to identify the source. Chitinase-like molecules YM1 and YM2 are produced in murine lungs during Th2-type immune responses and are commonly observed as crystals at the site of parasitic and fungal infections.[71] Their role is not clearly understood; however, *in vitro* assays have revealed that YM1 and YM2 are chemotactic for eosinophils, T lymphocytes, and polymorphonuclear leukocytes.[72] Additional markers of M2 macrophage activation include tissue remodeling and immunoregulatory genes, such as matrix metalloproteinases 2 and 7, and adenosine A3 receptor.[73] Interestingly, during *C. neoformans* central nervous system infection, monocyte-derived macrophages, but not microglia, exhibit the M2 markers MR and YM1.[74]

Macrophages have a unique plasticity that allows them to alter their phenotype depending on the cytokine microenvironment.[75–77] When polarized toward an M1 phenotype in the presence of IFN-γ, macrophages can re-polarize to an M2 phenotype when stimulated with IL-4. Similarly, M2-polarized macrophages can re-polarize toward a functional M1 phenotype in the presence of IFN-γ,[75,77,78] signifying the critical importance of the local cytokine milieu in driving macrophage polarization. IL-4 and IFN-γ have been shown to cross-regulate each other as conditions conducive to IFN-γ production inhibit IL-4 production.[79] IFN-γ production by Th1-type T cells and NK cells can stimulate M1 macrophage activation in a signal transducer and activator of transcription (STAT) 1-dependent manner.[80] IL-4 and/or IL-13 induces M2 macrophage activation by stimulating the STAT6 pathway.[65,70,81–83] One of the most commonly used markers for M2 macrophage activation, Arg1, is considered a context-dependent marker as it can be induced by STAT6 as well as STAT3, which has been associated with regulating M2 macrophage polarization.[84–86] Arg1 competes with iNOS for the substrate L-arginine and produces L-ornithine and urea while iNOS and L-arginine yield NO and citrulline.[87] The Arg1-to-iNOS ratio is often considered an indicator of macrophage polarization phenotype; however, the regulatory network *in vivo* has proven to be quite complex.[58] Depletion of L-arginine in peritoneal-derived macrophages pretreated with IL-4 *in vitro* prevents the enzymatic activity of iNOS to produce NO; however, this inhibition of NO production can be overcome by adding supplementary L-arginine.[88,89] Therefore, conditions conducive to the induction of alternatively activated macrophages can inhibit classical macrophage activation.[90]

Fungal pathogens, such as *C. neoformans*,[7,74,91–93] *H. capsulatum*,[94] *P. brasiliensis*,[95,96] *Coccidioides immitis*,[97] *C. albicans*,[98] and *B. dermatitidis*,[99] are sensitive to the NO produced by M1 macrophages. However, many fungi use various mechanisms to avoid being killed by NO.[100,101] In the case of *H. capsulatum*, macrophages can efficiently phagocytize the fungus but are unable to kill it until activated by cytokines produced by activated T cells.[102,103] *P. brasiliensis* is capable of inhibiting macrophage NO production by an unknown mechanism, resulting in deficient killing of the fungus.[99,104,105] *B. dermatitidis* is believed to directly inhibit the enzyme iNOS, thus preventing NO production by macrophages.[99] *Coccidioides* has the ability to suppress NO production; however, NO does not seem to be required for clearance, indicating that there is another mechanism important for host defense.[106,107] *C. albicans* and *C. neoformans* are also thought to mediate NO suppression by downregulation of macrophage iNOS RNA.[108,109] In *C. albicans*, deletion of the gene *CaYHB1* results in decreased virulence due to NO sensitivity, nevertheless, mice still succumb to systemic infection,[98] indicating that killing by RNS may not be critical to protection against systemic *C. albicans* infections. Conversely, inhibition of iNOS by *C. neoformans* results in M2 macrophage activation and progression of disease,[7,110–113] thus, avoiding M1 macrophage activation altogether. *Pneumocystis* can inhibit functional NO by preventing iNOS from forming a homodimer that is required for NO production.[114,115] In contrast to other fungal pathogens, *Pneumocystis* can be cleared by the activation of M2 macrophages.[62,114] The subsequent sections of this review focus on macrophage polarization during fungal infections, principally *C. neoformans* and *Pneumocystis* species, as the majority of research in this area has been done with these prototypical model yeasts.

"CLASSICAL" WARRIORS IN HOST DEFENSE

A number of fungal respiratory pathogens enter the host via inhalation of the infectious propagule from the environment into the lung alveoli. Here the organisms are capable of proliferation and often disseminate to other organs, particularly when the host's immune system is incapable of curtailing the infection. *C. neoformans* is a model organism for the study of protective M1 macrophages and associated Th1-type immune responses that aid in resolution of disease. *C. neoformans* has been traditionally viewed as an opportunistic pathogen that causes disease predominantly in immunocompromised individuals, such as AIDS patients.[116,117] *C. neoformans* is generally inhaled by the host and, in healthy individuals, the yeast can be cleared. Conversely, in immunocompromised individuals and in some otherwise healthy individuals with no discernable underlying condition, the cryptococci can disseminate to the central nervous system, resulting in potentially fatal meningoencephalitis. Alveolar macrophages are generally one of the first cell types to encounter *C. neoformans*, and the interactions between these immune cells and the yeast can determine the outcome of the infection.[118,119]

Protection against *C. neoformans* is largely mediated by Th1-type cell-mediated immune responses which are characterized by cytokines including IFN-γ, TNF-α, IL-2, and IL-12.[91,110,120–126] These conditions promote M1 macrophage activation and production of NO and other antimicrobial factors. Yet, infection with *C. neoformans*, including the highly virulent strain H99, results in Th2-type-polarized cytokine

responses; production of IL-4, IL-5, and IL-13; and M2 macrophage activation.[91,92,112,127–129] These macrophages are able to easily phagocytize the cryptococci but are not efficient killers of the yeast.[6,7,111,130] *C. neoformans* cells can survive within M2 macrophages and use them as a protective niche to evade recognition and killing by the host.[131] To illustrate, IFN-$\gamma^{-/-}$ mice given an experimental pulmonary infection with *C. neoformans* have a propensity to gravitate towards an M2 phenotype and generate a Th2-type cytokine environment within the lungs, correlating with increased intracellular fungal growth within macrophages and more severe pulmonary infection (**Table 1**).[110,111] Conversely, when infected with *C. neoformans*, IL-$4^{-/-}$ mice have a reduced fungal burden within host macrophages and a decrease in markers for M2 activation.[111] Furthermore, mice with macrophage-specific ablation of IL-4Rα have a significantly increased survival rate and decreased pulmonary fungal burden as compared with that of wild-type (WT) mice.[132] These conditional knockout (KO) mice also have an increase in iNOS and a decrease in Arg1, YM1, and MR in pulmonary tissue as compared with what occurs with WT mice.[132] This further demonstrates the role of IL-4 in inhibition of M1 macrophage–polarizing conditions during *C. neoformans* infection. Experimental pulmonary infection with *C. neoformans* strain 52D results in a chronic infection in C57BL/6 mice, but the infection is eventually resolved in BALB/c mice.[111,133] Inoculation with *C. neoformans* strain 52D in the chronic model with C57BL/6 mice results in a dynamic shift from Th2- to Th1-type cytokine production at 5 weeks after inoculation. This shift surprisingly does not aid in fungal clearance, suggesting that the initial Th2-type cytokine response allows for the establishment of a chronic fungal infection, which is not overcome by subsequent increases in IFN-γ and TNF-α.[111] Additionally, deletion of the M2-polarizing cytokine IL-13 results in decreased expression of M2 macrophage activation markers, less lung eosinophilia, reduced production of Th2-type polarizing cytokines, and increased survival as compared with infected WT mice and IL-13-overexpressing transgenic mice (**Table 1**).[91] Taken together, both IL-4 and IL-13 facilitate M2 macrophage polarization during infection with *C. neoformans*, resulting in a defective anti-cryptococcal immune response.

The role of TLRs in cryptococcal infection is controversial as studies have demonstrated conflicting data regarding the necessity of TLRs, such as TLR2 and TLR4. TLR2 recognizes a wide range of bacterial, fungal, and viral pathogen-associated molecular patterns, including lipopeptides, peptidoglycan, zymosan, and hemagglutinin.[34] TLR4 primarily recognizes bacterial lipopolysaccharide, fungal o-linked mannans, and glucuronoxylomannan.[134] Both of these TLRs signal through the TIR (Toll/IL-1 receptor) domain–containing adaptor molecule MyD88 (myeloid differentiation primary response gene 88)-dependent pathway, which drives the induction of inflammatory cytokines.[34] Macrophages with deficient TLR2 expression or MyD88 ablation exhibit reduced production of TNF-α when cultured with *C. neoformans in vitro*.[135] Furthermore, TLR$2^{-/-}$ and MyD$88^{-/-}$ mice show a decreased expression of TNF-α, IFN-γ, and IL-12p40 transcripts in the lungs, brain, and spleen during infection with *C. neoformans*, a result which may indicate that the TLR2/MyD88 pathway is important for host defense against *C. neoformans* infection.[135] The lack of production of these Th1-type cytokines, particularly IFN-γ, could suggest a defect in M1 macrophage activation, which may explain their increased susceptibility to cryptococcal infection. Other studies have shown that these receptors may not be required or are only marginally helpful for overcoming *C. neoformans* infection.[136,137] However, the consensus is that the TLR-associated adaptor protein MyD88 is important for protection not only against *C. neoformans* but also against other fungal pathogens.[47,135,137,138] Another receptor, TLR9, has recently been implicated in M1 macrophage activation and protection in BALB/c mice inoculated with *C. neoformans* strain 52D.[130] TLR9 is found on the endosomal membrane within host immune cells, including dendritic cells (DCs), plasmacytoid DCs (pDCs), monocytes, and macrophages in mice and pDCs and B cells in humans. This receptor recognizes unmethylated pathogen CpG DNA following its ingestion and degradation by the phagocyte.[139,140] TLR9 specifically interacts with cryptococcal CpG DNA in DCs, resulting in the secretion of IL-12p40 and the expression of co-stimulatory molecule CD40.[141] Cryptococcal infection in mice with TLR9 deletion leads to decreased production of M1-polarizing IFN-γ and TNF-α compared with WT mice. Additionally, TLR$9^{-/-}$ mice produce more of the M2-polarizing cytokine IL-4, a condition that correlates with decreased infiltration of leukocytes into the lungs and ultimately survival (**Table 1**).[130] More compelling is that TLR$9^{-/-}$ mice have increased gene expression of M2 markers Arg1 and FIZZ-1 and decreased expression of iNOS at 3 weeks post-infection,[130] suggesting that cryptococcal degradation and recognition of the CpG DNA by TLR9 is necessary for protection against *C. neoformans*. On the contrary, scavenger receptor A (SRA or CD204) is a receptor on immune cells, including macrophages, that senses a broad range of ligands expressed by *C. neoformans*,[142–144] including heat-shock proteins.[145] However, SRA has not been implicated in the activation of macrophages in response to the yeast.[146] Deletion of SRA in mice leads to decreased production of Th2-type, M2 macrophage–associated cytokines IL-4 and IL-13 by pulmonary leukocytes.[147] Additionally, following inoculation with *C. neoformans*, SRA$^{-/-}$ mice have an increase in iNOS transcripts and a decrease in Arg1 transcripts within pulmonary macrophages; however, lack of SRA expression is not sufficient for macrophage fungicidal activity.[147]

As infection with virulent *C. neoformans* strains elicit a non-protective, Th2-type immune response, a need remained for an infection model that allowed for the study of protective anti-cryptococcal immune responses. Consequently, the highly virulent *C. neoformans* strain H99 was genetically engineered to express murine IFN-γ and was designated H99γ.[148] This modified strain elicits protective immune responses in BALB/c, A/Jcr,[7,148,149] and C129 mice (unpublished data), thus providing a model for characterizing protective immune responses against *Cryptococcus* (**Table 1**). Furthermore, immunization

Table 1 Influence of genetic modification and/or treatment of mice on macrophage activation phenotype and disease progression

Modification/treatment	MΦ polarization	Effect on disease progression	References
Cryptococcus			
IFN-γ$^{-/-}$	M2	Th2-type immune response, increased intracellular growth within MΦ, decreased transcripts for CXCL9 and CXCL10 and increased expression of CCL22	111
IL-4$^{-/-}$	M1	Decreased intracellular growth, increased transcripts for CXCL9 and CXCL10, decreased gene expression for CCL22	111
MΦ-specific IL-4R$^{-/-}$	M1	Increased survival, decreased pulmonary fungal burden	132
TLR9$^{-/-}$	M2	Decreased pulmonary leukocyte infiltration, decreased survival, defects in granuloma formation, increased intracellular yeasts, defects in Th polarization	130
H99γ infection	M1	Th1-type immune response, increased pulmonary leukocyte infiltration, decrease in pulmonary fungal burden, increased survival	7
H99γ immunization + *C. neoformans* H99	M1	Th1-type immune response, increased pulmonary leukocyte infiltration, decrease in pulmonary fungal burden, increased survival, increased STAT1 phosphorylation	6
T1/ST2$^{-/-}$ (IL-33R)	↓M2	Decreased fungal burden, decreased dissemination, increased survival, decreased eosinophilia, diminished goblet cell hyperplasia and mucus hyper-secretion, decreased serum IgE	179
IL-13$^{-/-}$	M1	Decrease in Th2-polarizing cytokines, decreased serum IgE, decreased mucus production, decreased eosinophilia	91
Scavenger receptor A$^{-/-}$	M1?	Decrease in Th2-type cytokine production, increased iNOS transcripts and decreased Arg1 transcripts in pulmonary MΦ, not sufficient for fungicidal activity	147
Mannose receptor$^{-/-}$	Unknown	Defects in adaptive immune response, increased susceptibility	193
Pneumocystis			
Src TKO	M2	Increased inflammatory cell recruitment, increased cytokine (including IL-33) and chemokine production, increased MΦ killing of the yeast	173
Recombinant IL-33	M2	Reduction in pulmonary fungal burden, increased CCL17 production	62
Caspase 9 inhibition	Unknown	Reduction in MΦ apoptosis, decreased pulmonary fungal burden and inflammation, increased survival	163
Mannose receptor$^{-/-}$	Unknown	Increased pulmonary phagocyte recruitment, increased pathology, accumulation of glycoproteins, equal susceptibility to WT mice	172
STAT4$^{-/-}$ (C57BL/6 mice)	↑M2	No difference in fungal burden compared with WT, initial decrease in Th1- and Th2-type cytokine production, increased CCL17 production, elevated *Pc*-specific antibody levels, increased Th2-type cytokines in draining lymph nodes	182
STAT4$^{-/-}$ (BALB/c mice)	↓M2	Increased fungal burden compared with WT, prolonged decrease in Th1- and Th2-type cytokine production, decreased CCL17 production, impaired Th2-type cytokines in draining lymph nodes	182
Sulfasalazine	M2	Reduced disease severity, reduced inflammation, increased MΦ phagocytosis, promotion of Th2-type immune response	164
Aspergillus			
TLR9$^{-/-}$	Unknown	Th2-type immune response, decreased organ fungal burden	138
Serum amyloid P	↓M2	Decrease in STAT6 phosphorylation, attenuated *A. fumigatus*-induced allergic airway disease	192
Histoplasma			
Histone 2B antibody	Unknown	Increased phagocytosis by MΦ, inhibition of intracellular growth, reduced fungal burden, increased survival	194,195
SOD3$^{-/-}$	Unknown	Increased susceptibility of *H. capsulatum* to PMN and M1-produced ROS, decreased virulence, increased survival of the host	196

↓, decrease in macrophage activation markers; ↑, increase in macrophage activation markers;?, evidence suggests M1 activation but is inconclusive; Arg1 arginase-1; CCL17, chemokine ligand 17; CXCL, C-X-C motif chemokine ligand; H99γ, IFN-γ-producing *Cryptococcus neoformans* strain; IFN-γ interferon-gamma; IgE, immunoglobulin E; IL, interleukin; IL-4R, IL-4 receptor; iNOS, inducible nitric oxide synthase; M1, classically activated macrophage; M2 alternatively activated macrophage; MΦ, macrophage; *Pc*, *Pneumocystis*; PMN, polymorphonuclear leukocyte; ROS, reactive oxygen species; SOD3, superoxide dismutase 3; Src TKO, Src-family tyrosine kinase triple knockout; STAT, signal transducer and activator of transcription; T1/ST2, unique IL-33 receptor; Th2, T helper type 2; TLR9, Toll-like receptor 9; WT, wild type.

with H99γ affords 100% protection against subsequent challenge with WT *C. neoformans* H99.[6,148] In both primary infection with H99γ and secondary *C. neoformans* H99 challenge of mice immunized with H99γ, there is a decrease in fungal burden, an increase in survival, and an increase in M1 macrophage activation as compared with mice given a primary inoculation with the *C. neoformans* H99 or a pulmonary challenge with *C. neoformans* H99 following mock immunization with heat-killed *C. neoformans* H99 (HK*C.n.*).[6,7,148] Pulmonary macrophages from mice immunized with H99γ (protected) and challenged with *C. neoformans* H99 have an increase in iNOS transcripts, an increase in NO production, and a decrease in transcripts for Arg1, YM1, FIZZ-1, and MR as compared with mice immunized with HK*C.n* (non-protected).[6] In addition, studies have indicated that the M1 macrophages induced by the IFN-γ-producing *C. neoformans* strain are likely activated via STAT1 signal transduction pathways, as total STAT1 and phosphorylated STAT1 protein, along with transcripts for STAT1-regulated genes, are increased in macrophages from protectively immunized mice.[6] These findings suggest that the induction of M1 macrophages, which are associated with protective immune responses to *C. neoformans*, is STAT1 mediated. Thus stimulation of the STAT1 pathway could provide a novel approach to anti-cryptococcal immune therapies and may be important for other fungal infections where M1 macrophages are protective.

THE "ALTERNATIVE" IN HOST RESPONSES

Like *C. neoformans*, *Pneumocystis jiroveci* (formerly known as *Pneumocystis carinii*, which is currently the name of the *Pneumocystis* species used in rats while *P. murina* is the species used in mice) is an opportunistic fungal pathogen most commonly associated with AIDS patients.[150,151] Infection with *P. jiroveci* in immunocompromised individuals can result in life-threatening pneumonia. Fortunately, the incidence of *Pneumocystis* pneumonia (PCP) among AIDS patients has decreased with the introduction of highly active antiretroviral therapy[152]; nevertheless, PCP remains a concern. The mortality rate for AIDS patients at medical facilities during two retrospective studies was approximately 10–11% and is as high as 29% in one study when intensive care was required.[153,154]

The role of T cells is a debated topic. Studies have shown that $CD4^+$ T cells have a critical role in host defense against *Pneumocystis*, because depletion of $CD4^+$ T cells increases the susceptibility to PCP.[155–157] However, contradictory studies suggest that resolution of an infection with *Pneumocystis* can occur despite persistent $CD4^+$ lymphocyte depletion[158,159]; therefore, it may be inferred that other immune cells, such as macrophages, are essential for resolving PCP. Studies suggest that alveolar macrophages contribute to an effective anti-*Pneumocystis* host response[10,160–163] and serve as the downstream effector cell for $CD4^+$ T cell-dependent clearance,[164] although much of the evidence is circumstantial. Enhanced *Pneumocystis* pathogenesis is associated with a reduction in alveolar macrophage numbers,[165] an effect which may be due to apoptosis induced by the organism.[166] Reduction of macrophage apoptosis in rats and mice via inhibition of caspase 9 during infection with *Pneumocystis* is associated with decreased pulmonary fungal burden, increased phagocytosis, and host survival.[163] The improvement of disease resolution when macrophage apoptosis is prevented indicates that the presence of pulmonary macrophages and, therefore, prevention of apoptosis is critical to host defense.

The M2 macrophage phenotype is associated with the C-type lectin receptor MR, which recognizes the carbohydrate group mannose, a structural component of fungal cell walls.[167] MR has been implicated in the mediation of cytokine production, including IL-6, TNF-α, MCP-1, GM-CSF, IL-1β, IL-12, and IL-10, and is capable of recognizing numerous fungi, including *C. neoformans*, *C. albicans*, and *P. jiroveci*.[167] Human alveolar macrophages recognize opsonized *Pneumocystis* organisms largely via MR, resulting in binding and phagocytosis of the fungus.[168,169] MR is also found on various other cell types, including DCs and, though less abundantly, M1 macrophages. In the murine model of PCP, induction of IL-1β, IL-6, and TNF-α transcripts, as well as an influx of alveolar macrophages, is associated with clearance of the organism.[170] Use of an MR-blocking ligand or siRNA silencing of MR in human macrophages *in vitro* promotes the release of TNF-α to unopsonized *P. jiroveci*, which is not released under WT conditions in response to the unopsonized fungus.[171] MR KO mice infected with *Pneumocystis* were no more susceptible to PCP than WT mice.[172] However, MR KO mice exhibited a greater influx of phagocytes into the lung alveoli during infection, and this increase in pulmonary infiltrates was accompanied by greater pathology, including accumulation of glycoproteins which are normally cleared by cells expressing MR (**Table 1**).[172] Taken together, MR^+ macrophages recognize *Pneumocystis* and may aid in regulation of pro-inflammatory TNF-α and subsequent phagocyte infiltration to the lungs, thus preventing deleterious inflammatory responses during *Pneumocystis* infection.

Recognition of *P. murina* by alveolar macrophages leads to activation of Src-family tyrosine kinases (SFKs). Recently, a study by Nelson *et al.*[173] showed that mice deficient in the SFKs Hck, Fgr, and Lyn (termed Src triple knockout or Src TKO mice) demonstrate augmented clearance of *P. murina* in the mouse model of PCP (**Table 1**). However, Src TKO mice had increased cytokine and chemokine levels, as well as increased inflammatory cell recruitment to the lungs.[173] In addition, the alveolar macrophages of Src TKO mice more efficiently killed *P. murina* as compared with macrophages from infected WT mice.[173] Further study of PCP in the Src TKO mice revealed that clearance of *Pneumocystis* is associated with enhanced M2a macrophage activation, as evidenced by increased levels of FIZZ-1, Arg1, and YM1 transcripts in the lungs at day 3 post-infection as compared with what occurred with WT mice.[62] In addition, while there was no increase in IL-4 and IL-13, there was an increase in IL-33,[62] which can work synergistically with IL-4 and IL-13,[174] thus suggesting that IL-33 may have a role in enhancing M2a macrophage activation during *Pneumocystis* infection. Other studies have indicated that IL-33 may have a

role in amplifying the polarization of M2 macrophages and potentially contributes to airway inflammation.[175] Activated NK T cells are capable of inducing alveolar macrophages to produce IL-33, further activating NK T cells, as well as ILCs, to produce IL-13.[176] Intranasal administration of chitin in mice resulted in the increased expression of IL-25, IL-33, and thymic stromal lymphopoietin, resulting in the production of IL-5 and IL-13 by ILC2s as well as M2 macrophage activation.[65] The study also showed that chitin stimulation resulted in ILC2-mediated suppression of Th1- and Th17-type cytokine responses and increased eosinophil infiltration, further demonstrating a link between IL-33 and the cytokine microenvironment in the lung during fungal-induced inflammation.[65] In humans, chemoattractant receptor CRTH2$^+$ ILCs were shown to respond to IL-33 and IL-25 (IL-17E) by the production of IL-13.[177] Pretreatment of macrophages with recombinant IL-33 (rIL-33) and IL-13 enhanced *in vitro* killing of *P. murina* as compared with macrophages pretreated with IL-13 alone, IL-33 alone, IFN-γ alone, or untreated macrophages.[62] Furthermore, intraperitoneal injections of rIL-33 in mice during infection led to a reduction in pulmonary fungal burden and to increases in markers of M2a activated macrophages and their by-products, such as CCL17/thymus and activation-regulated chemokine that can lead to recruitment of Th2-type T cells (**Table 1**).[16,62,178] Alternatively, IL-33 signaling has been shown to have a detrimental role during infection with *C. neoformans* and is upregulated during infection with the yeast.[179] Deficiency in T1/ST2, the unique IL-33R, resulted in reduced M2 activation and decreased fungal burden and dissemination of *C. neoformans*. The results of these studies further exemplify how infection with different fungal organisms requires variations in the host's immune response to prevent the progression of disease.

An important point to remember is that not all mouse strains produce the same results during experimental infections. A recent study examined the role of STAT4 during the protective immune response against pulmonary infection with *Pneumocystis* using both C57BL/6 and BALB/c mice with STAT4 deletion (**Table 1**). STAT4 is activated by IL-12 signaling which, along with the transcription factor T-bet, is required for optimal development of Th1-type T cells.[180] STAT4 has also been shown to antagonize Th2 development.[181] C57BL/6 *Stat4*$^{-/-}$ mice exhibited an increase in transcripts for M2 macrophage activation marker FIZZ-1 while BALB/c *Stat4*$^{-/-}$ mice had decreased levels of FIZZ-1, and neither group of KO mice showed a difference in mRNA levels for the M1 marker iNOS as compared with WT mice.[182] Interestingly, BALB/c *Stat4*$^{-/-}$ mice were more susceptible to PCP, suggesting that the intact M2 macrophage activation in the C57BL/6 *Stat4*$^{-/-}$ mice may contribute to protection.[182]

Overall, evidence suggests that the polarization of alveolar macrophages toward an M2 phenotype is conducive to the induction of protective immune responses against *Pneumocystis*. Although M2a macrophages appear capable of directly killing *Pneumocystis* yeasts, it may be that the anti-inflammatory environment promoted by M2a macrophages prevents the development of over-exuberant pulmonary inflammatory responses. In the mouse model of PCP, IL-33 appears to have a role in the protective immune response. Immune cells, such as macrophages and DCs, and alveolar epithelial cells have been implicated as sources of IL-33 mRNA.[62,183] IL-33, in turn, induces the production of IL-13 by ILCs,[176] further stimulating M2 macrophages and the progression of a Th2-type immune response. The suppression of inflammatory damage during this response may be an indirect mechanism of protection; however, additional research is necessary.

FUTURE STRATEGIES TO COMBAT FUNGAL PATHOGENESIS

Many pathogens induce an environment within the host that favors their own proliferation and dissemination. A potential tactic for designing novel treatments is to develop strategies for overcoming these pathogen-mediated effects so as to induce a more microbicidal environment. Therapeutics targeting modifications of the host response rather than the pathogen would limit selective pressure on the microbe that can lead to drug resistance and increased virulence. The studies discussed in this review have indicated that macrophage polarization has a major role in the outcome of infection. Therapeutics targeting macrophage activation to shift polarization toward an antifungal phenotype may provide a novel method for treatment of fungal infections.

In the case of *Pneumocystis* pneumonia, treatments that reduce pulmonary inflammation and promote the polarization of macrophages toward an M2a phenotype would be beneficial in slowing and/or stopping disease progression. Macrophages treated with IL-33 and IL-13 have an increased fungicidal effect on *Pneumocystis* compared with M1 macrophages induced by IFN-γ.[62] Intraperitoneal injection of rIL-33 in mice led to greater pulmonary clearance of the yeast,[62] indicating that IL-33 has potential as a novel immunotherapy for PCP. Additionally, sulfasalazine, a potent anti-inflammatory drug, enhances the clearance of *Pneumocystis* from the lung in the PCP-Immune Reconstitution Inflammatory Syndrome (IRIS) model, correlating with the promotion of M2 macrophage activation.[164] Sulfasalazine is normally used to treat inflammatory illnesses, such as Crohn's disease[184] and rheumatoid arthritis,[185] by altering macrophage and T-cell responses and inhibiting nuclear factor-κB.[186,187] During infection with *Pneumocystis*, sulfasalazine treatment resulted in reduced severity of disease, decreased inflammation, and promotion of M2 macrophage activation. The M2-polarized macrophages displayed enhanced phagocytosis and clearance of the fungus,[164] further supporting a role of M2 macrophages in protection against PCP.

Skewing of the immune response and subsequent macrophage polarization phenotype is a technique a number of fungal pathogens use to aid in host invasion and colonization. Studies have shown that M2 polarization can be shifted to M1[35,188]; however, it is not known whether the macrophages are being re-polarized or if newly infiltrating macrophages become M1 polarized. Nevertheless, the local cytokine milieu is perhaps the

final determinant of macrophage polarization phenotype. Studies examining plasticity of tumor-associated macrophages have indicated that administering IFN-γ or targeting nuclear factor-κB have re-polarized M2 macrophages to M1 macrophages *in vivo*.[189,190] A recent study has demonstrated that macrophages polarized toward an M2 phenotype with IL-4, as observed during infection with *C. neoformans*, can be overcome by the addition of IFN-γ *in vitro*, resulting in a phenotypic switch to functional M1 macrophages.[78] These re-polarized macrophages were fully capable of producing NO and were fungicidal, thus inhibiting proliferation of the yeast.[78] Therefore, strategies for inducing M1 macrophage activation and/or inhibiting M2 macrophage activation during infection could prove to be a novel and effective method of inducing host protection against *Cryptococcus* and other fungal infections. For example, treatment of macrophages with kojic acid, a secondary metabolite of fungal species, including *Aspergillus* and *Penicillium*, results in increased ROS production but not NO.[191] More investigation to characterize the macrophage activation phenotype and to determine the anti-fungal activity of kojic acid is needed. Serum amyloid P has recently been shown to inhibit M2 polarization via an FcγR-dependent mechanism in cultured macrophages.[192] The effect led to the downregulation of M2 markers, a decrease in STAT6 phosphorylation, and attenuated *A. fumigatus*-induced allergic airway disease,[192] suggesting that inhibition of M2 polarization shows promise for treatment of *Aspergillus* and other pulmonary fungal infections that result in allergic airway disease. Additionally, studies in our lab have suggested that STAT1 has a role in mediating M1 macrophage activation and subsequent protection against *C. neoformans*[6]; therefore, induction of the IFN-γ/STAT1 signaling pathway could provide a potential mechanism for induction of M1 macrophages.

The stimulation of M1 macrophage activation and/or the prevention of M2 macrophage activation have the potential to provide protection against a myriad of fungal infections, including *C. neoformans*, *H. capsulatum*, and *A. fumigatus*. Induction of pro-inflammatory responses by M1 macrophages, as well as the production of ROS and RNS, contributes to eradication of the pathogen and resolution of disease. However, induction of M2 macrophages, but not M1 macrophages, is conducive to clearance of *Pneumocystis* infections. Dampening of pro-inflammatory responses that limit PCP-associated damage, along with the anti-*Pneumocystis* activity of M2 macrophages, allows the host to resolve the infection. Therefore, discovery and utilization of therapeutics that induce polarization of macrophages toward an anti-fungal phenotype must consider the pathogen in question. Targeting the host's immune response rather than the pathogen could provide a much needed, novel approach to the treatment of fungal infections.

ACKNOWLEDGMENTS

This work was supported by research grant W911NF-11-1-0136 from the Army Research Office of the Department of Defense and 2RO1 AI071752 from the National Institute of Allergy and Infectious Diseases (NIAID) of the National Institutes of Health (NIH) (to F.L.W. Jr). Thanks to K. Wozniak, A. Cardona, and G. Pizzola for critical reading of the manuscript.

DISCLOSURE

The authors declared no conflict of interest.

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